



Safety Evaluation of Branched-Chain Amino Acids in *Centropomus Viridis* Brain Cells: *In Vitro* Assay.

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ABSTRACT

Branched-chain amino acids (BCAAs), composed of L-leucine, L-isoleucine, and L-valine, are essential amino acids in the nutrition of various animal species, including marine fish. Traditionally considered precursors of protein synthesis, BCAAs also act as key signaling molecules and metabolic modulators. Although its physiological role has been widely studied, information on its toxicity and safety, especially in fish cell systems, remains limited. The species *Centropomus viridis*, due to its high dietary requirements of amino acids, represents a relevant model to explore the effects of BCAAs on brain cells in an *in vitro* environment. Immortal cultures of *Centropomus viridis* brain cells were established from juvenile specimens, following standardized protocols of enzymatic disintegration and seeding in 96-well plates. The cells were kept in L-15 medium supplemented with 10% fetal bovine serum and antibiotics, incubated at 25 °C without CO₂. The treatments consisted of exposure to BCAAs solutions (L-leucine, L-isoleucine, and L-valine) at concentrations of 50, 100, 250, 500, 1000, 2500, 5000 and 10000 µg/mL, instantly prepared from sterile stocks. Negative controls (cells without BCAAs) and positive controls were included. Cell viability was assessed using the MTT assay, adding 10µL of MTT solution (5 mg/mL) per well and measuring absorbance at 570 nm after 4 hours of incubation. Additionally, trypan blue exclusion was performed by incubating the cells with the dye for 5 minutes and counting viable and non-viable cells under light microscopy. The evaluation criteria included percentage of viability with respect to the negative control and observation of cell morphology. BCAA treatments even at concentrations of 1000, 2500, 5000 and 10000 µg/mL did not produce significant alterations in cell viability, with percentages greater than 95% compared to the negative control. No

morphological changes or increase in trypan blue exclusion were observed. At the maximum concentration evaluated (5000 and 10000 µg/mL), no decrease in viability was detected (up to 95% of live cells), without reaching criteria of significant cytotoxicity or evident morphological damage. The positive control showed a reduction in viability of less than 60%, validating the sensitivity of the experimental system. Therefore, the cellular safety limit for BCAAs in this line is above 5000 and 10000 µg/mL, with no appreciable adverse effects above this threshold. The results obtained coincide with the few previous reports in mammals, where BCAAs present low toxicity even at high concentrations. The absence of relevant cytotoxic effects in brain cells of *Centropomus viridis* suggests a wide tolerance to these amino acids, in accordance with the physiological needs of marine species with high protein requirements. However, the literature indicates a lack of controlled studies in humans and other species, which makes it difficult to extrapolate these data to broader clinical and dietary contexts. This study demonstrated that branched-chain amino acids are harmless and safe for brain cells of *Centropomus viridis* under *in vitro* conditions, even at elevated concentrations of up to 10000 µg/mL. The methodology used, based on MTT assays and exclusion of trypan blue, provides robust criteria for the toxicological evaluation of nutritional supplements in marine fishes. These findings contribute to knowledge about food safety in aquaculture and highlight the need to expand research to other cell systems and species, as well as to generate comprehensive metabolic and toxicological data to establish accurate recommendations on the use of BCAAs in animal and human feed.

KEY WORDS: BCAAS, MTT assay, Cytotoxicity. Safety and Toxicity

INTRODUCTION

Fish are the most diverse group of vertebrates, accounting for approximately 95% of all vertebrate species, with teleost making up half and inhabiting almost all aquatic environments (Nelson et al., 2006; Nelson et al., 2016; Vaktskjold, 2018). This variety is reflected in its anatomy, ecology, behavior, and genome (Volf, 2005). Positioning fish as ideal models for investigating systems related to metabolic regulation, cell growth and development in vertebrates (Volkoff et al, 2003). Scientific research has identified relevant molecular mechanisms and metabolic pathways, such as the highly conserved food intake regulation system among teleost fish and mammals (Volkoff et al, 1999). However, not all observed responses are completely comparable, largely due to the paucity of studies in fish.

