UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
DEPARTAMENTO DE BIOQUÍMICA

EFEITOS DA MAL NUTRIÇÃO PROTÉICA SOBRE O METABOLISMO DA GLICINA EM CEREBELO DE RATOS DURANTE O SEU DESENVOLVIMENTO

KARINE BRESOLIN DE SOUZA
Orientador: Prof. Dr. Marcos Luiz Santos Perry
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Introdução

Diversas mudanças tem sido observadas no Sistema Nervoso Central de várias espécies animais submetidos a dietas deficientes em proteínas. A severidade destas mudanças está relacionada com o período do desenvolvimento em que a restrição nutricional for imposta e com a duração da deficiência alimentar. Outras evidências indicam que a malnutrição imposta ao rato no período de lactação, provoca modificações morfológicas, químicas e funcionais no cérebro (29, 19).

Os níveis protéicos mais utilizados na literatura correspondem a 8% para dieta hipoprotéica e 25% para a dieta normoprotéica ou controle. O nosso laboratório adotou há vários anos este modelo de malnutrição proteica para estudar diferentes parâmetros neurobioquímicos e comportamentais (15, 25, 16).

O Sistema Nervoso Central (CNS) utiliza aminoácidos de diferentes formas, dependendo da idade, região cerebral e seu transporte. O aminoácido glicina é utilizado mais rapidamente por cultura de astrócitos que outros aminoácidos. A glicina, além de substrato energético, participa de reações de síntese sendo precursor metabólico para vários compostos, como bases púricas, creatina, serina, tripeptídio glutationa e o ácido δ-aminolevulínico. No cérebro a glicina também funciona como neurotransmissor inibitório e modulador dos receptores glutamatérgicos tipo NMDA. O metabolismo da glicina ocorre através de duas enzimas, o Sistema de Clivagem da Glicina (ou Glicina Sintase), presente em cérebro, fígado, rins e placenta, e Serina Hidroximetiltransferase. Daly et al. (7) e experimentos realizados em nosso laboratório (Fagundes et al., 2001) propõe que o Sistema de Clivagem da Glicina é a principal via de oxidação deste aminoácido.

Além da massa, o crescimento de um órgão como o cérebro pode ser acompanhado pela relação proteína/DNA, a qual pode estimar o tamanho de suas células constituintes, e também a quantidade de DNA, que pode refletir o número de células de um órgão, inclusive o cérebro. Considerando isso, Winick e Noble (23) observaram que a malnutrição protéica precoce em ratos, levava a uma redução permanente no número de células do cérebro (conteúdo de DNA), embora o seu tamanho (relação proteína DNA) não fosse afetado. Com a malnutrição protéica imposta após o desmame, o número de células permanecia inalterado e o tamanho das mesmas, diminuído. Em 1991, Azzolin et al. (25), verificaram que a concentração de DNA é maior em cerebelo de ratos normonutridos (25% de proteínas na dieta) de sete e quinze dias comparado aos animais submetidos à malnutrição protéica com mesma idade. Porém, aos vinte e um dias, este parâmetro foi maior nos malnutridos.

Os animais experimentais utilizados neste estudo foram alimentados com três tipos de dietas isocalóricas: 25% de proteínas (grupo controle); 8% de proteínas; e 8% de proteínas (sem adição de L-metionina). A metionina é necessária para o crescimento e desenvolvimento normal de mamíferos. Esse papel essencial é devido a utilização desse aminoácido na síntese de proteínas e S-adenosilmetionina, portanto o grupo de animais alimentado com dieta 8% de proteínas sem adição de metionina foi submetido a uma malnutrição mais severa.

Neste estudo nós investigamos os efeitos da malnutrição protéica sobre o metabolismo da glicina em cerebelo de ratos com 7, 21 e 75 dias de vida pós-natal, assim como determinamos a concentração de DNA nos cerebelos dos animais experimentais.

STUDY OF DEVELOPMENTAL EFFECTS OF PROTEIN MALNUTRITION ON GLYCINE METABOLISM IN CEREBELLUM OF RATS

Karine B. de Souza, Ana Maria Feoli, Adriane H. Krüger, Marcelo R. de Souza, Carolina T. Perry, Liane N. Rotta, Diogo O. de Souza and Marcos L. S. Perry.

