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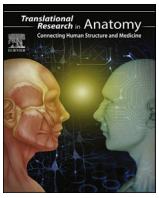

Morphoquantitative effects on striated skeletal muscle of Wistar rats (*Rattus norvegicus*) subjected to a diet utilized in young children from rural Mozambique

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ABSTRACT

Mozambique is a country of sub-Saharan Africa where about 55% of the population lives below the absolute poverty line with less than one meal a day hardly surviving based on by donations. Food insecurity and precarious nutrition, especially in children, are factors that induce to levels of 44% of chronic malnutrition (CD) in infants. The CD is responsible for one third of deaths in children under five years. The aim of this study was to evaluate the morphoquantitative effects in gastrocnemius muscle of Wistar rats fed with a diet utilized by people from rural areas of Mozambique. We used 75 Wistar rats weighing approximately 300 g divided in three groups: nourished or control (N), malnourished (D), and Mozambique or experimental group (M), measured at birth and at weaning. The animals were kept under the same housing conditions, temperature, humidity and light, but with different diets depending on the group: Group N with normal protein diet (20% casein), Group D with hypo-proteic diet (5% casein), and Group M with Mozambique diet. In all groups we evaluated the body mass at birth and weaning, and collected the right gastrocnemius muscle of male pups at weaning for analysis. Serial sections of 10 μm were performed in a cryostat prior to histology techniques of hematoxylin and eosin, picrosirius, NADH-tr and analysis in transmission electron microscope. Statistical evaluation was determined by analysis of variance (ANOVA) and Tukey tests. Significant differences were found between groups N, D and M. In group M were observed a great variation of body mass that was approximately similar to group D; Group M also showed the same changes in muscle fiber which exhibited round-shaped contours, and predominance of type III collagen, similarly to malnourished group (D). Ultra-structurally, animals from Mozambique displayed a disorganization of the Z lines of sarcomeres, myofibrils disruption, decreased cross-sectional area and a smaller proportion of glycolytic and glycolytic-oxidative fibers. Additionally, the animals from M group revealed higher percentage and cross-sectional area identical to group D in respect to oxidative fibers.

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1. Introduction

It is estimated that worldwide, from 2004 to 2008, about 208,000 children died by protein-calorie malnutrition and 1.327 million newborns revealed mental retardation by iodine deficiency [3].

Mozambique is a country of sub-Saharan Africa where about 55% of the population lives below the absolute poverty line with less than one meal a day. The diet of rural areas of Mozambique is mainly constituted by leaves, peanut, cassava, maize, etc. Chronic malnutrition (CD) is prevalent in Mozambique particularly in

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children. The symptoms of CD are associated with stunting, vitamin A deficiency, anemia, and other nutrition-related disorders [1] that can lead to a high risk of death. Additionally, CD is reportedly related with secondary complications like hypoglycemia and concomitant co-infections such as bacteremia, acute diarrhea, oral candidiasis, and HIV/AIDS [2].

In the Republic of Mozambique, child malnutrition is considered a serious public health problem as it affects more than a third of the child population. CD is responsible for one third of deaths in children under five years old. One in each seven children dies in the first year of life, and 1 in each 5 die before reaching five-years-old. The CD with an average rate of 44% is also responsible for the low productivity in the country, absence in the work, susceptibility to disease, low physical and intellectual levels [3]. Such numbers led to several procedures & policies implemented by the Government of Mozambique, which launched in 2010, an official strategy of multisector action to reduce absolute poverty in the country (PARPA).

Although specific policy priorities need to be determined at country level, many countries of sub-Saharan Africa agreed recently that much must be done in research focusing on the development of effective community nutrition interventions [4]. In this concern, there is an intense effort from the government of Mozambique & other African countries to design diets using local foods in order to reduce the prevalence of CD.

Recent reports in the country demonstrate that few information is currently available regarding the nutritional requirements for the development of a balanced diet, using native cultures, for supplementation of children affected by CD [5].

