Letícia de Campos Franzoni

Leucina versus isoleucina no tratamento da encefalopatia hepática: Ensaio clínico randomizado e duplo-cego

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutor(a) em Fisiopatologia em Clínica Médica

Orientador: Prof. Dr. Fernando Gomes Romeiro

Coorientador: Prof. Dr. Carlos Antônio Caramori

Botucatu
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Lista de Abreviaturas

AA – arachidonic acid

AC – arm circumference

ADP – Adenosine diphosphate

APMT – adductor pollicis muscle thickness

ATP – Adenosine Triphosphate

α-KG – α-ketoglutarate

αKGDH – α-ketoglutarate dehydrogenase complex

BCAA – Branched chain amino acids

BCKA – branched-chain ketoacids

BMI – Body mass index

Ca^{2+} – calcium

CAMA – correct mid-arm muscle area

CBF – cerebral blood flow

CO2 – Carbon dioxide

ECD – ethyl cysteinate dimer

EEG – Electroencephalogram

GHD – glutamate dehydrogenase

GLN – glutamine

GLU – glutamate

HE – hepatic encephalopathy

hGDH1 and hGDH2 – glutamate dehydrogenase isoenzymes in humans

HGS – handgrip strength

HPLC – high-performance liquid chromatography

HRQoL – higher scores indicating better health-related quality of life

LEHR – low energy and high-resolution

MAMC – The mid-arm muscle circumference

MELD – the Model of End Stage Liver Disease

Mn2 – manganese

MPT – mitochondrial permeability transition

NAD – Nicotinamide adenine dinucleotide

NADP – Nicotinamide adenine dinucleotide phosphate

NCT – numerical connection test

NH4+ – ammonia

OPA – ortho phthalaldehyde

PDH – pyruvate dehydrogenase complex

ROIs – Regions of interest

ROS – reactive oxygen species

SF-36 – Medical Outcomes Study 36-item Short-Form General Health Survey

SPECT – Brain Single Photon Emission Computed Tomography

SPM12 – Statistical Parametrical Mapping 12

TCA – tricarboxylic acid

TCLE – Termo de consentimento livre e esclarecido

TSF – triceps skinfold

ABSTRACT

Introduction: Branched chain amino acids (BCAA) are part of hepatic encephalopathy (HE) treatment and lead to brain perfusion increasing. As they are composed by three different amino acids, the proportions of each one vary in the clinical trials, making difficult to clarify which substance is more involved in brain perfusion and other significant endpoints. The aim of this study was to compare clinical and brain perfusion effects obtained by supplementation of leucine versus isoleucine for HE treatment. **Methods:** Fifty outpatients with cirrhosis and HE were randomized to receive oral supplements containing 30 g of leucine or isoleucine on a daily basis for one year. Clinical evaluations and nutritional assessment were performed bimonthly. Brain Single Photon Emission Computed Tomography (SPECT) and dynamic brain scintigraphy were performed pretreatment and at 1, 8 and 12 months of supplementation. Twenty-seven subjects concluded the study (16 taking isoleucine and 11 taking leucine). **Results:** Increasing in brain perfusion was observed only in the isoleucine group. The increase was documented at 8 months of treatment by both SPECT and brain scintigraphy ($p<0.001$ and $p= 0.05$, respectively) and by SPECT at the 12th month ($p <0.05$). It was associated with a significant HE improvement at 8 and 12 months in this group ($p=0.008$ and 0.004, respectively), which was less clear in the leucine group ($p=0.313$ and 0.055, respectively). Conclusions: Isoleucine supplementation allowed achieving a better impact on HE manifestations and brain perfusion of patients with cirrhosis. The results suggest that patients with HE should receive more isoleucine than leucine.

Keywords: Hepatic encephalopathy, liver cirrhosis, leucine, isoleucine

Introduction

Hepatic encephalopathy (HE) has a harmful impact on chronic and acute liver illnesses and affects more than one third of patients with cirrhosis. After overt episodes, the one-year mortality of these patients varies from 42 to 64%, which is even worse than the values observed in other complications such as ascites and variceal bleeding (1-3). Hyperammonaemia caused by hepatic failure is the chief disturbance in HE physiopathology, when the conversion of ammonia ($NH4^+$) to glutamine (GLN) is fully activated in extrahepatic tissues. Ammonia detoxification in the brain increases intracellular water into astrocytes due to GLN osmoregulation (4, 5). Furthermore, the high permeability of blood-brain barrier facilitates the influx of $NH4^+$ to the brain under inflammatory conditions, which worsens HE grade (6).

HE treatment is based on controlling the trigger factor that lead to neurological impairment and managing $NH4^+$ production and/or absorption from the gut, using disaccharides or antibiotics as the main medications. Since many patients do not achieve enough improving, branched chain amino acids (BCAA) and drugs aiming to increase $NH4^+$ conversion to urea are often added as adjuvant therapies, allowing some patients to accomplish a better control of HE manifestations.

Among all these medications, BCAA have the less understood mechanism of action. They are composed by three amino acids: leucine, isoleucine and valine. The role of these substances on HE treatment is well established from data of systematic reviews and meta analysis (4). Even so, the amount of BCAA and the proportion of each amino acid that patients should receive is still a matter of controversy, because all clinical trials until now used them together, making impossible to analyze the influence of each one on HE manifestations. Of note, BCAA are not only linked to HE improvement but also to increased cerebral perfusion (7).

The key disturbance in aminoacidemia related to HE is $NH4^+$ accumulation, which is present in more than 80% of patients according to the diagnostic methods adopted (8). The main $NH4^+$ source in patients with cirrhosis is the intestinal catabolism of GLN from the blood, with a lesser role of intestinal bacteria production (9-11). In normal adults, the daily amount produced is approximately 1000 mmol (12). When the conversion of $NH4^+$ to urea is insufficient due to hepatic insufficiency and/or portosystemic shunting, the excessive $NH4^+$ increases GLN production in skeletal muscles and brain (13). Since most patients with cirrhosis present moderate to severe malnutrition and consequently loss of muscle mass, the conversion of $NH4^+$ to GLN occurs predominantly in brain and this condition worsens the HE grade (14, 15).

The chief extrahepatic detoxifying process in mammals is the glutamine synthetase activity (16) and begins with glutamate (GLU) and $NH4^+$ being converted to GLN (Eq. 1). It requires GLU provided from BCAA catabolism in skeletal muscles, where BCAA and α-ketoglutarate (α-KG) can be converted to GLU and branched-chain ketoacids (BCKA) (Eq. 2) (17).

$$GLU + NH4^+ + ATP \rightarrow GLN + ADP + Pi + H^+ \quad \text{(Eq. 1)}$$

$$BCAA + \alpha - KG \rightarrow GLU + BCKA \text{ (Eq. 2)}$$

Once hyperammonaemia occurs, it causes a decrease in extracellular BCAA and a higher release of BCKA from the muscle (13). Of note, the reversible nature of these reactions makes the substrates and products concentrations the main regulators of this process (13). Until this point, the patient would have high levels of $NH4^+$, GLN and BCKA, but lower levels of BCAA in the extracellular fluid, leading to a lack of BCAA all over the body.

In this setting, GLU is more converted to GLN than to alanine, because the lack of BCAA and the increased demand for GLU (13). In theory, the reduction in alanine formation in the muscle leads to increasing in both substrates: GLU and pyruvate. In turn, pyruvate is irreversibly converted to acetyl-CoA by the pyruvate dehydrogenase complex (PDH) in the tricarboxylic acid (TCA) cycle (Figure 1).

An interesting property of PDH is that during the five reactions to form acetyl-CoA the substrates concentration is kept very high, preventing the theft by other enzymes that could need Acetyl-CoA. As PDH keeps the acetyl-CoA from pyruvate, pyruvate carboxylase would not be able to catalyze the carboxylation of pyruvate to form oxaloacetate, leading to a TCA cycle with plenty acetyl-CoA but lacking oxaloacetate (18). Once oxaloacetate deficiency occurs, pyruvate is carboxylated to produce it, but the lack of free acetyl-CoA would preclude this repair, requiring BCAA to keep the cycle working.