Departamento de Bioquímica. Instituto de Ciências Básicas da Saúde-UFRGS/Porto Alegre, RS-Brazil.

Running title: Glycine Metabolism in Protein Malnourished Rat Cerebellum

Reprint requests to: Professor Dr. M. Perry, Departamento de Bioquímica/ Instituto de Ciências Básicas da Saúde-UFRGS, Rua Ramiro Barcelos, 2600, anexo, CEP 90035-003 Porto Alegre/RS, Brazil. *e-mail*: mlsperry@zipmail.com.br

Abstract

Malnutrition is a worldwide problem affecting millions of unborn and young children during the most vulnerable stages of brain development (1). All restriction of protein during the perinatal period of life can alter the development of mammalian fetus and have marked repercussions on development of the Central Nervous System (CNS). The brain is vulnerable to protein malnutrition with altered morphologic and biochemical maturation, leading to impaired functions. The focus of this study is to investigate [U-^{14}C]glycine metabolism in malnourished rats submitted to pre- and postnatal protein deprivation (diet: 8% protein with addition and without addition of L-methionine) on glycine metabolism of rats (normonourished group: 25% protein). It was observed that protein malnutrition alters oxidation to CO_2, conversion to lipids and protein synthesis from [U-^{14}C]glycine in cerebellum of malnourished rats without addition of L-methionine on a diet at 7 and 21 days of postnatal life. Our results also indicate that protein malnutrition causes a retardation in the normally ordered progression of brain development, and the malnourished groups have smaller cells, reduction in cell numbers and smaller cerebellar weight comparing to the control group.

Key words: Protein Malnutrition, Glycine Metabolism, Cerebellum.

Introduction

There is no longer any doubt that a quantitative deficit or a qualitative unbalance of food intake produces alterations in the nervous system ontogeny and function (2). Several ontogenic steps of brain development, including neuronal proliferation and migration, brain growth spurt and myelination are altered by nutritional insults, resulting in long-lasting or even permanent deleterious effects (4, 5).

In precocial animals (such as chick and guinea pig), the cerebellum is well developed at birth, whereas in atricial animals (such as rodents and man), which are helpless at birth, cerebellum is immature and its histogenesis and morphogenesis mainly occur during postnatal life. At birth (which occurs after a 21-day gestation) only around 3% of cells found in adult cerebellum are already present (2).

The glycine amino acid is a metabolic precursor for the synthesis of various low molecular weight compounds like purine bases, creatine, serine, tripeptide glutathione (γ-glutamylcysteinylglycine) and δ-aminolevulinate. In the CNS it serves additional functions as an inhibitory neurotransmitter and a modulator of NMDA receptors (5, 6). Although glycine metabolism in the brain remains to be clarified, there are at least two enzymes detected in astrocytes: the Glycine Cleavage System (GCS) (E.C. 2.1.2.10) (7) and the Serine Hydroxymethyltransferase (E.C. 2.1.2.1) (8). Efforts to detect these enzymes in neurons were not successful. Astroglial cells can degrade glycine by the GCS (9, 10). This enzymatic system is apparently lacking in neurons as it can not be detected either immunohistochemically (9) or by the generation of radioactively labeled CO_2 from [^{14}C]glycine in neuronal cultures (10). Sato et al. (9) have shown that the GCS in CNS

occurs only in astrocytes. Dringen et al. (11) demonstrated that astroglial cells are able to synthesize creatine, serine and glutathione using glycine as precursor. Bixel et al. (4), in a study of branched-chain amino acid and glycine consumption by astroglia-rich rat brain cell cultures, have found that the consumption half-life was 8 days of culture for leucine and 16 days for valine, while glycine was fully cleared from the culture medium within 4 days.

Methionine, required for the synthesis of S-Adenosylmethionine (SAM). In the lack of methionine the protein synthesis and SAM formation can be affected. SAM is essential for the synthesis of glutathione and participates in the synthesis of many compounds that contain methyl groups (12).