Several studies have been performed worldwide, using experimental animal models, to evaluate the effects of malnutrition in different tissues, organs and systems. Zeman [6] reported that the body weight and survival rate of female mice pups subjected to a low protein diet (6% casein) during pregnancy were lower compared to those from females fed with diet containing normal quantity of protein (24% casein) during the same gestational period.

Knowing that the nutritional status is important in determining tissue growth in organisms, it has been demonstrated that the undernourished condition can be reproduced in laboratory animals by different methods [7].

Currently, there is a lack of information available evidencing the efficacy of the diet used in rural areas of Mozambique, fact that itself justifies the present study. It is believe that the findings from this research may add information for implementation of a supplementary diet as well as improvement of the diet used in the rural areas, optimizing -protein gain, increasing the quality of life and decrease mortality in child affected by CD in various regions of the African continent.

Hence, considering the existence of a correlation between muscle development and nutrition, and knowing that malnutrition affects negatively this development, this study aimed to evaluate the morphoquantitative aspects of the development of the gastrocnemius muscle (MGc) in Wistar rats (*Rattus norvegicus*) submitted to a diet based on native plants of rural Mozambique.

2. Material and methods

2.1. Project design and sample collection

All experiments were performed in accordance with the ethical principles of the Ethics Committee of the School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil (Protocol 2223/2011).

Seventy five Wistar rats (*Rattus norvegicus*) of either sex weighing 280–320 g were mated over a period of ten days without restrictions of the specific diets utilized for each group: Nourished Group (N= Control group): composed by animals fed with AIN-93G[2] normal protein diet (20% casein); Malnourished group (D) consisting of animals receiving AIN- 93G1 hypoproteic diet (5% casein); Group of Mozambique (M) consisting of animals receiving diet of Mozambique. The water supply was "*ad libitum*" for all groups (Table 1). All animals used in this study were kept under housing conditions of the vivarium of the Institute of Biomedical Sciences (temperature of 22 ± 2 °C and light/dark cycle of 12 h), University of São Paulo, Brazil.

From the first day of the breeding the standard diet of vivarium was changed and the animals were offered *"ad libitum"* water and rodent chow diet (AIN- 93G) to animals of N and D groups and Mozambique diet to M group. All the three diets were prepared in specialized laboratory (Rhoster Industria & Commercio Ltda, Brazil).

After a mating period of 10 days the females were separated from the males and placed in individual cages according to the diet during pregnancy and lactation.

For experimental purposes, only farrow of ten animals were sampled. The body weight (g) [using digital scale (Mars model)] and size (cm) of the puppies were measured at birth and weaning (21 days). In order to eliminate hormonal variation in the result, it was decided to standardize the technique using only male offspring.

Twenty one male pups were distributed according to their diets in groups N, D and M. Each group was formed by 7 animals from which 5 animals were used for light microscopy and 2 animals for transmission electron microscopy analysis (Fig. 1).

The animals were placed in a chamber and euthanized by carbon dioxide (CO_2) inhalation. The gastrocnemius muscle (MGc) was exposed and collected by a lateral longitudinal incision in the skin of the pelvic limb. The Muscle was then fixed longitudinally by the common calcaneus tendon in a wooden and his belly surrounded by sticky adhesive (glue), and covered by a small amount of special inclusion mean for freezing biological tissues (Killik - EasyPath®, São Paulo, SP, Brazil). This procedure was important for maintaining the muscle in the desired position (longitudinal). Talc neutral powder (Tragacanth® Sigma) was added on the surface of the specimens, to prevent possible artifacts resulting from low temperature of the freezer. Posteriorly, the specimens were completely covered by the inclusion mean and immediately immersed in a stainless steel beaker containing isopentane, which was previously immersion-stored in liquid nitrogen (temperature of approximately −150 °C). The samples were then stored in a "freezer" at −80 °C (Thermo Form: −86C freezer ULT).