Anatomical differences between fish and mammals include brain and gastrointestinal morphology, as well as the presence of specific organs in fish that do not exist in mammals (Nelson, 2006). In addition, within the fish group itself, there are anatomical and functional variations between species (Hoskins & Volkoff, 2012). For example, carnivores and omnivores have a stomach, pyloric caeca's, and short, straight intestines, while herbivores and detritivores usually lack a stomach and cecus and have long intestines (Leknes, 2015). Comparative research has focused mainly on species such as salmonids and cyprinids. As for the detection of nutrient levels in the vertebrate brain, this mechanism is essential for the regulation of food intake and energy expenditure (Berthoud & Morrison, 2008; Lieder et al 2017). Branched-chain amino acids (BCAAs), a group made up of valine, leucine, and isoleucine, perform fundamental nutritional functions such as protein building blocks and nutritional signals that induce physiological responses (Hutson et al, 2005). They account for about 20% of total protein intake, and unlike other amino acids, BCAAs bypass liver metabolism, while the other 17 amino acids are primarily metabolized in the liver. Thus, circulating BCAAs regulate protein synthesis and degradation, as well as insulin secretion. In addition, BCAAs are important energy substrates in the brain, being rapidly metabolized in neuronal cell cultures even in the presence of other substrates such as glucose (Yudkoff et al, 2005). This transamination occurs primarily in astrocytes, which release branched chain ketoacids into the extracellular fluid to be absorbed and metabolized by neurons (Garelick & Keneddy, 2011).

Valine, leucine, and isoleucine are then identified as branched-chain amino acids due to their lateral carbon skeletons, called aliphatic because they are composed only of hydrogen and carbon without benzene rings **Table 1**, (Sperringer et al, 2017). Although they have similar

structures, they exhibit subtle differences in the conformation and hydrophobia of their side chains. For example, leucine is more common in helices, while valine and isoleucine predominate in leaves, which explains leucine's involvement in helical zippers (Sperringer et al, 2017). These three amino acids have very similar three- or four-carbon side chains and molecular weights (leucine and isoleucine: 131.17 g/mol; valine: 117.15 g/mol) **Table 1**, and together they make up about 33% of the body's amino acids, concentrated especially in skeletal muscle where they act as structural elements and nitrogen stores (Murthy & Hertz 1987).

Table 1. Main physical-chemical characteristics of branched-chain amino-acids (BCAAs).

	L-Leucine	L-Isoleucine	L-Valine
CAS Registry Numbers	71000-80-1	73-32-5, 1160211-67-5	72-18-4, 7004-03-7
Molecule			
IUPAC name	(2S)-2-amino-4-methylpentanoic acid	(2S)-2-amino-3-methylbutanoic acid	(2S,3S)-2-amino-3-methylpentanoic acid
Molecular formula	C ₆ H ₁₂ NO ₂	C ₆ H ₁₂ NO ₂	C ₆ H ₁₂ NO ₂
Molecular weight	130.168 g/mol	117.148 g/mol	131.175 g/mol
Solubility in water	0.041 mg l ⁻¹	10 mg l ⁻¹	9.5x10 ³ mg l ⁻¹

Complete catabolism of BCAAs requires a series of enzymatic reactions, most of which occur in the mitochondria, giving acetyl-CoA as the product. After protein intake, BCAAs pass into the systemic circulation and more than 60% are metabolized in skeletal muscle. The first step in their peripheral metabolism is deamination by the enzyme branched-chain aminotransferase (BCAT), producing branched-chain ketoacids that can be incorporated into proteins or act as energy substrates in skeletal muscle and in the brain.

The estimated mean requirements (RAS) for leucine, isoleucine and valine are 34, 15 and 19 mg/kg/day, respectively. In the United States, the average daily intake of BCAAs is about three times higher for men and twice as high for women compared to recommended values. No studies have been reported on adverse events associated with normal diets or with supplemental doses up to 9.75 g, so a Tolerable Upper Intake Level (UL) has not been established due to a lack of dose-response data. Studies on acute or chronic oral administration of BCAAs have shown no adverse effects, even with doses up to 60 grams (Yudkoff, 2005).

There are currently few reports on the brain metabolism of BCAAs. Experiments have been conducted on primary cultures of astrocytes exposed to pathophysiological concentrations of BCAAs, where enzymes such as BCAA

transaminase and branched-chain ketoacid dehydrogenase are activated in brain mitochondria. Several studies have shown that BCAAs, individually or in mixture, can promote

signaling associated with increased mitochondrial biogenesis, including upregulation of PGC-1 α and numerous downstream targets related to mitochondrial function. However, the paucity of data in humans and the difficulty of controlling for variables in feeding studies limit the physiological relevance of these findings (Sperringer et al 2017).