Glycine was the amino acid more oxidized in cerebellum (13), which is a structure much susceptible to malnutrition and its development occurs mainly during postnatal life (2). Based on the data available this study was made to evaluate the effects of protein malnutrition on the development of the glycine metabolism and DNA concentration in rat cerebellum.

Materials and Methods

Chemicals: Chloroform, formic acid and methanol were obtained from Merck SA, Porto Alegre, Brazil. Hyamine hydroxide was purchased from J. T. Baker Chemical Company, Phillisburg, NJ, USA, and [U-^{14}C]glycine was from Amersham International (Berkinghamshire, UK).

Animals: Albino Wistar rats were obtained from Departamento de Bioquímica / UFRGS, and were maintained at 22°C / 12 h light-dark cycle. Female Wistar rats were fed with

diets containing 25% or 8% protein (with or without addition of L-methionine) during pregnancy and lactation. Litter size was limited to 8 pups per mother.

Diets: 1) 25% protein (control group), 2) 8% protein and 3) 8% protein without addition of L-methionine. Isocaloric diets, salts and vitamins as recommended by the Association of Official Analytical Chemists (14) and previously described by our group (15, 16) were used, and water *ad libitum.*

For the measurement of oxidation to CO_2, incorporation to lipids and protein synthesis from [U-^{14}C]glycine 7, 21 and 75-day-old rats were used and killed by decapitation. The cerebellum were isolated, weighed and cut into 0.3 mm slices using a McIlwain tissue chopper. Between 50-60 mg of tissue slices were incubated in: 1.0 ml Krebs Ringer bicarbonate buffer, pH 7.4 + 5m*M* glucose + 0,2 m*M* glycine + 0.5 uCi [U-^{14}C]glycine. Incubations were carried out in flasks after contents were gassed with a 95% O_2: 5% CO_2 mixture for 30 seconds and then sealed with rubber caps. The slices were incubated at 35°C during 1 hour in a Dubnoff metabolic shaker (60 cycles/min) according to the method of Dunlop et al. (17). Adding 0.2 ml 50% TCA through the rubber caps the incubation was stopped. Then 0.2 ml of 2*M* NaOH was injected into the central wells. The flasks were shaken for further 30 minutes at 35°C to trap CO_2. After, the contents of the central wells were transferred to vials and assayed for CO_2 radioactivity in a liquid-scintillation counter. The flask contents were homogenized, transferred to tubes and were washed three times with 10% TCA. The lipids were extracted with chloroform-methanol (2:1). The chloroform-methanol phase was evaporated in vials and the radioactivity was measured. The precipitate resulting after washing with chloroform: methanol (2:1) was dissolved in concentrated formic acid and radioactivity was measured. This radioactivity

represents protein synthesis from [U-^{14}C]glycine. All the results were expressed considering the initial specific activity of the incubation medium. The CO_2 production rate as well as the incorporation of radioactivity into lipids and protein was constant through 30, 60 and 90 minutes of the incubation period. The protocol concerning this research was used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil. The DNA concentration was determined by the method of Burton (18). Data were analyzed statistically by ANOVA and by the Duncan multiple-range test and by the Student's t-test when indicated, with the level of significance set at $p < 0.05$.

Results

In this study we investigated the effects of protein malnutrition on oxidation to CO_2 , incorporation to lipids and protein synthesis from [U-^{14}C]glycine in 7, 21 and 75-day-old rats cerebellum. As shown in figure 1, at 7 and 21days of postnatal life the malnourished group without addition of L-methionine had [U-^{14}C]glycine oxidation was significantly higher than the control group, as well as the conversion to lipids and proteins synthesis from [U-^{14}C]glycine at this ages (fig. 2 and 3). However the malnourished group with addition of L-methionine presented no difference from the control group in all ages studied.. And in the adult rats (75 days) there was no difference between the three groups on these parameters.

As it can be observed in table 1, all groups at all ages studied have cerebellar weight statistically different, and the degree of malnutrition was inversely proportional to the cerebellar weights at all ages studied.