2.2. Light microscopy

After being removed from the freezer, the samples were initially kept in a cryostat cold chamber (Leica CM 1850) at −25 °C for approximately 30 min (considered period of adaptation to the new

Table 1
Composition of standard diets (normal protein), low protein (hypoproteic) and mozambique (M).

Diet AIN-93G	Normal (N)	Hypoproteic (D)	Mozambique (M)
Humidity	8.4%	7.8%	4.75%
Crude Protein	20% (casein)	5.2% (casein)	16.25% (vegetable)
Ether extract	7.2%	7.0%	24.16%
Fibrous Material	4.1%	5.0%	0.96%
Mineral material	2.8%	2.8%	3.31%
Calcium	0.56%	0.5%	–
Phosphorous	0.26%	0.3%	–

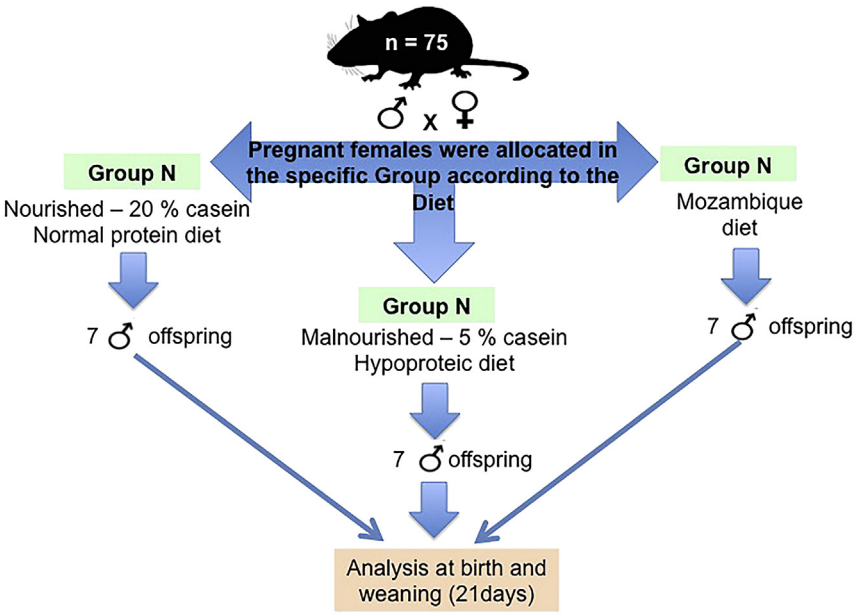

Fig. 1. Experimental design of the flowchart adopted for obtaining the three experimental Groups.

temperature). Cross sections of MGc (10 μm thick) were obtained, according to stereological criteria established by Howard and Reed [8]. The sections were later accommodated in glass slides and stored at room temperature for histology and histochemistry staining methods. Hematoxylin-eosin (HE) stain was used to illustrate the cyto-architecture of the MGc tissue [9], and Picrosirius for analysis of collagen fibers under polarized light [10].

2.3. Histochemistry

The Nicotinamide adenine dinucleotide Tetrazolium reductase (NADH -tr) technique was used to characterize different types of muscle fibers according to metabolic and functional parameters trough histochemical reaction of NADH -tr (Sigma, N8129) following the protocol established by Barnard et al. [11].

2.4. Morphometric analysis of the MGc

The NADH -tr histochemical method was used to determine the area of cross-sections of muscle fibers (ASTF) Type I (O, oxidative), IIA (GO, oxidative and glycolytic) and IIB (G, glycolytic). For each animal of each group (N, D and M) were obtained the ASTF of ten O, GO and G fiber types totalizing 50 fibers of each type. Image photographs was made through a stereoscopic microscope (Carl Zeiss MicroImaging, Stemi® SV6 model) coupled to a digital camera (Power Shot A640, Canon, Chine). Image processing was performed in computerized imaging equipment (Axiovision Rel. 4.6, Göttingen, Germany). The results were expressed in tables (average ± standard deviation). All results were analyzed using Anova and Tukey statistical tests with significance level of $p < 0.05$.