REAGENTS AND MEDIAS

All procedures and protocols of this research as far as possible were carried out under the approach and guidelines of non-animal models and methods used in the field of biomedical research. the replacement, reduction and refinement of animal testing (the "Three Rs") of Directive 2010/63/EU on the protection of animals used for scientific purposes (Organization WT, 2020; Perice du Sert et al 2020). The following chemicals were all from Sigma-Aldrich (St Louis, MO, USA): MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Cat. No. M2128); methylthiazolyldiphenyl-tetrazolium bromide (CAS No. 298-93-1, Cat. No. M2128); bovine serum albumin (BSA, Cat. No. A9418). Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher, Cat. No. 11965092); Leibovitz-15 (L-15) medium (Thermo Fisher, Cat. No. 11415064); antibiotic/antimycotic solution (100 \times , Thermo Fisher, Cat. No. 15240096); 2.5% trypsin (Thermo Fisher, Cat. No. 15090046); 1 \times trypsin-EDTA solution (Thermo Fisher, Cat. No. R001100); penicillin-streptomycin (100 \times , 5000 U/ml, Thermo Fisher, Cat. No. 15070063); and fetal bovine serum (FBS, Thermo Fisher, Cat. No. 10082147) were all obtained from Gibco BRL (Grand Island, NY, USA).

Compounds and material

The BCAAS (L-valine; L-leucine; and L-isoleucine), pharmaceutical grade and purity not less than 98.5% purchased from Wuxi Jinghai Amino Acid Co., Ltd (brand: Aminowill) Co., LTD; Donggang, Wuxi, Jiangsu; China. The initial solutions were sterilized by filtration using 0.22 μ m filters (Millipore®, USA), except for those prepared in dimethyl sulfoxide (DMSO), which were sterilized after being diluted in culture medium. The preparation of the dilutions for use was carried out fresh from the initial solutions in culture medium without phenol red with 10% fetal bovine serum, without the concentration of the solvent exceeding 0.5% in any case. The Stock solutions of each amino acid individually and together were prepared at the

time of application up to a maximum concentration of 10000 mg/mL prior to experimental use.

2.2. Cell Line and maintenance

To carry out the present study, a cell line established, developed, characterized and immortalized in our laboratory from the marine fish *Centropomus viridis* was used (Avalos-Soriano et al, 2021). One vial with 1x10⁶ cell transfer 2. has certain characteristics that make it a biological model for the performance of the morphological studies carried out in this study, such as its wide and flat cytoplasm and the high density of cytoplasmic organelles. It is a cell line from the primary brain culture of *C. viridis* March 2022. They have been widely used in studies to evaluate the antioxidant capacity of agro-industrial residues and their brain origin also makes them a good, recommended model for neurotoxicological studies.

Equipment

- Laminar flow hood biosafety level 2
- CO₂-free Incubator, 26-30°C
- Inverted binocular microscope 4X, 10X and 20X objectives
- Thermostatic bath at 37°C
- Leibovitz (L-15) Culture Medium,
- Dulbecco's Modified Eagle Medium (DMEM), with and without phenol red
- Anti-Anti (10000 U/mL - 10000 μ g/mL)
- Fetal bovine serum (FBS).
- Saline phosphate buffer (PBS), pH = 7.2-7.4
- Trypsin 2.5% (p/v)
- Trypsin-EDTA (ethylenediamineacetic acid, 200 mg/L)

2. PROCEDURE

Cultures were started from a vial containing 1x10⁶ cells cryopreserved in liquid nitrogen. The cells were frozen in DMSO in fetal bovine serum with 10% (v/v) DMSO. After rapid thawing in a bath at 37 °C, the cells were seeded in 75 cm² plates with L-15 culture medium with 4.5 g/L glucose, 1% (v/v) anti-anti and 10% (v/v) fetal bovine serum, kept in an incubator at 27 °C and humidified atmosphere without CO₂. For routine cell maintenance, subcultures were performed once or twice a week, depending on the cell type, performing dilutions 1:3 - 1:5 of the starting culture. The cells were trypsinized from the culture surface using trypsin/trypsin-EDTA 0.25% (v/v) and resuspended in fresh culture medium for reseeding. In all cases, the cells used in the experiments were between the third and tenth pass after thawing. To carry out the different treatments with the compounds, exact seedings were carried out on different culture surfaces, depending on the experimental approach and the cell type used, the volumes of cell suspension necessary were determined by direct counting and by the

trypan blue exclusion method of the number of viable cells per milliliter in a Neubauer chamber.