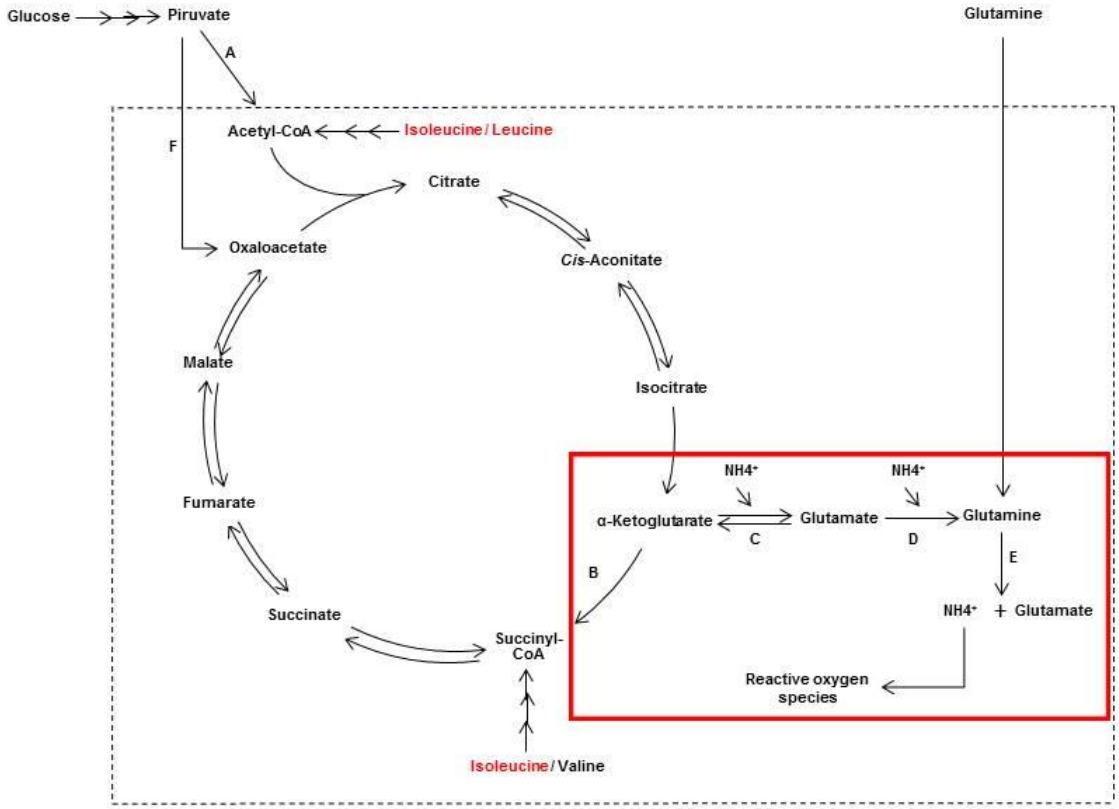

Figure 1. Schematic representation of relevant metabolic reactions related to hyperammonaemia and branched chain amino acids. **A**= Pyruvate dehydrogenase, **B**= α-Ketoglutarate dehydrogenase, **C**= Glutamate dehydrogenase, **D**= Glutamine synthetase, **E**= Phosphate activated glutaminase, **F**= Pyruvate carboxylase, **(→→→)**= Multiple steps, **(-------)**= Mitochondrial membrane. Some of the most important steps related to this study are showed in red.

Therefore, the aim of this study was to evaluate data related to HE and $NH4^+$ effects on the body, with special attention to cerebral and muscle findings, as well as the serum levels of leucine and isoleucine. The variables assessed were HE grade, quality of life, lab tests, handgrip strength measures, cerebral blood flow and cerebral perfusion of patients with cirrhosis stricken by HE who were receiving leucine or isoleucine in a randomized, double-blind trial.

Methods

Patients with cirrhosis and HE aged more than 18 years were invited to participate. Patients with other neurological diseases, hepatocellular carcinoma, prior liver transplantation or acute-on-chronic hepatic failure were excluded, as well as those who were already taking BCAA or had started/changed medications for HE treatment in the last week. The local Ethics Committee approved the study (protocol number 4334/2012), which was conducted according to the Declaration of Helsinki and its revisions. All the subjects signed the informed consent.

The diagnosis of liver cirrhosis was established from liver biopsy or from clinical and complementary exams. Clinical and nutritional evaluations were carried before the supplementation and bimonthly, including psychometric testing, anthropometric evaluation and handgrip strength measures.

HE was graded according to the West Haven criteria proposed by Amodio et al. and endorsed by the American and European Guidelines (19-21). Grade 0 comprised only subjects without clinical signals of HE. Grades I and II were categorized according to the neurological manifestations described in the current Guidelines (20, 21), documenting each finding in a specific form. Since only outpatients were recruited, those with HE Grades III and IV were not included.

Randomization plan

The subjects were randomized in a 1:1 basis. The randomization plan was obtained from a proper website, and a list for 50 patients receiving two different treatments was created (22). The amino acids packets were only marked with the legends “A” or “B” and the manufacturer was the only person who knew which amino acid the subjects were receiving, assuring that all the assessments were blinded during the trial. After defining the group that each patient would belong to, the supplement “A” or “B” was given in packets of 10 g.

Each packet contained 10g L-leucine or 10g L-isoleucine plus 5g maltodextrin, 1.8g maltitol and 1.2g of flavoring (Ajinomoto® and Basecol Mix Indústria e Comércio de Alimentos LTDA, Brazil). When the subjects started the supplementation, they received 180 packets and were reevaluated every other month to receive this same amount of supplements. The suggested schedule was to take 3 packets daily mixed in 200 mL of juice or dairy drinks ingested during breakfast, lunch and an evening snack for 12 months. At every new appointment, the adherence to treatment was checked by interviewing the subjects and counting the amount of any remaining packet. In addition, monthly phone calls were made to assure that the subjects were taking the supplement according to the trial schedule.

Before the amino acid supplementation, the subjects were submitted to clinical exams, psychometric testing, anthropometry assessment, handgrip strength measures, brain SPECT, dynamic brain scintigraphy, electroencephalogram (EEG) and lab tests. The clinical exam was especially determined on grading HE and ascites. Laboratory exams included venous $NH4^+$ and the tests needed to calculate both the Model of End Stage Liver Disease (MELD) score and the Child-Pugh classification.

Electroencephalogram

Interictal EEG was performed with a 32-channel recorder (Nihon Kohden, Tokyo, JPN). Electrodes were positioned as stated by the 10-20 international system of electrode placement. The exam was performed within 20 minutes, while hyperventilation and photic stimulation were both applied. A specialist who are not aware about the supplement given analyzed the result. Electroencephalographic signs of HE on the background activity were used to categorize the results in normal or abnormal (23). EEG was done just before the treatment in order to diagnose Grade 0 HE.

Single Photon Emission Computed Tomography (SPECT)

The subject was kept in supine position and free from audiovisual stimulus for at least 20 min before performing the brain SPECT acquisitions. Caffeine, alcohol and stimulants were not allowed since the day before the exam. Each patient received 1110 MBq (30 mCi) of 99mTc-bicisate ethyl cysteinate dimer (ECD) through a peripheral vein one hour before the acquisition. A dual-headed gamma camera (Millennium MG system, General Electric, UK) was used to acquire the images. The photopeak were centered at 140 keV with a 20% energy using low energy and high-resolution (LEHR) collimators. The acquisition was done in a 128 x 128 matrix over 360º rotation performing 65 frames of 25 seconds. Butterworth high pass filter of fifth order with cutoff frequency of 0.5 cycles/min was applied to perform the filtered backprojection reconstruction (24, 25).

A specialist who was not aware about the supplement given did the SPECT analysis. The data were examined using SPM12 (Statistical Parametrical Mapping 12, Wellcome Department of Cognitive Neurology, University College, London, UK) under Matlab 8.5 (Mathworks Inc., Sherborn, MA, USA). Each pixel was thresholded at 10% of the mean pixel intensity across all slices. SPECT scans were standardized by a 12 parameters affine

transformation in order to apply them on a template and to reformate the data to 8-bit images of 128 x 128 x 128 voxels, each 3 x 3 x 3 mm^3 voxel size. Spatially normalized images were then smoothed by a Gaussian filter (FWHM 3.0 mm). SPECT was performed at 1, 8 and 12 months of treatment.