As observed in figure 4, at 7 days of postnatal life both malnourished groups had the DNA concentration two times smaller than the control group. At 21 days old the DNA concentration in the malnourished group without addition of L-methionine is two times higher than the control group. The malnourished group with addition of L-methionine was statistically higher than the control group and smaller than the malnourished group without addition of L-methionine. And at 75 days the DNA concentration in the malnourished group without addition of L-methionine is higher than the control group and the other malnourished group. At this age the malnourished group with addition of L-methionine has no difference to control group.

Discussion

In recent work of our group (13) we measured the CO_2 production from glycine using [1-^{14}C]glycine because the GCS releases the radioactive marker at carbon 1 as CO_2. We have previously showed that this cleavage is about 80 times greater than the oxidation of [2-^{14}C]glycine in 14-day-old rat cerebellum and the main route of glycine oxidation in many brain regions of rat CNS was the GCS. The contribution of glycine oxidation through serine (by Serine Hydroxymethyltransferase) is practically insignificant in various CNS regions of 14-day-old and adults rats (60 days) (7, 13). Accordingly, we can conclude that in our study the glycine oxidation was almost exclusively through the GCS.

In order to synthesize neutral lipids, glycine must be previously converted to serine (by Serine Hydroxymethyltransferase), which then could sequentially produce pyruvate, acetyl-CoA plus CO_2, and finally neutral lipids and CO_2. Fagundes et al. (13) showed that the lipids synthesized from glycine and serine in CNS were predominantly phospholipids, and Rotta et al. (20) found that glycine was the poorest substrate to lipid synthesis in CNS and this could be explained by the fact that the acetyl-CoA synthesis from serine has no physiological significance.

As observed at 7-day-old rats, independent of group studied, the glycine metabolism is higher than in the other ages. Rotta et al. (20) found that with the increase of animal age the glycine oxidation decreased contributing to maintain the ammonia at non-toxic levels. Fagundes et al. (13) observed in 14-day-old rat cerebellum a higher protein synthesis and conversion to lipids, and this parameters decrease with increase of animal age, this was due to the fact that this region presents a basically post-natal development, and the period of most rapid cerebellum growth occurs between 0 and 15 days of life (19).

It is important to refer that the postnatal development of cerebellum involves both neuronal and astrocytic cell proliferation (2). Rotta et al. (20) found that the great decrease of the glycine oxidation (astrocytic) with increase of animal age probably it is not due to changes of neuron/astrocytes ratio, but could be due to the appearance of some putative enzymatic inhibitor or to a decrease of the enzyme levels.

Animals subjected to prenatal malnutrition alone have been shown to have a 15% reduction in total brain cell number at birth (22). Interestingly, animals subjected to purely postnatal malnutrition show a similar 15-20% reduction in cell numbers at

weaning (23). On the other hand, animals deprived both prenatally and postnatally show a 60% reduction in total brain cell number by weaning (24), like in the present study. Thus, the latter produces a significantly greater effect than the sum of effects produced during either the pre- or postnatal period of cell proliferation (3).

Azzolin et al. (25) found that the cerebellar DNA concentration was higher in normally fed 7- and 15-day-old rats than in malnourished rats of the same age, whereas at 21 days of postnatal life it was higher in the malnourished rats, this shows a delay in cellular division in the cerebellum of rats fed with a low-protein diet. In agreement, the present data on the DNA concentration in the control group at 7 days (fig. 4) was two times higher than both of the malnourished groups, this was probably because the malnourished groups presented higher velocity of cellular division compared to control group at the same age causing a smaller cell number in this groups. At 21 days of postnatal life the malnourished groups showed a high DNA concentration compared to the control group, a possible explanation of this is that the cells of the malnourished groups are smaller. The same is observed in the malnourished group without addition of L-methionine at 75-day-old which showed a higher DNA concentration comparing to the control group and the malnourished group with addition of L-methionine.

In experiments of our research group Azzolin et al. (25) observed that protein malnourished rats (8% protein on a diet) had lower body weights on the day of birth. In agreement with the present paper (table 1) the malnourished groups have smaller cerebellar weight compared to the control group, the greater the malnutrition was, the smaller was the cerebellar weight.