To carry out the present work, the established Cerebral cell line of *Centropomus viridis* was obtained, and characterized previously by the laboratory molecular biology of CIAD-Mazatlán (34). The experiments were done with transfer subcultures no less than a tenth. Cultures were maintained at 27°C in a humidified without CO₂ atmosphere with medium changes every two days.

EXPERIMENTAL DESIGN

The experimental design, the biological model and the parameters to be evaluated are three factors involved in the quality and specificity of the data obtained in studies using *in vitro models* (Donato et al, 1995). In the present work, a methodological approach has been proposed in which the conditions of exposure to the compound under study have been evaluated, using a battery of tests aimed at assessing the main targets of toxicity **Figure 1**.

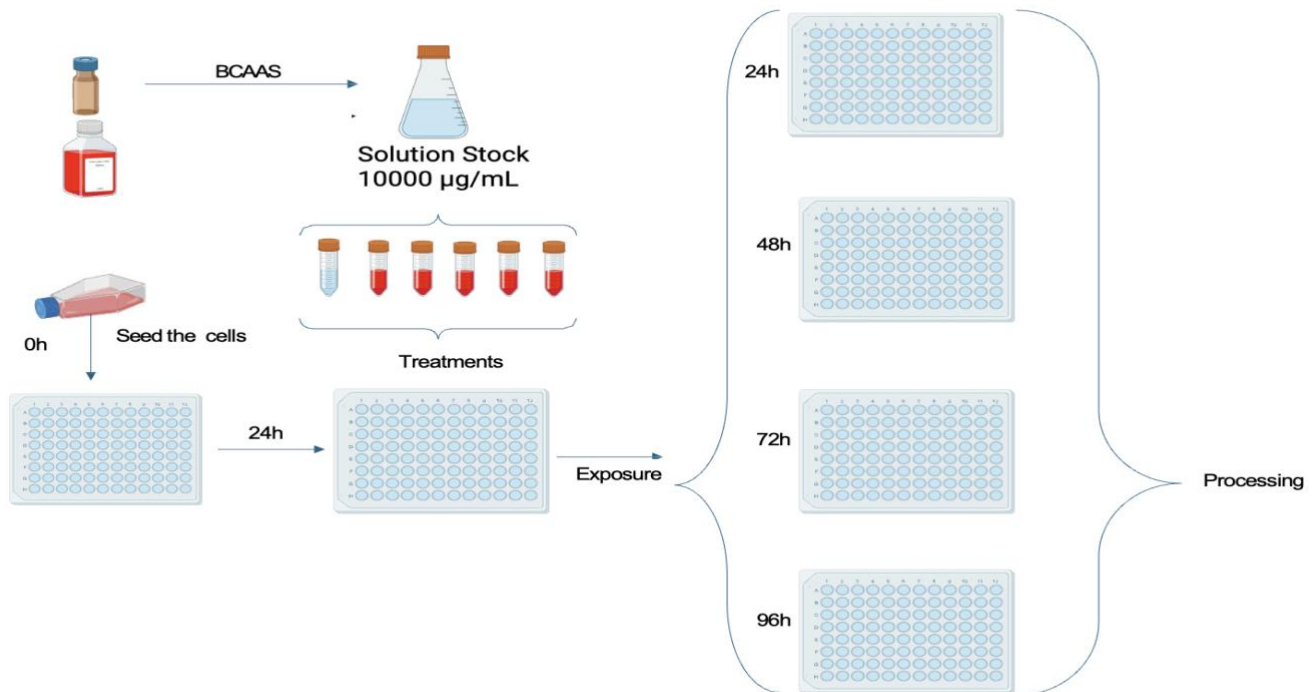


Figure 1. Experimental design of safety and toxicity studies. In methodological approaches, cells remain exposed to BCAAS treatments for the established time. After treatment, the cells are processed according to the experimental trial.

CRITICAL EXPOSURE

Cerebral cells were seeded at a density of 1×10^4 cells per well in 96-well plates (Corning Glass Works, Corning, NY, USA). For culture, Leibovitz L-15 medium supplemented with 10% fetal bovine serum (FBS) was used, constituting the basal medium (BM). Once the cultures reached the period of exponential growth, the cells were subjected to various concentrations of branched-chain amino acid treatments. The dilutions used were prepared just before use, starting from a stock solution of 10,000 $\mu\text{g}/\text{mL}$ in culture medium supplemented with 1% anti-anti and 10% SFB. During the trial, the cells remained in contact with the compounds under these experimental conditions for a period of 24 hours. At the end of the exposure period, the viability of the cells was evaluated by spectrophotometry, allowing the effect of the treatments applied to be determined (**Fig. 1**).