2.5. Transmission electron microscopy

For transmission electron microscopy techniques, the animals were perfused with fixative solution of Karnovsky (2% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4), immediately after euthanasia and samples with 2 mm thickness were collected from the gastrocnemius muscle (MGc). The samples were immersed in Karnovsky solution for a period of 2 h at 4 °C prior to wash in cacodylate buffer, post-fixed in osmium tetroxide (OsO4) and treated with uranyl acetate solution (0.5%). Posteriorly, the samples were immersed in propylene oxide solution, followed by dehydration in increasing ethanol series (70% to absolute alcohol) and absolute propylene oxide. After dehydration, the specimens remained for 4 h in a mixture of resin (Low Viscosity Average Embebbing Spurr's Kit, Electron Microscopy Sciences, USA) and propylene oxide in the ratio 1: 1 and kept under stirring at room temperature for gradual infiltration of the resin material. Then, the mixture was replaced by pure resin, where the specimens remained for 12–18 h under stirring at room temperature. Dried samples remained in the resin mixture and propylene oxide per 1 h followed by replacement in pure resin, where they remained for 1 h in an oven at 37 °C for total evaporation of the fluids. The inclusion mean was performed was performed by placing the material into rectangular molds filled with pure resin and maintained for five days in an oven at 60 °C, in order to achieve the polymerization of the block. The blocks thus obtained were trimmed in thin sections (400 nm thickness) by an ultra microtome (Ultracut R, Leica Microsystems - Germany) and were subsequently stained with toluidine blue 1% for verifying the integrity of the material under light microscopy. Subsequently, the selected area of interest were sectioned in ultrathin sections (40 nm thick) by an ultramicrotome, which were harvested in appropriate 200 mesh copper screen (Sigma-Aldrich, USA) and counterstained with uranyl acetate solution (4%) for 3 min, washed with distilled water, counterstained with aqueous lead citrate (0.4%) for 3 min and again rinsed in distilled water (Reynolds, 1963).

The slides were analyzed and photographed in a transmission electron microscope (FEI Morgagni 268 (D FEI) Netherlands) at Department of Anatomy, Faculty of Veterinary Medicine and Animal Science, University of São Paulo (VCI - Anatomy/FMVZ -USP).

3. Results

3.1. Weight and size

Table 2 shows the data of the weight and size of the animals at birth and weaning (21 days). There was a significant difference

between groups ($p < 0.001$). At birth, the animals of the Mozambique group exhibit higher weight compared to nourished and malnourished groups. However, the animals of nourished group weighed more than malnourished animals. At 21 days the nourished animals were the heaviest, followed by the Mozambique and finally malnourished animals. Table 2 also shows significant difference ($p < 0.001$) relatively to the length at birth and at 21 days. At birth, animals of Mozambique exhibit similar length with malnourished animals, both smaller than the length of nourished animals. At 21 days there was a significant difference between groups ($p < 0.001$); the nourished animals were larger in length, followed by Mozambique and finally by malnourished animals.

3.2. Histology

The muscles from the animals of the three groups (N, D and M) showed structural differences. In group N, muscle fibers were polygonal-shaped, with numerous nuclei heavily stained and crowd to the periphery of the cell. A well-defined relatively thick endomysium gave a compact aspect to the sample (Fig. 2A). The animals of group D showed sparse cells exhibiting different shapes and sizes: from small round to oval or even irregular-shaped; their nuclei even crowded to the periphery were slightly more elongated and less stained compared to the N group. The endomysium was very thin and difficult to be visualized in certain cells that furthermore lost their contours (Fig. 2B). The muscle fibers of group M were fashioned in a similar pattern of the group N although the cells reveal round-shaped contours with the peripheric very well defined; however, the enlarged nuclei were less stained compared to the group N. The endomysium, even well organized, was thinner than the one presented in the nourished animals (Fig. 2C). The Picrosirius Stain highlighted the perimysium formed almost exclusively by type I collagen fibers (red, orange and yellow colours) in the animals of group N (Fig. 2D). In D and M groups, however, the perimysium showed predominance of type III collagen fibers (green colour) (Fig. 2E and F, respectively).