Zamanhof et al. (26) found reduced cell division in all fetal malnourished organs studied, including the brain, and similar changes were seen in the placenta itself. Prenatal / postnatal malnutrition acts delaying cell division with prolongation of total cell cycle time. Studies by Shimada et al. (27) showed that prenatal malnutrition significantly extended the generation time of matrix cells in mice which presented malnutrition, resulting in a 14% prolongation of the generation time of these neuron precursor cells, thus resulting in a decreased production of neurons. In this study we have show clearly that protein malnutrition has caused modifications in velocity of cerebellar cellular division, causing a diminution on cells number and on cells size.

The protein malnutrition can alter the activity of enzymes and interfere with protein synthesis and protein structure and, thereby, it also interfere with the proper incorporation of lipids into various brain structures (21). The present study shows that the cerebellum is partially protected against the effects of protein malnutrition, this can explained why the malnourished group with addition of L-methionine has not shown modifications on glycine metabolism at all ages studied and it also explains the fact that the malnourished groups have not presented modifications on glycine metabolism at 75-day-old compared to the control group. However this does not mean that many specific alterations are not occurring.

Malnutrition could affect CNS development, however plasticity phenomena or compensatory mechanisms could produce functional restoration (28).

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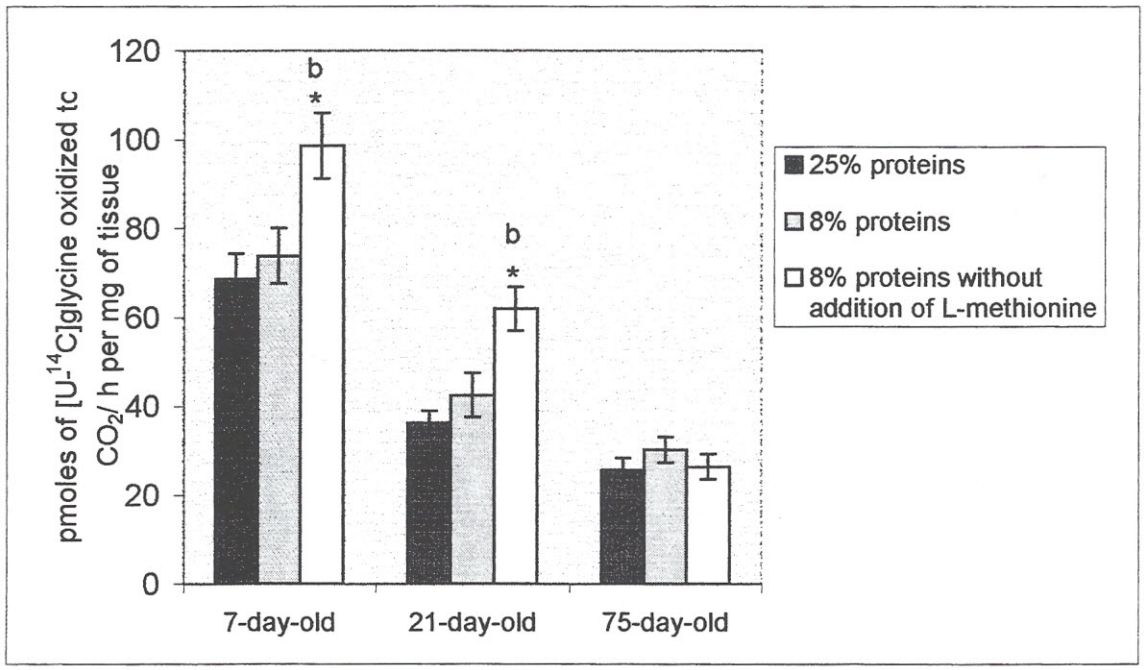

Fig.1.Oxidation of [U-^{14}C]glycine in cerebellum slices from 7, 21 and 75-day-old rats. The cerebellum were incubated with 0,2 m*M* of glycine and 0,5 µCi [U-^{14}C]glycine according to materials and methods. Values are expressed as mean ± SEM. *n* in each group is 12. Results are expressed in pmol of [U-^{14}C]glycine oxidized to CO_2. h^{-1} mg of $tissue^{-1}$. $p<0.05$.