Dynamic brain scintigraphy

Dynamic brain scintigraphy was also acquired with the patients in supine position. Immediately before the acquisition, the subjects received 1110 MBq (30 mCi) of 99mTc through a peripheral vein. Images including head, neck and proximal thorax were acquired by dual-headed gamma camera (Millennium MG system, General Electric, United Kingdom) equipped with LEHR, photopeak centered at 140 keV and 20% energy. Dynamic set of 40 frames (3 seconds per frame) were acquired in a 64 x 64 matrix in anterior view. Digital images were analyzed in MatLab 8.5. Dynamic images were visually assessed and only the first 20 images obtained from the 99mTc achieving the carotids were selected to analysis (1 minute of cerebral blood flow). Regions of interest (ROIs) from hemispheres and carotids were manually defined and applied by the operator. The size of ROIs was defined to include an entire hemisphere and each ROI was mirrored and applied to the contralateral one. ROIs of carotids constituted by 2 x 2 pixels were defined to avoid include thyroid and salivary glands.

Average count activity *versus* time curves from those regions were plotted and these curves were used to assess the initial slope of the curve, corresponding to the 99mTc arrival (26, 27). It was also performed at 1, 8 and 12 months of treatment, in the same week that the SPECT exam was done.

Nutritional assessment

Nutritional status was evaluated through anthropometric measures and handgrip strength (HGS). Anthropometric measurements collected were weight, height, arm circumference (AC), triceps skinfold (TSF) and adductor pollicis muscle thickness (APMT). Body mass index (BMI) was calculated from the dry weight divided by the square of the height [WHO, 2005]. The mid-arm muscle circumference (MAMC) and the correct mid-arm muscle area (CAMA) were obtained from AC and TSF (28-30) . Handgrip strength (HGS) from non-dominant hand was registered by a dynamometer (Saehan grip - Saehan

Corporation, SK). Three attempts were required, and the higher value was registered. The nutritional assessment was done bimonthly, at the same day that the clinical evaluations.

Quality of life survey

Quality of life was assessed using the Medical Outcomes Study 36-item Short-Form General Health Survey (SF-36) validated for Portuguese (31). The questionnaire consists of 11 questions and 36 items which comprises 8 scales subdivided into two categories: physical component summary and mental component summary. Physical component summary is composed by physical functioning, role-limitation physical, bodily pain and general health. Mental component summary is composed by vitality, sociaunctioning, role-limitation emotional and mental health. SF-36 scales ranges from 0 (lowest) to 100 (highest), with higher scores indicating better health-related quality of life (HRQoL). This survey was applied before the supplementation and at 12 months of treatment.

Serum levels of BCAA

Serum levels of leucine and isoleucine from the subjects before and after the trial were obtained by high-performance liquid chromatography (HPLC) (SCL-10Avp control system, RF-10AXL fluorescence detector, Shimadzu, JPN). Chromatographic separation was achieved on Shim-pack ISC-07/S1504 Na type sulfone group analysis column (4 mm x 150 mm i.d., 7 μm, Shimadzu, JPN). The column was operated at 25◦C. An ammonia trap column Shim-pack ISC-30/S0504 for trapping Na type ammonia also was used (4mm x 50 mm i.d.).

The aqueous mobile was Amino Acid Mobile Phase Kit, Na Phase (Shimadzu, JPN) consisting of phase A: buffer trisodium citrate dehydrate (19.6g), ethanol (55.3g), perchloric acid (60%), octanoic acid (0.1 ml) and water (0.9067 Kg), phase B: buffer trisodium citrate dehydrate (19.6g), boric acid (12.4g), sodium hydroxide (5.2g), octanoic acid (0.1 ml) and water (0.9679 Kg), phase C: sodium hydroxide (4g) and water (0.4992 Kg). The injection volume was 20 μl. Flow rate was set at 0.6 ml/min (phase A) and 0.2 ml/min (phase B and C).

The sample was diluted in NaS buffer pH 2.2 and filtered by GV Millex (Millipore™, USA) before infusion in equipment. Shimadzu LC-10A/C-47A system was applied to data analysis using ortho phthalaldehyde (OPA) derivatization method.

Statistical analysis

Descriptive analysis was presented as median and interquartile ranges. Pre-treatment comparisons between the groups were assessed by Mann-Whitney U test and the individual results throughout the trial were evaluated by the Wilcoxon U test. The software Sigma Stat 3.5 (Systat Software Inc., San Jose, CA, USA) for Windows was used for the analysis.

The assessment of brain perfusion was done using the software SPM 12. Paired *t* test was used to compare acquisitions at 1, 8 and 12 months of treatment with those before the supplementation. The anatomic visualization of the brain perfusion results from all patients of each group was obtained by a t statistic image, which was constructed and projected onto a standard high resolution T1-weighted Magnetic Resonance Image (32). The statistical significance level was defined as $p < 0.05$ for all the assays, and some additional analysis with $p < 0.01$ and $p < 0.001$ were also performed for the SPECT comparisons throughout time.

Results

Eighty-one outpatients were invited to participate. Sixty had the inclusion criteria without any exclusion criteria. Nine subjects died before starting the trial and one lost follow-up, so the supplementation began with 50 individuals (26 in the leucine group and 24 in the isoleucine group). Fifteen subjects were excluded from the leucine group (5 for adverse events such as nausea and vomiting, 5 for losing follow up, 3 for not completing the study protocol, 1 for cirrhosis complications and 1 for liver transplantation). Eight subjects were excluded from the isoleucine group (3 died, 3 for not completing the study protocol, 1 for losing follow up and 1 for liver transplantation). Thus, 11 subjects in the leucine group and 16 subjects in the isoleucine group finished the study protocol after 12 months of supplementation. The subjects' characteristics are summarized in Table 1.

Table 1 – Main characteristics of the sample (n=26)

Variables	Values	p
Men/women – leucine group	8/3	0.152
Men/women – isoleucine group	12/4	
Age – leucine group	61.00 (52.50 – 65.75)	0.767
Age – isoleucine group	57.00 (52.00 – 62.00)	
MELD – leucine group	12.00 (8.50 - 15.50)	0.535
MELD – isoleucine group p	11.00 (9.50 - 12.50)	
Child-Pugh – leucine group	8.00 (6.00 – 10.00)	0.218
Child-Pugh – isoleucine group	7.00 (6.50 – 8.50)	
HE Grade – leucine group	1 (0 – 1)	0.909
HE Grade – isoleucine group	1 (0 – 1)	
NCT – leucine group	70.00 (62.00 - 169,50)	1.0
NCT – isoleucine group	85.00 (57.50 - 154,25)	

MELD= model for end stage liver disease; HE = hepatic encephalopathy; NCT= number connection test. HE grading was performed according to the West Haven criteria. Data are expressed as median (1^{st} quartile – 3^{rd} quartile).

In the SPECT analysis, there were no differences on cerebral perfusion after 1 month of treatment, and the results obtained at 8 and 12 months of treatment were not significantly different from the baseline for patients receiving leucine supplements (non-showed data). In contrast, a significant increase in cerebral perfusion was registered in the isoleucine group at 8 months of supplementation. Comparisons between the images obtained at this time and those from the baseline were so dissimilar that additional assessments were performed to evaluate the results using $p<0.001$ (Figure 2).