EXTENDED EXPOSURE

To conduct the studies by prolonged exposure (**Fig. 1**), brain cells were seeded at a confluence of 80% (1×10^4 cells/well) in a plate of 96 wells (Corning Glass Works, Corning, NY, USA) in Leibovitz L-15 medium supplemented with 10% FBS (basal medium, BM). Two days later, when the cells were within the period of exponential growth, they were exposed to the concentrations of the branched-chain amino acid treatments. Dilutions were prepared prior to application from a base solution of 10,000 mg/mL in culture medium supplemented with 1% anti-anti- and 10% SFB; After 72 hours, the contents of the wells were replaced by new culture with the corresponding concentration of branched-chain amino acids in each case. Under these conditions, the cells remained in culture for an additional 48 hours, reaching a total of 96 hours in contact with BCAAS treatments. After this exposure period, cell viability was evaluated by spectrophotometry.

Evaluation of Safety BCAAS on C. viridis Brain Cells

Evaluation of the safety of BCAAs on cerebral cells. To determine in vitro safety, the effect of BCAAs on cell viability and proliferation was evaluated.

Cytotoxicity assay by MTT

Thiazolyl blue tetrazolium bromide (MTT) was dissolved in PBS to a final concentration of 5 mg/mL and sterilized by filtration through a 0.22 μm . The MTT assay was carried out by modification of the original Mosmann (1983) (Mosmann, 1983). and protocol p29.1. MTT-Based Cytotoxicity Assay Freshney (Culture of animal cells, 2010). Brain cells (3×10^4) were seeded in a flat-bottom 96-well plate, using 10 columns in the middle of the plate (80 wells per plate), starting at column 2 and ending at column 11, 200 μL of growth medium were added to the 8 wells in column 1 (to blank the plate reader); column 12 helped keep column 11 moist and minimized edge effect. The plate was incubated in a humid environment at 27°C, so that the cells were in the exponential growth phase when BCAAS treatments were added. A 10000 $\mu\text{g}/\text{mL}$ stock solution in L-15 medium was freshly prepared and subsequent dilutions in L-15 medium to obtain the final concentrations. BCAAS treatments were added only to wells in columns 3-10 to a final concentration of 5.0 50, 100, 250, 500, 1000 2500, 5000 and 10000 $\mu\text{g} / \text{mL}$. Base medium without BCAAS was added to the wells at columns 2 and 11, these cells were used as controls. After applying the treatments, the plates returned to incubation for a period of 72 h at 27°C. To estimate the number of surviving cells, the plate was fed with 200 μL of L-15 medium without phenol red and 50 μL of MTT was added to all wells of columns 1-11. The plate was protected from light with aluminum foil and finally incubated for 4 h.

After removing the medium and MTT from the wells, the MTT-formazan crystals were dissolved by adding 200 μL of DMSO to all the wells of columns 1 to 11, subsequently, 25 μL of glycine buffer (0.1M glycine, 0.1M NaCl, pH 10.5) were added to all wells containing DMSO. Finally, the absorbance was determined at 570 nm in a Microplate Spectrophotometer Epoch™ 2 Biotek Inc. The growth of treated cells was compared with that of untreated cells. Each of the six concentrations was examined in six replicate wells and two controls ($n=48$), and the number of viable cells per well was determined (Mosmann, 1983; Denizot & Lang, 1983).

STATICAL ANALYSIS

The data from the studies were collected and graphed using the® Microsoft Excel® 2007 (version 12.0) software (Microsoft Corporation, USA) EC50 values from baseline cytotoxicity studies were calculated using GraphPad Prism 10.0.0 for macOS, Boston, Massachusetts USA, www.graphpad.com. To carry out a statistical analysis that would allow detecting significant differences between the treated samples and the control samples, at least three independent experiments were carried out, varying the final total number depending on the parameter evaluated and the type of methodology used for their study. For each parameter studied, the normality and homoscedasticity tests were performed by Shapiro-Wilk and Levene, respectively. Based on the information obtained in these tests, the data were analyzed as follows: If the samples could be considered homogeneous and normal, the normalized data were analyzed using an ANOVA, followed by a post hoc Bonferroni or Games Howell test, in each case the most appropriate. The differences were considered significant at a p-value ≤ 0.05 . If the samples were not homogeneous or did not present a normal distribution, the Kruskal-Wallis's test was used, analyzing the differences between the pairs using the Mann-Whitney U test. In this case, the probabilities were adjusted by the Bonferroni correction. The differences were considered significant at a p-value ≤ 0.05 .