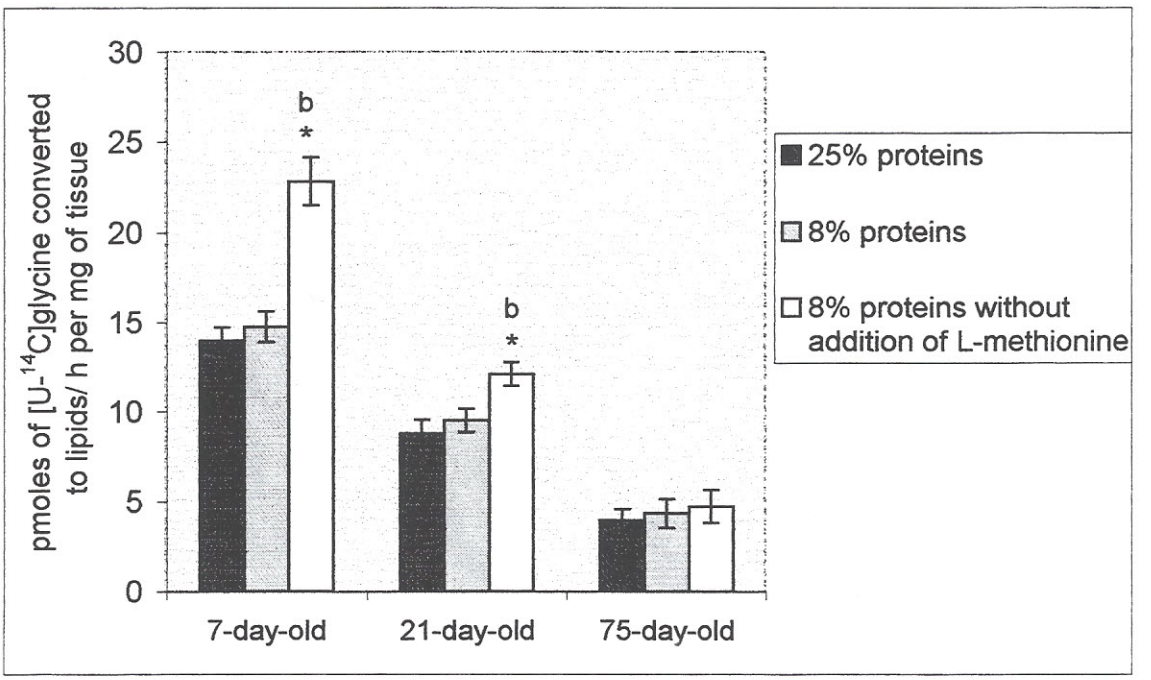

Fig.2. Conversion of [U-^{14}C]glycine to lipids in cerebellum from 7, 21 and 75-day-old rats. The cerebellum slices were incubated with 0,2 m*M* of glycine and 0,5 µCi [U-^{14}C]glycine according to materials and methods. Values are expressed as mean ± SEM. *n* in each group is 12. Results are expressed in pmol of [U-^{14}C]glycine converted to lipids . h^{-1} mg of $tissue^{-1}$. $p<0.05$.