3.3. Ultrastructural analysis (MET)

Under the MET, the muscles of the animals of the group N revealed a well defined morphology with myofibrils and Z lines well aligned; the spaces between myofibrills were preserved and contained sarcoplasmic organelles, such as mitochondria. The ratio of the width between the bands A and I was approximately 3:1 (Fig. 3A and B). In the group D were observed myofibrils broken or with misaligned Z lines. There was a slight increase of the A band and a significant increase of the band I (Fig. 3C–E). Broken myofibrils and misalignment of the Z lines was also observed in the animals of group M, as well as, enlarged spaces between myofibrils some of them filled with large mitochondria (Fig. 3F and G). Group M also revealed the bands A and I of the sarcomere similar to the Group D, with an approximate ratio of 2:1 between the bands A and I.

3.4. Cross-sectional area of muscle fibers (ASTF)

The results obtained for cross-sectional area of muscle fibers (ASTF) *glycolytic*, *oxidative* and *glycolytic-oxidative* of different groups indicated that the M group presented the lowest ASTF when compared to N and D groups (Table 3). No significant difference was observed between M and D groups relatively to *oxidative* and *glycolytic-oxidative* fibers. It was noted that the ASTF of *glycolytic* fibers was higher in the group D with no significant differences in this type of fibers between the ASTF of the control (N) and the group M. For the *oxidative* fibers, the lowest ASTF was observed in the group N, whereas no significant differences were observed between the ASTF of D and M groups.

4. Discussion

Some reports have demonstrated that muscle tissue is directly affected in CD. The measurement of the DNA in muscle and liver of patients suffering from kwashiorkor confirmed that morphological changes occur mostly in the muscle than in the liver [12]. Supporting this evidence [13], concluded that in the Kwashiorkor there is a greater tissue protein depletion in muscle tissue that constitutes half of the body mass. In this study, we chose the muscle tissue since its growth and development is reportedly determined by the nutritional status of the individual [7].

Children need a greater amount of calories and nutrients for their growth and development, fact that itself makes them particularly susceptible to malnutrition. When ingested nutrients are insufficient for the functioning of the body, it uses sequentially the energy stored in the glucose of the tissues and blood, the glycogen of the liver and muscles and, finally, liver and muscle proteins.

As is well known, to face the problem of malnutrition, additionally to the ingestion of some vitamin complexes, it is essential to adopt a balanced diet in order to maintain the essential amount of water in the body and prevent weight loss [5].

Information on the long-term effects of child malnutrition still inconsistent as the time the child was subjected to malnutrition should be considered. In addition, it is known that when malnourished children are treated in time, there is a complete recovery of the liver and muscles, as well as the immune system [15,16]. However, in some cases, recovery is just impossible; especially in cases that are observed serious damage of nutrient absorption from the intestines and various degree of mental impairment, symptoms that are associated with the time that child was malnourished, the degree of malnutrition and the age at which the disease began [3].

The indicators of CD in Mozambique are weight and height for age [3]. In order to evaluate how much the diet of Mozambique would be able to change the animal body weight, and whether it would or not associated with malnutrition, were decided to measure the weight and body length during the study period. In the parameter length of the animals, our results are in accordance with the Zeman [6] findings, who reported that the body weight and survival rate of female mice pups subjected to low protein diet (6% casein) was smaller than those of females fed with diet containing

Table 2
Weight (g) and average length (cm) of the three groups at birth and with 21 days old.

		Nourished	Malnourished	Mozambique
Weight	At birth	6.61 ± 0.59	5.50 ± 0.23	7.38 ± 0.38
	21 days	59.08 ± 4.25	23.15 ± 4.04	35.49 ± 3.01
Length	At birth	5.96 ± 0.11	5.24 ± 0.04	5.37 ± 0.10
	21 days	12.80 ± 0.56	9.30 ± 0.53	10.28 ± 0.42

*Data expressed on average and standard deviation.