RESULTS

To determine the safe of BCAAS, we used a fish brain cell line, which we exposed to the different for specific periods of time, then using the MTT technique, cell viability was determined.

Effect of BCAAs on Cerebral cell line of Centropomus viridis

We evaluated the possible toxic effect of BCAAS, in the brain cell line of *C. viridis*, which we exposed to treatments at different concentrations during specific periods, then using the MTT technique, cell viability was determined. A very important characteristic to consider mentions that Branched-Chain Amino Acids (L-Leucine, L-Valine, and L-Isoleucine) belong to the food grade (API). Obtained by bacterial fermentation and intended to supplement infant milk formulas. They must contain not less than 98.0 and not more than 102.0% of alpha amino acids. were provided by Wuxi Jinghai Amino Acid Co., Ltd. (brand: Aminowill) the effect on the viability of brain cells, as we can see in **Figure 2** BCAAS at the different concentrations evaluated did not present a toxic effect on the viability of brain cells exposed during a period of 48, 72 and 96 hours with the respective treatment, indicating that they do not have a cytotoxic effect on this cell line (**Fig. 2-5**).

Effect of BCAAS on brain cells proliferation

As we can see in **Figure 5-7** the BCAAS did not present a toxic effect on this type of cells in any of its concentrations, on the contrary, a small tendency to increase the percentage of viable cells can be seen, but this effect was not statistically significant. indicating that none of the BCAAs affected the viability of brain cells in their different concentrations evaluated.

To confidently assess the effects of BCAAS on the molecular mechanisms responsible for regulating the mTOR pathway, we first determined concentrations that minimally affect the viability of *C. viridis* brain cells. Treatments of BCAAS 10000, 5000 mg/mL. and 1000 mg/mL. did not reduce cell viability compared to untreated cells. Lower concentrations of BCAAS did not appreciably affect cell viability (**Fig. 3-5**). according to values inferred from dose-response curves elaborated from experimental data. Therefore, the effects of BCAAS on the molecular pathways regulating mTOR activity were evaluated at concentrations ranging from 500 to 1000mg/mL.

In the evaluation of safety in the *in vitro* model, it was decided to study the three BCAAs together and individually to determine if their safety is modified. Treatments were administered at different concentrations, with no cytotoxic effects observed even in treatments of high concentrations.

The results obtained at 48 and 72 hours of exposure to the different concentrations of BCA treatment showed a percentage of viability like the control group in all treatments (**Fig. 3-4**). At present there are no reports of biosafety of the BCAAs, in similar cell lines, however, we observe that at concentrations of 2500, 5000 and 10000 mg/mL, considered high concentrations, did not alter the cell viability, indicating that they are not toxic. This indicates that branched-chain amino acids can be considered safe at concentrations as high as 10000 mg/mL for *Centropomus viridis* brain cells grown *in vitro*.

Proliferation is another of the tests carried out in the bioevaluation of *in vitro* safety, because many organic synthesis reactions generate impurities, intermediates, solvents, related substances; these can be carcinogenic and promote cell proliferation (Miller & Strömmland, 1999). When determining the effect of BCAAs on cell proliferation of the cell line of *Centropomus viridis*, proliferation kinetics like that of the control group was observed. This indicates that BCAAs do not have a carcinogenic effect on this cell line (**Fig. 6-9**). The results of the bioevaluation in the *in vitro* system that we used suggest so far that the branched chain amino acids at concentrations evaluated for the exposure period of 48 and 72 hours did not modify the safety of the molecules, having zero cytotoxic and carcinogenic activity.

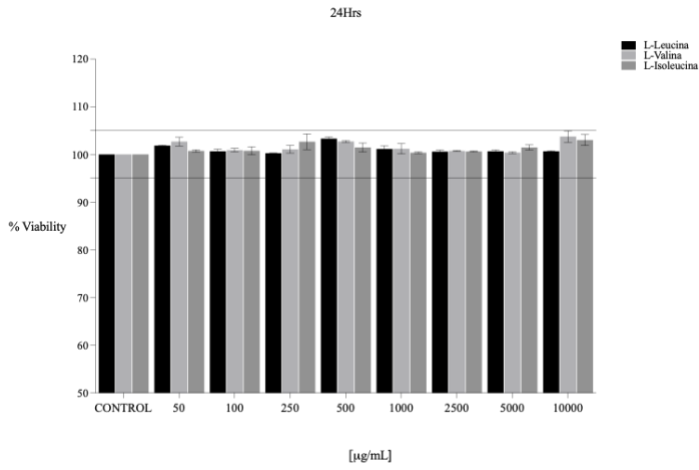


Figure 2. Effect of branched-chain amino acids on the viability of brain cells after being incubated with treatments for 24 hours. Each bar represents the average ± standard deviation of each group. *Difference with respect to the control group $p < 0.05$