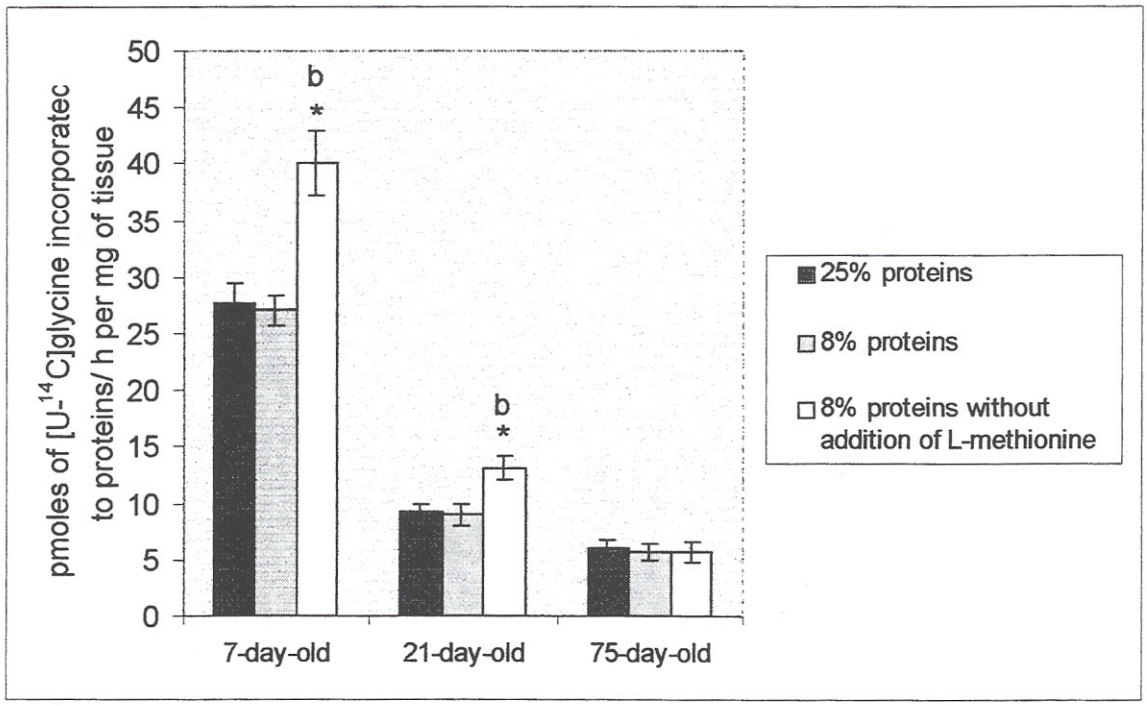

Fig.3.Incorporation of [U-^{14}C]glycine to proteins in cerebellum from 7, 21 and 75-day-old rats. The cerebellum slices were incubated with 0,2 m*M* of glycine are expressed in pmol of [U-^{14}C]glycine incorporated to proteins. h^{-1} mg of $tissue^{-1}$. $p<0.05$.