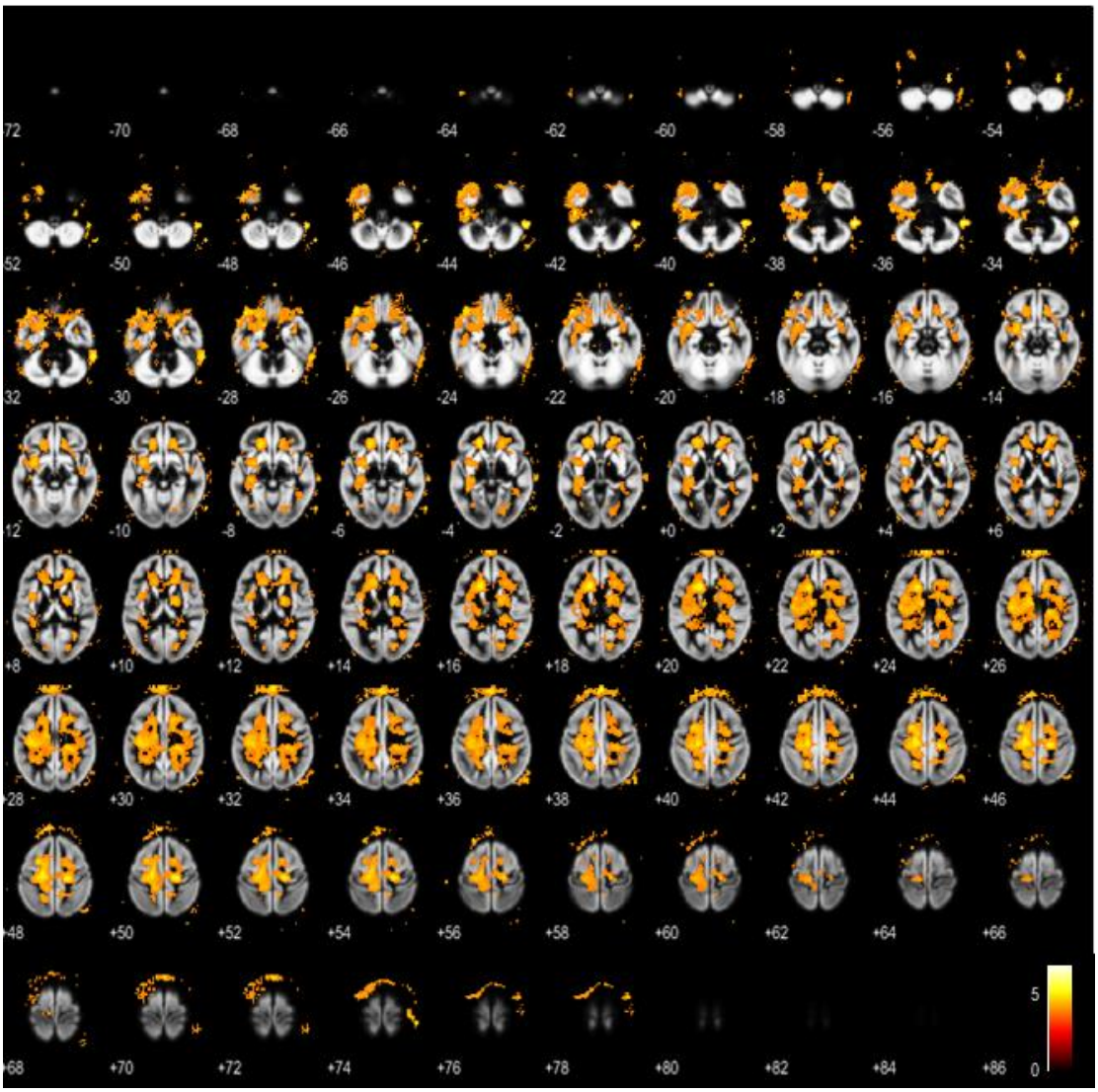

Figure 2. Voxel-wise paired *t* test comparison of cerebral perfusion between baseline and 8 months of isoleucine supplementation. Differences observed by brain SPECT are shown as color regions (346329 mm^3), depicting the areas that had perfusion increasing during the supplementation ($p<0.001$).

This significant effect of isoleucine was also documented at 12 months of treatment. The difference at this time was inferior when compared to the results obtained at 8 months of supplementation, as seen in figure 3 ($p=0.05$). These analyses pointed to which parts of the brain had the higher cerebral perfusion increase at 8 and 12 months of isoleucine supplementation, such as some basal nuclei regions.

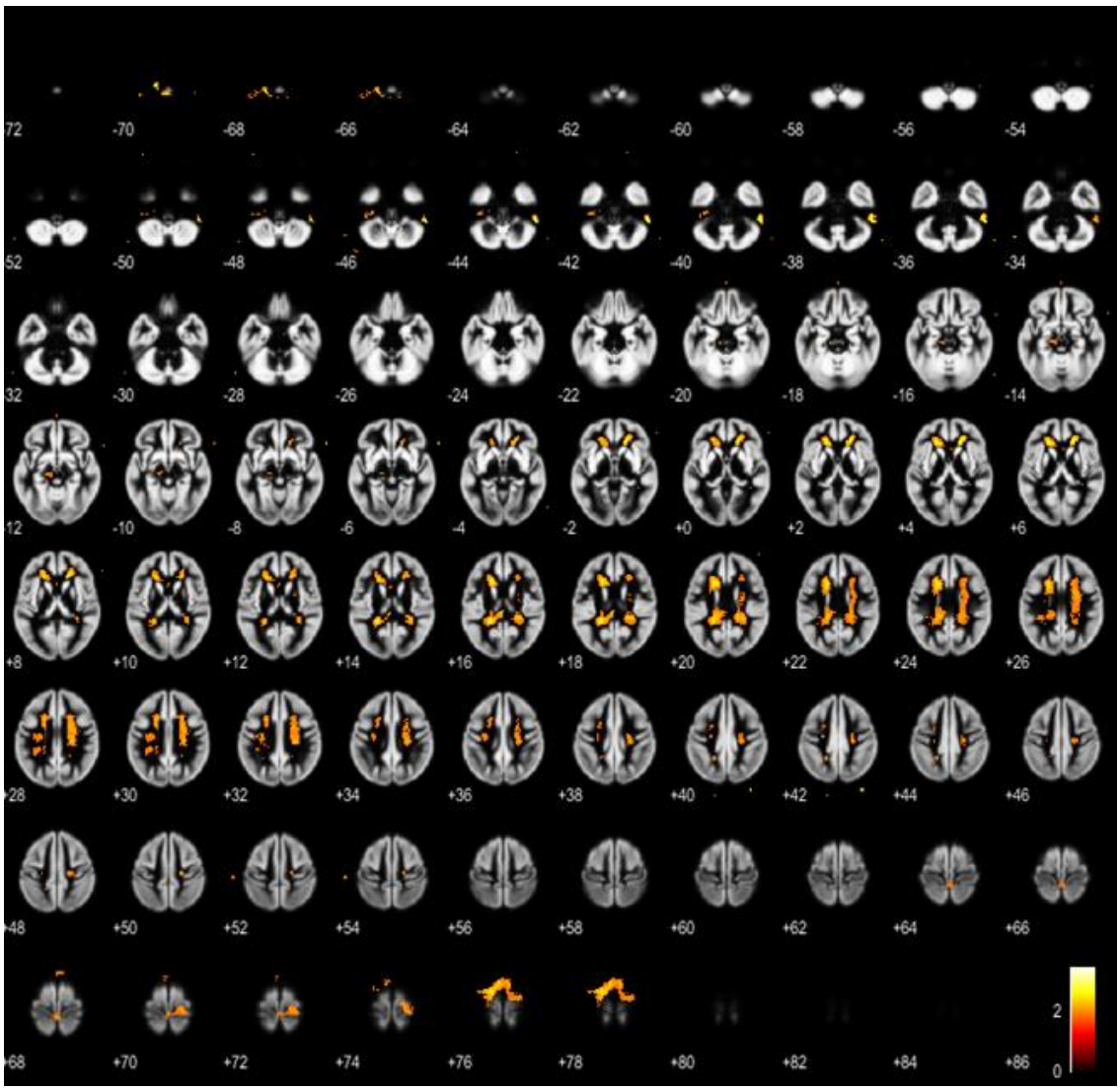

Figure 3. Voxel-wise paired *t* test comparison of cerebral perfusion between baseline and 12 months of isoleucine supplementation. Differences observed by brain SPECT are shown as color regions (48033 mm^3), depicting the areas that had perfusion increasing during the supplementation (p=0.05).

To analyze the brain scintigraphy results, the slope of the ascending curves of carotids of both sides was measured in order to quantify the cerebral blood flow. Again, the results obtained at the first month were not different from those before the trial, and the results obtained at 8 and 12 months of treatment were not significantly different from the baseline for patients receiving leucine supplements (non-showed data). On the other hand, a clear enhancement of cerebral blood flow at 8 months of supplementation was observed in the isoleucine group, which was shown by the increase of slope curves of both hemispheres at this moment. However, in brain scintigraphy analysis it did not achieve the significance level at the 12^{th} month (Table 2).