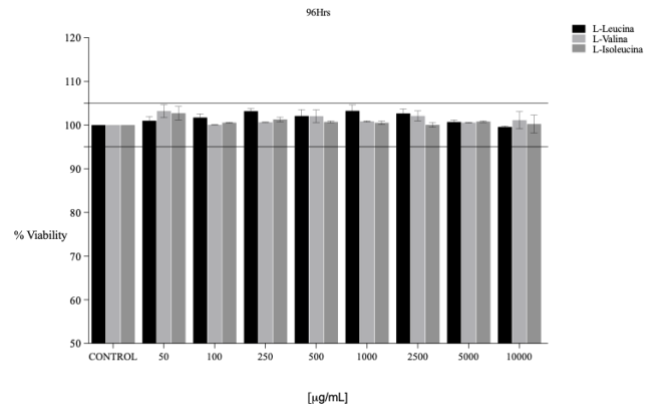


Figure 5. Effect of branched-chain amino acids on the viability of brain cells after being incubated with treatments for 96 hours. Each bar represents the average ± standard deviation of each group. *Difference with respect to the control group $p < 0.05$.

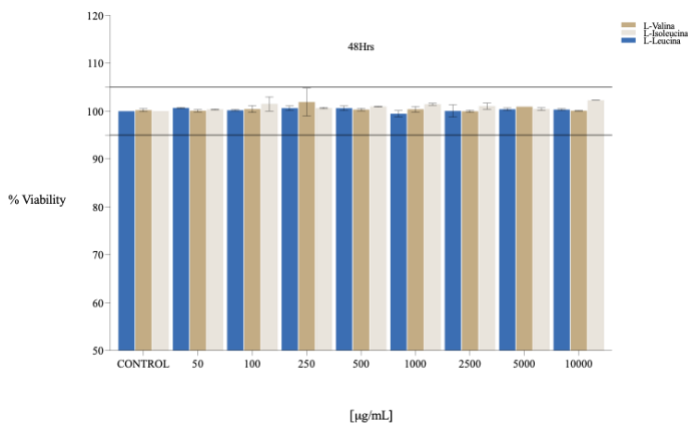


Figure 3. Effect of branched-chain amino acids on the viability of brain cells after being incubated with treatments for 48 hours. Each bar represents the average ± standard deviation of each group. *Difference with respect to the control group $p < 0.05$.

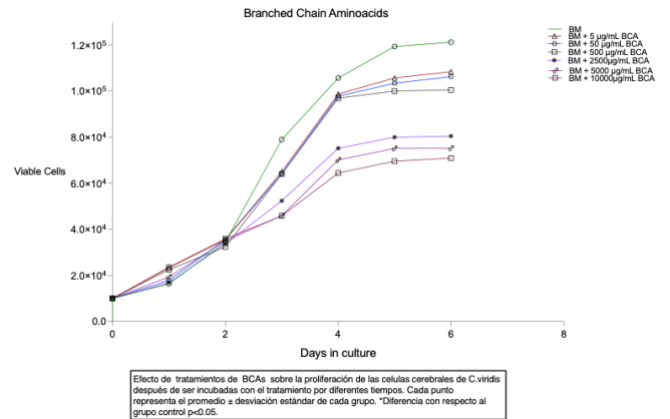


Figure 6. Effect of BCAAs on the proliferation of brain cells of *Centropomus viridis* after being incubated with the treatment for different times. Each point represents the average ± standard deviation of each group. *Difference with respect to the control group $p < 0.05$.