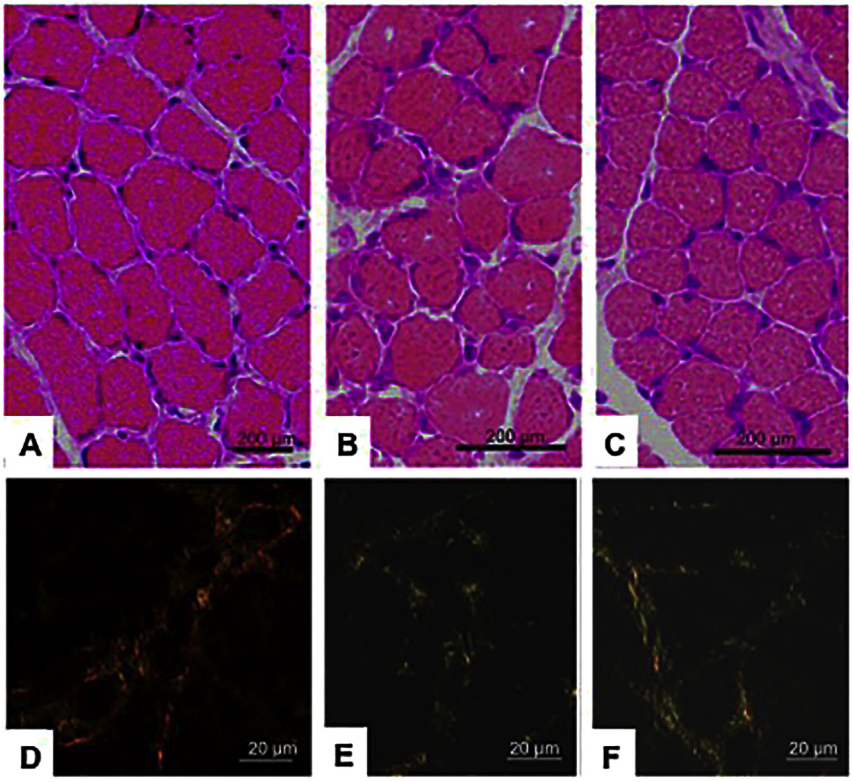

Fig. 2. **(A–C)**: photomicrographs of the gastrocnemius muscle of rats. Hematoxilin and eosin (HE) staining. **(A)** Nourished (Group C), demonstrating the polygonal aspect of muscle fibers and their dense arrangements, with regular and well defined endomysium. Note the large number of flattened nuclei. **(B)**: Malnourished (Group D), demonstrating cells of different sizes and shapes, with a spacing between them. Observe the enlarged nuclei, which were irregularly distributed. **(C)**: Mozambique (Group M), highlighting muscle fibers compacted with rounded contours. Observe the dilated peripheral few nuclei and the extremely slender endomysium. **(D–F)**: Picrosirius staining, analyzed by polarized light. **(D)**: Group N, showing the perimysium, with a predominance of collagen fibers type I (red, orange and yellow). **(E)** and **(F)**: Group D and M, respectively, showing the perimysium, with a predominance of type III collagen fibers (green).

24% casein (protein levels) during pregnancy. Additionally [15], fed rats with low percentage of protein (malnourished), and reported a reduction in weight (53% less) than the control animals of the same age, thus confirming our findings. In albino female rats fed with malnourished diet during mating, gestation and birth, the puppies weight was about 50% lower compared to the control group [17]. Based on the findings of the above mentioned studies compared to the results presented here, we can explore the possibility that the decrease of weight during growth observed in animals of Mozambique group may probably due to lack of protein in the diet similarly to malnourished animals, although the weight of the animals of Mozambique was relatively higher than the malnourished. Were believed that our results corroborate well with [18] reports who stated that “any organism once malnourished no longer have more tissues to be degraded that can reflect in a large difference in the body mass”.