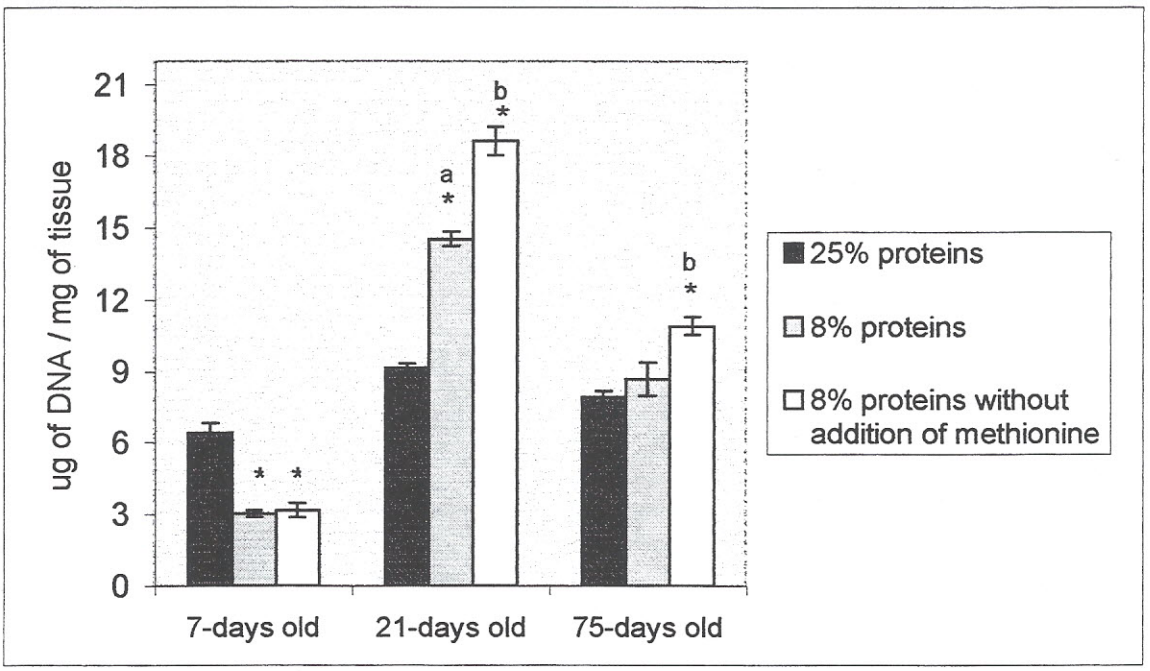

Fig.4. Concentration of DNA per mg of cerebellum of 7, 21 and 75-day-old rats. Value are expressed as mean ± SEM. *n* in each group is 18. $p<0.05$.

Table 1 –Effects of malnutrition on cerebellar weight of rats

Age days	25% protein	8%protein	8%protein without addition of L-methionine
7	64.57 ± 1.65	55.88 ± 0.74 * a	48.50 ± 0.53 * b
21	202.88 ± 2.63	188.25 ± 2.62 * a	171.00 ± 4.05 * b
75	285.14 ± 3.33	232.89 ± 4.21 * a	181.80 ± 3.10 * b

Legend:

* Statistically different from the control group (25% protein) ($p<0.05$).

a: Statistically different from the group fed with diet 8% protein *without* addition of L-methionine ($p<0.05$).

b: Statistically different from the group fed with diet 8% protein *with* addition of L-methionine ($p<0.05$).

25% protein = Control group: diet containing 25% protein (casein) *with* addition of L-methionine;

8% protein = Malnourished group: diet containing 8% protein (casein) *with* addition of L-methionine;

8% protein without addition of L- methionine = Malnourished group: diet containing 8% protein (casein) *without* addition of L-methionine.

Conclusões

- O grupo malnutrido sem adição de L-metionina (malnutrido mais severamente) apresentou alterações estatisticamente significantes no metabolismo da glicina comparado ao grupo controle, enquanto que o grupo menos malnutrido não apresentou tais diferenças. Afirmando que quanto mais severa for a malnutrição maiores serão os danos causados. Este grupo também não mostrou recuperação na concentração de DNA por peso de tecido aos 75 dias de vida pós-natal, ao contrário do grupo menos malnutrido.
- Aos sete dias de vida pós-natal os grupos malnutridos mostraram metade da quantidade de células comparados ao grupo controle (fig. 4), provavelmente devido ao atraso na seqüência de eventos do crescimento cerebral. Já aos 21 dias essa situação se inverte e os grupos malnutridos mostraram maior concentração de DNA por peso de tecido comparados ao grupo controle, devido provavelmente ao fato de as células dos grupos malnutridos serem menores. Aos 75 dias de vida pós-natal o grupo 8% de proteínas (com adição de L-metionina) mostrou uma recuperação quanto a concentração de DNA, o que não ocorreu com o grupo 8% de proteínas sem adição de L-metionina devido ao fato de este ser o grupo mais severamente malnutrido.
- O presente estudo mostrou diminuição no peso cerebelar de ambos os grupos malnutridos, em todas as idades estudadas o que confirma a diminuição no tamanho e no número das células dos animais submetidos a malnutrição protéica. Outro fato que

confirma esta afirmação é que quanto mais severa for a malnutrição protéica menor será o peso cerebelar, o que pode ser visto na tabela 1.

- Nosso estudo mostrou que quanto mais severa for a malnutrição protéica maiores serão os danos, também podemos afirmar que o cerebelo é parcialmente protegido da malnutrição protéica, o que explica o fato de o grupo malnutrido com adição de L-metionina não ter apresentado modificações no metabolismo da glicina em todas as idades estudadas, e explica também o fato de os animais malnutridos adultos não apresentarem modificações no metabolismo deste aminoácido em comparação ao controle.