Table 2 – Dynamic brain scintigraphy analyses comparing the slope of the ascending curves obtained from carotids and brain hemispheres. The comparisons were performed between pretreatment values and the results at 8 and 12 months of supplementation

		Amino acid					
		Leucine group (n= 11)			Isoleucine group (n= 17)		
		Pretreatment	8 months	12 months	Pretreatment	8 months	12 months
CA	R	72.85 (67.46 – 73.39)	76.57 (61.08 – 79.22)	69.44 (57.37 – 73.53)	72.03 (62.94 - 76.96)	75.54 (71.22 - 81.54)	71.90 (60.24 – 79.60)
	L	71.24 (58.68 – 78.83)	72.83 (58.47 – 75.88)	63.74 (59.91 – 73.53)	66.50 (54.59 - 75.19)	70.84 (57.67 - 77.90)	71.49 (55.91 - 80.06)
BH	R	59.90 (41.13 – 65.33)	60.87 (46.48 - 63.75)	52.83 (39.83 – 57.72)	50.22 (38.78 - 61.62)	62.76 (48.66 - 75.37)*	56.42 (30.81 – 65.27)
	L	60.65 (39.80 - 64.09)	56.14 (47.17 – 63.38)	55.84 (35.17 – 61.59)	49.18 (30.78 - 56.66)	59.77 (44.86 - 73.79)#	52.01 (40.69 - 66.17)

CA= carotids; BH = brain hemispheres; R= right; L= left. Data are expressed as median (1st quartile – 3rd quartile).
*p=0.05; #p<0.029.

The clinical evaluation findings at 8 and 12 months are described in Table 3. There were no significant changes in the MELD score during the trial. However, the ability of the subjects to complete the NCT improved in both groups. Reductions in Child-Pugh classification and HE grade were also documented, but did not achieve the significance level in the leucine group. In contrast, both Child-Pugh classification and HE grade decreased at 8 and 12 months in the isoleucine group. Of note, ammonia levels did not change significantly during the trial.

Table 3 – Clinical evaluation findings and laboratory tests comparing pretreatment values with the results at 8 and 12 months of supplementation

	Amino acid					
	Leucine group (n= 11)			Isoleucine group (n= 17)		
Variables	Pretreatment	8 months	12 months	Pretreatment	8 months	12 months
MELD	12 (8.50 – 15.50)	11 (8 – 12.75)	12 (8 – 12.75)	11 (9.50 – 12.50)	11.50 (9.5 – 13)	12 (10.50 – 13.50)
Child-Pugh	8 (6 – 10)	7 (5.25 – 7.75)	7 (6.25 – 7.75)	7 (6.50 – 8.50)	6 (5 – 6.50) *	6 (5 – 7.50)
HE Grade	1 (0 – 1)	0 (0 – 1)	0 (0 – 0) §	1 (0 – 1)	0 (0 – 0) §§	0 (0 – 0) §§§
NCT	70 (62 – 169.50)	56 (51.25 – 103.50) $^{\#\#\#}$	68 (51.50 – 88) $^{\#}$	85 (57.50 – 154.25)	57 (43.75 – 88.25) $^{\#\#\#}$	54 (36 – 122.50) $^{\#\#}$
$NH4^{+}$ (μM/L)	52 (27.75 – 78.25)	66.50 (44 – 94)	77 (46.5 – 114.25)	58 (26.50 – 95.50)	55 (13.75 – 94.50)	34 (20.75 – 66)

MELD= model for end stage liver disease; Child-Pugh = Child-Pugh classification; HE = hepatic encephalopathy; NCT= number connection test; $NH4^{+}$= ammonia. Data are expressed as median (1^{st} quartile – 3^{rd} quartile). * $p<0.001$; § $p=0.055$; §§ $p=0.008$; §§§ $p=0.004$; $^{\#}$ $p=0.019$; $^{\#\#}p=0.003$; $^{\#\#\#}$ $p<0.001$.

Body assessment measures are shown in Table 4. For most nutritional variables, there were no significant differences between pretreatment values and those measured at the 8th and 12th months, whereas TSF and HGS increased significantly in both groups.

Table 4 – Body assessment measures comparing pretreatment values with the results at 8 and 12 months of supplementation

	Amino acid					
	Leucine group (n= 11)			Isoleucine group (n= 17)		
Variables	Pretreatment	8 months	12 months	Pretreatment	8 months	12 months
Weight (kg)	82.20 (69.60 - 90.00)	92.40 (78.75 - 95.45)	91.60 (80.35 - 99.80)	69.70 (59.77 - 77.72)	77.95 (69.37 - 87.30)	75.60 (69.85 - 86.67)
BMI (kg/m²)	30.14 (25.10 - 34.53)	31.46 (29.07 - 36.84)	31.57 (30.32 - 36.79)	26.04 (24.87 - 28.61)	29.69 (26.83 - 32.31)	29.39 (27.44 - 31.30)
AC (cm)	30.00 (26.75 - 34.50)	34.00 (30.25 - 36.50)	32.50 (29.50 - 35.75)	30.25 (26.62 - 32.25)	31.00 (28.37 - 33.87)	31.75 (27.00 - 32.87)
TSF (mm)	17.00 (10.00 – 19.75)	25.00 (22.00 - 32.50)*	27.00 (20.75-33.62) *	16.50 (8.50-21.00)	22.00 (17.00-30.00) *	23.50 (15.00-28.50) *
MAMC (cm)	25.67 (22.04 - 27.84)	24.15 (22.17 - 27.37)	23.88 (22.71 - 25.43)	25.54 (23.86 - 26.99)	23.70 (21.50 - 25.84)	23.88 (22.35 - 25.03)
CAMA (cm²)	42.48 (30.42 - 53.49)	39.93 (29.19 - 49.65)	36.38 (31.22 - 41.86)	41.93 (35.34 - 48.31)	34.72 (26.83 - 43.17)	35.43 (29.78 - 40.58)
APMT (mm)	6.00 (4.50 – 9.00)	8.00 (6.50 - 9.00)	9.00 (7.00 - 10.00)	5.50 (4.75 - 7.25)	7.00 (5.00 - 9.00)	7.00 (6.00 - 8.00)
HGS (kg)	18.00 (16.50-22.00)	28.00 (22.00-38.75)§	28.00 (20.50-41.50)§§	25.50 (18.00-28.50)	33.00 (26.50-40.00) §§§	32.00 (26.50-41.00) §§

BMI = body mass index; AC = arm circumference; TSF = triceps skinfold; MAMC= mid-arm muscle circumference; CAMA= correct mid-arm muscle area; APMT= adductor pollicis muscle thickness; HGS= handgrip strength; kg= kilogram, kg/m² = kilogram per square meter, cm = centimeter, mm = millimeter. Data are expressed as median (1st quartile – 3rd quartile).

* p=<0.001; § p=0.010; §§§ p=0.002; §§§ p=0.001.

Table 5 presents the results of HRQoL. The data showed significant improvement of physical functioning scale at 12 months of treatment in both leucine (p=0.030) and isoleucine (p=0.008) groups. However, other items related to physical and mental components summary did not improve under supplementation.