Comparing the ASTF of glycolytic (G), glycolytic/oxidative (GO) and oxidative (O) fibers it was found that the animals of the M group exhibit lower diameter of the fibers compared to groups N and D. Previous studies [19] showed a decrease in muscle growth rate, size and number of muscle fibers of young rats subjected to severe protein restriction.

Histologically, the normal compacted appearance of cells with peripheral nuclei intensely stained observed in the animals of group N was fairly different from animals of group M and D, which exhibit a more round-shaped architecture. Some authors [20] have associated the round-shaped appearance and large intercellular space with muscle atrophy. A marked reduction in thickness of the fibers and a slight increase in interfascicular space was observed in the MGc of undernourished rats [21,22]. Reduction in the size of muscle fibers and increased interfascicular connective tissue in the sartorius muscle was also observed in Jamaican children aged 8–16 months of life who died due to DPC; in this children, the muscle fibers reduced to a size of fetuses aged 31th to 36th week of pregnancy [23]. Other authors [17] also found a reduction in thickness of fibers and slightly increased of interfascicular space in albino rats at 15 and 30 days of post weaning life. The endomysium of the cells of the group M of the present study were more slender than the one of N group suggesting the occurrence of a change in the size; However the most remarkable sign was observed in the thickness of the perimysium which increased significantly suggesting edema [18]. Edematous infiltration in the interfascicular septa and muscular atrophy were also reported by Ref. [24] who investigated the histology of the deltoid and trapezius muscles of children suffering from kwashiorkor. Increased space of the perimysium filled by connective tissue in some places was also observed by Ref. [25], who reported pathological processes involving muscles resulting in a regression toward an embryonic state of the protoplasmic tissue with proliferation of interstitial tissue. The decrease in the diameter of the fibers with a loss of cell borders and hence presenting irregular contours observed in groups M and D of this study corresponds to previously reports [17], in which becomes clear that during protein deprivation can occur during intrauterine life a delay in the differentiation and development of muscle fibers and the second motor neuron concurrently in the interstitial tissue, which causes diffuse masses of monocytes in interfascicular space and occasionally also in the fascicles themselves. Our evaluation under polarized light, corroborates with the

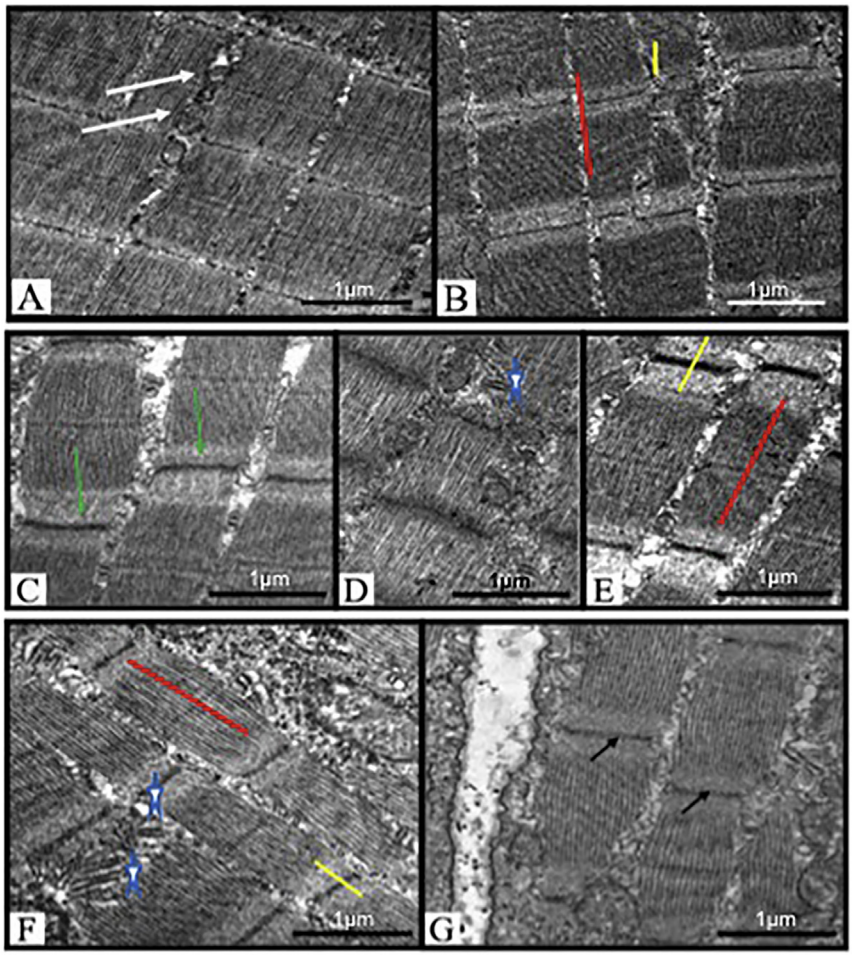

Fig. 3. Transmission electron microscopy of the gastrocnemius muscle of rats. **(A** and **B)**: Group N, showing well aligned myofibrils and Z lines, interfibrillar spaces preserved with sarcoplasmic organelles. Mitochondria (white arrows). **(C**–**E)**: Group D, denoting misalignment of Z lines (green arrows), with rupture of the myofibrils (star blue outline). Note slightly increases of band A and a significant increase of Band I. **(F** and **G)**: Group M, showing misalignment of the Z lines (black arrow) and rupture of the myofibrils (star blue outline). Observe different sizes of mitochondria in intermyofibrillar space and appearance of the bands A and I, similar to the Group D. Red Line: Band A. Yellow line: Band I.

Table 3
Cross-sectional area of muscle fibers (ASTF) (μm^2) of the gastrocnemius muscle of the three groups at birth and with 21 days old.

Type of fibers	Nourished (N)	Malnourished (D)	Mozambique (M)
Glicolitic (G)	16.3 ± 6.0	19.4 ± 2.1	10.3 ± 0.8
Glicolitic/Oxidative (GO)	8.9 ± 0.9	13.8 ± 2.1	9.2 ± 0.7
Oxidative (O)	4.4 ± 0.5	7.0 ± 0.9	7.5 ± 0.7

*Data expressed on average and standard deviation.

literature [18], which determined that the prevalence of this type of fibers (type III) in animals of D and M groups are probable indicative of a delay in collagen maturation in the muscles of these animals.

The misalignment of the Z lines, loss of myofilaments and few mitochondria of D and M groups, was also reported by Ref. [17], who although not observed changes in the mitochondria of malnourished animals, admitted that this fact may have been caused by artifacts in technique. The author also ensure that such preservation of mitochondria may have occurred because of the intensity and the short period of protein deprivation (puppies with 15 and 30 days of life), similarly to the experimental groups of our study that consisted of puppies of 21 days. The results found in the literature about the ultrastructure of muscle fibers corroborate with the findings of this study as it is possible to identify a large length of the band I in the animals of D and M groups, indicating an increase in sarcomere length and consequently decrease in the number of sarcomeres, features reported by many authors in cases of malnutrition.

5. Conclusions

From the results of this study along with epidemiological data and observation of local culture and traditions, it can be concluded that malnutrition brings not only social problems, but also irreversible physical effects. Structurally, the gastrocnemius muscle of malnourished animals revealed similarities with the animals who received the Mozambique diet both showing several changes that can perhaps be applied to other skeletal musculature. This results leads us to the reflection on the degree of functional impairment that is caused by protein deprivation. Knowing that the

Mozambique rural diet is made of leaves, peanut and Carbohydrates (maize, cassava, etc), were believed that the data from this study can be used in the short term, to generate changes in local culture and public policies (particularly from the Ministry of Health), seeking a supplementary diet, especially for children living in the rural areas of the country where the level of CD is high.

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