Table 5 – Health-related Quality of Life scores measured before and at the end of the trial

	Amino acid			
	Leucine group (n= 11)		Isoleucine group (n= 17)	
Scales	Pretreatment	12 months	Pretreatment	12 months
Physical functioning	40.00 (21.25-83.75)	90.00 (76.25-95.00) **	80.00 (50.00 - 97.50)	95.00 (90.00 – 100.00) *
Role-limitation physical	0.00 (0.00 – 50.00)	25.00 (0.00 – 100.00)	100.00 (50.00 – 100.00)	100.00 (50.00 – 100.00)
Bodily pain	100.00 (36.00 – 100.00)	84.00 (57.50 – 100.00)	84.00 (34.00 – 100.00)	100.00 (87.00 – 100.00)
General health	45.00 (26.00 - 77.00)	80.00 (39.50 - 88.50)	77.00 (51.00 - 91.00)	82.00 (68.50 - 92.00)
Physical component summary	165.00 (114.00 - 272.00)	261.00 (174.50 - 380.00)	301.00 (233.50 - 376.00)	370.00 (318.00 – 377.00)
Vitality	55.00 (20.00 - 85.00)	80.00 (37.50 - 95.00)	85.00 (55.00 - 92.50)	90.00 (82.50 - 97.50)
Social functioning	50.00 (25.00 – 100.00)	100.00 (68.75 - 100)	100.00 (73.75 – 100.00)	100.00 (87.50 – 100.00)
Role-limitation emotional	100.00 (18.75 - 100.00)	66.66 (0.00 – 100.00)	100.00 (100.00 – 100.00)	100.00 (78.00 – 100.00)
Mental health	60.00 (36.00 - 78.00)	80.00 (56.00 - 87.00)	80.00 (60.00 - 94.00)	88.00 (80.00 - 94.00)
Mental Component Summary	275.00 (96.75 - 365.50)	317.66 (169.75 - 384.00)	358.50 (299.25 – 378.00)	374.00 (303.50 – 391.00)

Data are expressed as median (1^{st} quartile – 3^{rd} quartile). Analyses of group B were performed with 15 individuals.
* p=0.030; ** p= 0.008.

Serum level analysis from subjects under leucine supplementation demonstrated significant increase of leucine (p=0.049) at 12 months of treatment, although levels of isoleucine did not change in this group. At this same time, patients under isoleucine supplementation achieved a markedly increase of both leucine (p=0.030) and isoleucine (p=0.003) serum levels, as presented in Table 6.

Table 6 – Amino acid serum levels (nM/L) measured before and at the end of the trial

	Amino acid			
	Leucine group (n= 11)		Isoleucine group (n= 15)	
Serum levels	Pretreatment	12 months	Pretreatment	12 months
Leucine	34,25 (23.12 - 45.47)	63,02 (30.21 - 120.41)*	27.94 (19.87 - 37.00)	40.14 (27.20 – 58.89)**
Isoleucine	21.21 (13.81 – 22.43)	21.70 (10.92 - 89.32)	14.39 (11.33 – 20.70)	88.52 (20.83 – 207.23) §

Data are expressed as median (1st quartile – 3rd quartile). The analyses of the isoleucine group were performed with 15 individuals because in two of them the leucine and isoleucine peaks were overlapped, precluding their quantifying. * p=0.049; ** p=0.030; §p=0.003.

Discussion

The results of this trial suggest that hyperammonaemia affects cerebral energy metabolism, in agreement to previous studies presented by Cooper and Plum (18), Ott and Vilstrup (33), Bessman and Bessman (34), Lai and Cooper (35). Furthermore, in the sample evaluated, isoleucine was able to reduce HE manifestations. The data demonstrate an increase of serum levels of BCAA at 12 months of supplementation in both groups, which could explain the gain of body assessment measures such as TSF and HGS after 8 and 12 months of supplementation.

Ammonia detoxification inside astrocytes causes an important influx of water to these glia cells due to GLN accumulation and the osmotic effect of this aminoacid (36). Concurrently, $NH4^+$ degradation into muscle cells seems to be linked to strength loss, which is proportional to HE severity (15, 37). Thus, the results of recovering strength, increasing TSF and ameliorating HE grading during isoleucine supplementation could not be explained unless considering a significant effect of BCAA on energy metabolism, thus decreasing cytotoxic brain edema and recovering muscle strength.

In the absence of kidney disease, urea production decreases $NH4^+$ levels without aminoacidemia disturbances, whereas conversion to GLN is only a temporary way to avoid toxic effects because GLN is converted to $NH4^+$ in enterocytes and other cells (17). It could lead to a vicious cycle between GLN and $NH4^+$ unless by the activity of the enzyme glutamate dehydrogenase (GDH) converting α-KG and $NH4^+$ to GLU. This enzyme is an important connection between aminoacid and carbohydrate metabolism (38). The reaction is reversible, but is another pathway to $NH4^+$ detoxifying when the substrates are increased (Eq. 3). It is also an additional source of GLU for $NH4^+$ conversion by the major detoxifying process through glutamine synthetase.

$$NH4^+ + \alpha - KG + NAD/NADP \rightleftarrows GLU \quad \text{(Eq. 3)}$$

There are two GDH isoenzymes in humans (hGDH1 and hGDH2). High levels of the first can be found in brain, liver and kidneys, whereas hGDH2 is expressed in astrocytes, testis and kidneys (38). Both isoenzymes are activated by L-leucine and ADP, especially hGDH2 (39). As the reaction is reversible, there is a debate whether GDH would favor $NH4^+$ decrease or release, because the high Km for $NH4^+$ would favor $NH4^+$ formation (40). Hyperammonaemia stimulates $NH4^+$ and α-KG degradation in the brain (41). Therefore, in astrocytes, the balance between $NH4^+$ and GLN could be also regulated by GDH activity leading to a higher formation of GLU. As hGDH2 depends on the presence of ADP and

leucine, both cofactors are important to keep the reaction generating GLU instead of $NH4^+$ and α-KG, explaining part of the benefits achieved by leucine supplementation and giving place to inquire whether isoleucine has a similar effect.

Zaganas et al. (2013) showed that the activities of hGDH1 and hGDH2 as $NH4^+$ scavengers were quite similar in high ADP concentrations, but not when this cofactor was lowered. In low ADP conditions the cooperative binding of $NH4^+$ as hGDH2 substrate increased, facilitating $NH4^+$ binding when another $NH4^+$ molecule is already linked to this multimeric enzyme. The authors mentioned that this difference of hGDH2 activity could be an advantage allowing it to work more efficiently during hyperammonaemia (38). Therefore, the reactions of glutamine synthetase (Eq. 1) and GDH (Eq. 3) have more in common than $NH4^+$ and GLU, because ADP generated by the first has a clear impact in GDH activity. In hyperammonaemia, both enzymes act in order to reduce $NH4^+$ levels: GDH provides GLU for the glutamine synthetase conversion of $NH4^+$ in GLN, a reaction that requires ATP and generates ADP. In turn, the ADP released favors more GLU production by hGDH2 in astrocytes, thus leading to more GLN accumulation in astrocyte mitochondria. Of note, both reactions requires energy.

Taken together, these findings on muscle and astrocytes metabolism suggest that $NH4^+$ toxicity is related to a disruption in cellular mechanisms involved in energy metabolism, especially in the brain. GDH activity depends on the amount of α-KG and energy from NAD/NADP provided by the TCA cycle, whereas glutamine synthetase activity requires ATP. However, excessive $NH4^+$ inhibits the α-ketoglutarate dehydrogenase complex (αKGDH) in this cycle (33), shifting α-KG to GLN production and decreasing the α-KG oxidation to SuccinylCoA and CO_2, thus contributing to curb aerobic energy production (35) (Eq. 4).

$$\alpha - KG \rightarrow succinyl - CoA + CO2 \text{ (Eq. 4)}$$

The previous step in TCA cycle is another evidence that energy metabolism is the key to understand how leucine and isoleucine can decrease HE manifestations, and it begins with α-KG formation. It is produced when isocitrate is oxidated to α-KG and CO_2 (Eq. 5 and 6), requiring a manganese ion binding to the carbonyl group before the decarboxylation. Thus, αKGDH inhibition by hyperammonaemia leads not only to GLN accumulation but also to an interruption in the TCA cycle at α-KG formation (Figure 1). In compensated cirrhosis, leucine, isoleucine and valine could provide Acetyl-CoA and Succinyl-CoA to keep the TCA

cycle running, but in advanced disease the lack of BCAA could lead to α-KG accumulation, because this substance would be no longer combined to BCAA to form GLU and BCKA in skeletal muscles (Eq. 2). Thus, the consequence to tissues that depends on aerobic processes to obtain energy, such as the brain, would be a decrease in manganese uptake at the previous TCA cycle step, leading to manganese accumulation in many regions, such as basal nuclei and cortical areas, a common finding in patients with HE and acquired hepatocerebral degeneration.

$$Isocitrate + NAD/NADP + Mn2 \rightarrow Oxalosuccinate \quad \text{(Eq. 5)}$$

$$Oxalosuccinate \rightarrow \alpha - KG + CO2 \quad \text{(Eq. 6)}$$

Since αKGDH is inhibited by unbalanced $NH4^+$ load, causing GLN accumulation, it might increase the concentration of α-KG and GLU, which is the substrate for $NH4^+$ conversion to GLN in extrahepatic tissues. Moreover, αKGDH inhibition is an additional cause to oxidative metabolism impairment in tissues that depends on aerobic processes to obtain energy, such as the brain (18, 35, 42), thus impairing ATP generation from TCA cycle. Despite the fact that GLN accumulation in astrocytes causes cytotoxic edema due to GLN osmotic effect (43), it could be not enough to explain the astrocyte swelling nor the manganese deposits in HE, which only could be explained by additional disturbances, such as metabolic impairment and oxidative stress.

Regardless the osmotic effect, increased concentration of GLN in the central nervous system is *per se* a source of toxic effects, because GLN inside the mitochondria is converted to $NH4^+$, which leads to reactive oxygen species (ROS) production, increasing mitochondria permeability and resulting in disturbances of mitochondria membrane. It is an additional source of oxidative metabolism impairment, which is known as the Trojan horse hypothesis (44-46). The oxidative stress induces mitochondrial permeability transition (MPT), increasing mitochondrial permeability pore with consequent organelle dysfunction and failure of ATP syntheses (46). Although the GLN osmotic effect is an important cause of cytotoxic brain edema, ATP depletion is responsible to a significant influx of ions and water into the astrocytes due to impairment of energy-dependent ionic pumps (47). It is considered a key component of cytotoxic brain edema in hyperammonaemia (46).

Even if brain perfusion control is not fully understood, there are important findings showing that cerebral blood flow (CBF) is regulated by astrocytes according to brain

metabolism, controlling vasoconstriction and vasodilation (48, 49). The GLU release by neurons raises intracellular Ca^{+2} in astrocytes, which active the production of arachidonic acid (AA) and vasodilators such as prostaglandins and epoxyeicosatrienoic acids. On the other hand, AA can be converted to 20-hydroxyeicosatetraenois acid in arteriolar smooth muscle, resulting in vasoconstriction. Therefore, the direction for vasodilation or vasoconstriction in the brain is mediated by metabolic conditions (48, 49).

The findings obtained in this trial suggest that astrocyte disfunction caused by hyperammonaemia is probably the cause of cerebral perfusional changes observed in HE. In the SPECT analysis, it was observed an increase of cerebral perfusion after isoleucine supplementation at 8 and 12 months of treatment as show in Figures 2 and 3. The slope of the ascending curves obtained from brain hemispheres, depicting the raise of the CBF, was an additional evidence of CBF improvement at 8 months of isoleucine supplementation (Table 2). Cytotoxic edema recovery seems to be a reasonable explanation to the increased perfusion at 8 and 12 months of treatment observed in the isoleucine group. However, it was observed a greater improvement of CBF after 8 months than 12 months of treatment (Figure 2-3, Table 2). The results of this trial are not enough to know how this effect was diminished at 12 months, and new studies are needed to elucidate this finding.

In the study of Iwasa, Matsumura (7) the authors used the same image technique applied in this study and they also observed a CBF improvement in patients with cirrhosis under BCAA supplementation. Nevertheless, all BCAA (valine, leucine and isoleucine) were administrated together, being difficult to distinguish the role of each one in this effect on cerebral perfusion.

The reason why the results from isoleucine supplementation were better than those obtained from leucine can only be understood analyzing the $NH4^{+}$ effects on TCA cycle. Isoleucine and leucine can both be converted to Acetyl-CoA, but isoleucine is also converted to Succinyl-CoA, providing additional carbon to this cycle at the step that αKGDH is inhibited by $NH4^{+}$ (42, 50-52). Due to this unique property of isoleucine, it is an ideal substrate for maintaining TCA as a source of energy even in the presence of hyperammonaemia, reestablishing oxaloacetate to form citrate from Acetyl-CoA (53). Thus, BCAA supplementation could recover the oxidative metabolism in neural cells, especially when a larger amount of isoleucine is given, because it could reduce the disruption in ion transportation through the cell membranes, maintaining the osmotic regulation and reducing the cytotoxic edema seen in HE.

In addition to this role in the TCA cycle maintenance, isoleucine is also involved in $NH4^+$ detoxification when α-KGDH is inhibited (50). This additional role of isoleucine could reduce GLN concentration in astrocytes, avoiding the cytotoxic edema. The evidence of this property of isoleucine was also given by the results obtained in this trial, because the subjects who received isoleucine presented increased serum levels of both isoleucine and leucine (Table 6). On the other hand, leucine supplementation lead to the increase only in this same aminoacid serum level (Table 6), probably due to fact that leucine only supplies Acetyl-CoA to TCA, requiring isoleucine to provide Succinyl-CoA and keep the cycle running (Figure 1) (50, 53).

In addition, both leucine and isoleucine supplementation improved quality of life related to physical functioning, probably by restoring energy generation and strength, as showed by HGS increase in both groups. It shows that some symptoms were reduced by the treatment, because almost all the subjects reported clinical improvement. Last, but not least, five subjects in the leucine group leaved the trial because they had nausea and vomiting. As it did not occurred to subjects in isoleucine group, this finding leads to the suspicion that isoleucine preparations can be more acceptable than leucine ones.

In conclusion, the results of this study showed that isoleucine supplementation allowed achieving a better impact on HE manifestations and brain perfusion of patients with cirrhosis, suggesting that when BCAA supplementation is given to them the amount of isoleucine should be larger than leucine.

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Compliance with Ethical Standards:

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: All procedures performed involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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References

1. Bustamante J, Rimola A, Ventura PJ, Navasa M, Cirera I, Reggiardo V, Rodes J. Prognostic significance of hepatic encephalopathy in patients with cirrhosis. J Hepatol 1999;30:890-895.

2. Stewart CA, Malinchoc M, Kim WR, Kamath PS. Hepatic encephalopathy as a predictor of survival in patients with end-stage liver disease. Liver Transpl 2007;13:1366-1371.

3. Jepsen P, Ott P, Andersen PK, Sorensen HT, Vilstrup H. Clinical course of alcoholic liver cirrhosis: a Danish population-based cohort study. Hepatology 2010;51:1675-1682.

4. Gluud LL, Dam G, Les I, Cordoba J, Marchesini G, Borre M, Aagaard NK, et al. Branched-chain amino acids for people with hepatic encephalopathy. Cochrane Database Syst Rev 2015:CD001939.

5. Holecek M. Branched-chain amino acids and ammonia metabolism in liver disease: therapeutic implications. Nutrition 2013;29:1186-1191.

6. Alonso J, Cordoba J, Rovira A. Brain magnetic resonance in hepatic encephalopathy. Semin Ultrasound CT MR 2014;35:136-152.

7. Iwasa M, Matsumura K, Watanabe Y, Yamamoto M, Kaito M, Ikoma J, Gabazza EC, et al. Improvement of regional cerebral blood flow after treatment with branched-chain amino acid solutions in patients with cirrhosis. Eur J Gastroenterol Hepatol 2003;15:733-737.

8. Al Sibae MR, McGuire BM. Current trends in the treatment of hepatic encephalopathy. Ther Clin Risk Manag 2009;5:617-626.

9. Nance FC, Kline DG. Eck's fistula encephalopathy in germfree dogs. Ann Surg 1971;174:856-862.

10. Weber FL, Jr., Veach GL. The importance of the small intestine in gut ammonium production in the fasting dog. Gastroenterology 1979;77:235-240.

11. Olde Damink SW, Jalan R, Redhead DN, Hayes PC, Deutz NE, Soeters PB. Interorgan ammonia and amino acid metabolism in metabolically stable patients with cirrhosis and a TIPSS. Hepatology 2002;36:1163-1171.

12. Walker V. Ammonia metabolism and hyperammonemic disorders. Adv Clin Chem 2014;67:73-150.

13. Holecek M, Kandar R, Sispera L, Kovarik M. Acute hyperammonemia activates branched-chain amino acid catabolism and decreases their extracellular concentrations: different sensitivity of red and white muscle. Amino Acids 2011;40:575-584.

14. Romeiro FG, Augusti L. Nutritional assessment in cirrhotic patients with hepatic encephalopathy. World J Hepatol 2015;7:2940-2954.

15. Merli M, Giusto M, Lucidi C, Giannelli V, Pentassuglio I, Di Gregorio V, Lattanzi B, et al. Muscle depletion increases the risk of overt and minimal hepatic encephalopathy: results of a prospective study. Metab Brain Dis 2013;28:281-284.

16. Cooper AJ. The role of glutamine synthetase and glutamate dehydrogenase in cerebral ammonia homeostasis. Neurochemical Research 2012;37:2439-2455.

17. Holecek M. Ammonia and amino acid profiles in liver cirrhosis: effects of variables leading to hepatic encephalopathy. Nutrition 2015;31:14-20.

18. Cooper AJ, Plum F. Biochemistry and physiology of brain ammonia. Physiol Rev 1987;67:440-519.

19. Amodio P, Montagnese S, Gatta A, Morgan MY. Characteristics of minimal hepatic encephalopathy. Metab Brain Dis 2004;19:253-267.

20. Vilstrup H, Amodio P, Bajaj J, Cordoba J, Ferenci P, Mullen KD, Weissenborn K, et al. Hepatic encephalopathy in chronic liver disease: 2014 Practice Guideline by the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver. Hepatology 2014;60:715-735.

21. Hepatic encephalopathy in chronic liver disease: 2014 practice guideline by the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases. J Hepatol 2014;61:642-659.

22. Dallal GE. Randomization.com. In. http://www.randomization.com/; 2013.

23. Amodio P, Marchetti P, Del Piccolo F, de Tourtchaninoff M, Varghese P, Zuliani C, Campo G, et al. Spectral versus visual EEG analysis in mild hepatic encephalopathy. Clin Neurophysiol 1999;110:1334-1344.

24. Morano GN, Seibyl JP. Technical overview of brain SPECT imaging: improving acquisition and processing of data. J Nucl Med Technol 2003;31:191-195; quiz 202-193.

25. Sunil HV, Mittal BR, Kurmi R, Chawla YK, Dhiman RK. Brain perfusion single photon emission computed tomography abnormalities in patients with minimal hepatic encephalopathy. J Clin Exp Hepatol 2012;2:116-121.

26. Matsuda H, Tsuji S, Shuke N, Sumiya H, Tonami N, Hisada K. A quantitative approach to technetium-99m hexamethylpropylene amine oxime. Eur J Nucl Med 1992;19:195-200.

27. Zaknun JJ, Leblhuber F, Schucktanz H. Value of cerebral blood flow quantification in the diagnosis of dementia. Nucl Med Commun 2008;29:260-269.

28. Callaway CW, Chumlea WC, Bouchard C, Himes JH, Lohman TG, Martin AD: Circumferences. In: Lohman TG RA, Martorell R ed. Anthropometric standardizing reference manual. Champaign: Human Kinetics, 1988; 39–54.

29. Harrison GG, Buskirk EK, Carter JEL, Ohmston JFE, Lohman TG, Pollock ML: Skinfold thicknesses and measurements technique. In: Lohman TG RA, Martorell R ed. Anthropometric standardizing reference manual. Champaign: Human Kinetics, 1988; 55-80.

30. Blackburn GL, Thornton PA. Nutritional assessment of the hospitalized patient. Med Clin North Am 1979;63:11103-11115.

31. Ciconelli RM, Ferraz MB, Santos W, Meinão I, Quaresma MR. Brazilian-Portuguese version of the SF-36. A reliable and valid quality of life outcome measure. Rev. Bras. Reumatol. 1999;39:143-150.

32. Song IU, Park JW, Chung SW, Chung YA. Differences in cerebral perfusion according to phenotypes of essential tremor: brain perfusion SPECT study using SPM analysis. Neurol Sci 2014;35:767-772.

33. Ott P, Vilstrup H. Cerebral effects of ammonia in liver disease: current hypotheses. Metab Brain Dis 2014;29:901-911.

34. Bessman SP, Bessman AN. The cerebral and peripheral uptake of ammonia in liver disease with an hypothesis for the mechanism of hepatic coma. J Clin Invest 1955;34:622-628.

35. Lai JC, Cooper AJ. Brain alpha-ketoglutarate dehydrogenase complex: kinetic properties, regional distribution, and effects of inhibitors. J Neurochem 1986;47:1376-1386.

36. Cichoz-Lach H, Michalak A. Current pathogenetic aspects of hepatic encephalopathy and noncirrhotic hyperammonemic encephalopathy. World J Gastroenterol 2013;19:26-34.

37. Augusti L, Franzoni LC, Santos LA, Lima TB, Ietsugu MV, Koga KH, Moriguchi SM, et al. Lower values of handgrip strength and adductor pollicis muscle thickness are associated with hepatic encephalopathy manifestations in cirrhotic patients. Metab Brain Dis 2016;31:909-915.

38. Zaganas I, Pajecka K, Wendel Nielsen C, Schousboe A, Waagepetersen HS, Plaitakis A. The effect of pH and ADP on ammonia affinity for human glutamate dehydrogenases. Metab Brain Dis 2013;28:127-131.

39. Shashidharan P, Clarke DD, Ahmed N, Moschonas N, Plaitakis A. Nerve tissue-specific human glutamate dehydrogenase that is thermolabile and highly regulated by ADP. J Neurochem 1997;68:1804-1811.

40. Adeva MM, Souto G, Blanco N, Donapetry C. Ammonium metabolism in humans. Metabolism 2012;61:1495-1511.

41. Faff-Michalak L, Albrecht J. Hyperammonemia and hepatic encephalopathy stimulate rat cerebral synaptic mitochondrial glutamate dehydrogenase activity specifically in the direction of glutamate oxidation. Brain Res 1993;618:299-302.

42. Bak LK, Waagepetersen HS, Sorensen M, Ott P, Vilstrup H, Keiding S, Schousboe A. Role of branched chain amino acids in cerebral ammonia homeostasis related to hepatic encephalopathy. Metab Brain Dis 2013;28:209-215.

43. Parekh PJ, Balart LA. Ammonia and Its Role in the Pathogenesis of Hepatic Encephalopathy. Clin Liver Dis 2015;19:529-537.

44. Bjerring PN, Hauerberg J, Frederiksen HJ, Jorgensen L, Hansen BA, Tofteng F, Larsen FS. Cerebral glutamine concentration and lactate-pyruvate ratio in patients with acute liver failure. Neurocrit Care 2008;9:3-7.

45. Albrecht J, Norenberg MD. Glutamine: a Trojan horse in ammonia neurotoxicity. Hepatology 2006;44:788-794.

46. Scott TR, Kronsten VT, Hughes RD, Shawcross DL. Pathophysiology of cerebral oedema in acute liver failure. World J Gastroenterol 2013;19:9240-9255.

47. Vlodavsky E, Palzur E, Shehadeh M, Soustiel JF. Post-traumatic cytotoxic edema is directly related to mitochondrial function. J Cereb Blood Flow Metab 2015.

48. Belanger M, Allaman I, Magistretti PJ. Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation. Cell Metab 2011;14:724-738.

49. Carmignoto G, Gomez-Gonzalo M. The contribution of astrocyte signalling to neurovascular coupling. Brain Res Rev 2010;63:138-148.

50. Ott P, Clemmesen O, Larsen FS. Cerebral metabolic disturbances in the brain during acute liver failure: from hyperammonemia to energy failure and proteolysis. Neurochem Int 2005;47:13-18.

51. Nissen JD, Pajecka K, Stridh MH, Skytt DM, Waagepetersen HS. Dysfunctional TCA-Cycle Metabolism in Glutamate Dehydrogenase Deficient Astrocytes. Glia 2015;63:2313-2326.

52. Holecek M. Three targets of branched-chain amino acid supplementation in the treatment of liver disease. Nutrition 2010;26:482-490.

53. Johansen ML, Bak LK, Schousboe A, Iversen P, Sorensen M, Keiding S, Vilstrup H, et al. The metabolic role of isoleucine in detoxification of ammonia in cultured mouse neurons and astrocytes. Neurochem nt 2007;50:1042-1051.