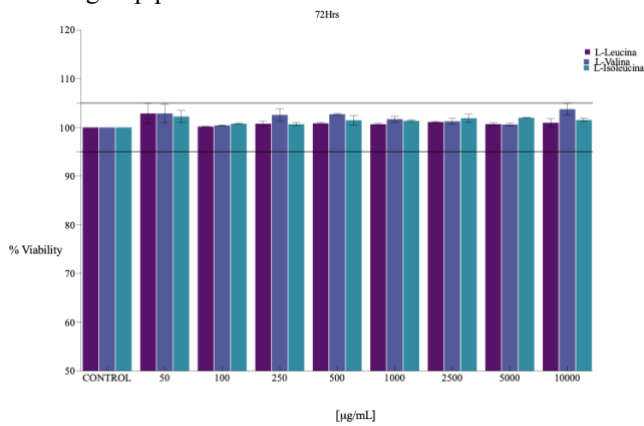


Figure 4. Effect of branched-chain amino acids on the viability of brain cells after being incubated with the treatments for 72 hours. Each bar represents the average ± standard deviation of each group. *Difference with respect to the control group $p < 0.05$

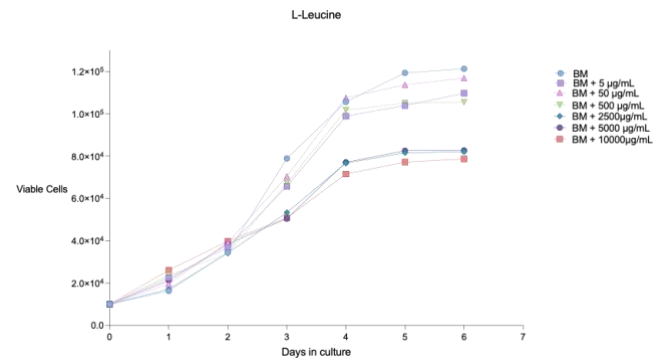


Figure 7. Effect of L-Leucine on the proliferation of brain cells of *Centropomus viridis* after being incubated with the treatment for different times. Each point represents the average ± standard deviation of each group. *Difference with respect to the control group $p < 0.05$.

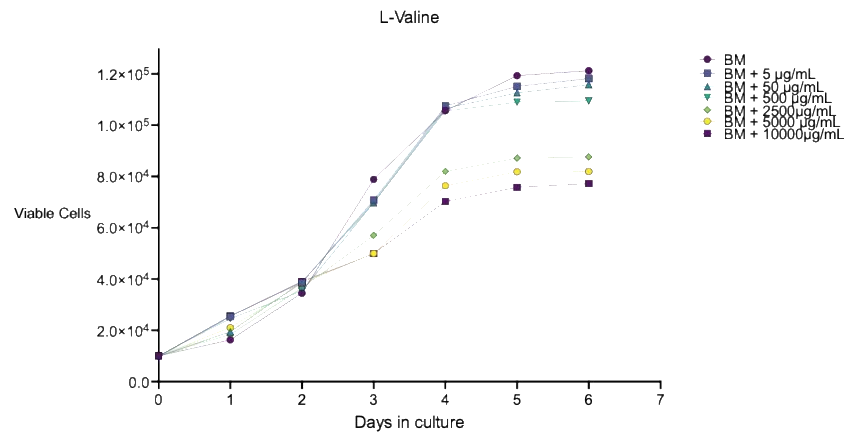


Figure 8. Effect of L-Valine on the proliferation of brain cells of *Centropomus viridis* after being incubated with the treatment for different times. Each point represents the average \pm standard deviation of each group. *Difference with respect to the control group $p < 0.05$.

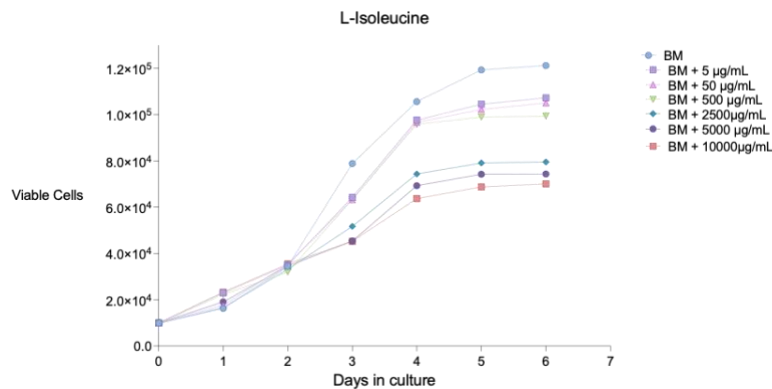


Figure 9. Effect of L-Isoleucine on the proliferation of brain cells of *Centropomus viridis* after being incubated with the treatment for different times. Each point represents the average \pm standard deviation of each group. *Difference with respect to the control group $p < 0.05$.

DISCUSSION

Amino acids that have long been considered simply precursors to protein synthesis are now recognized as exerting other significant influences, i.e. as precursors of essential molecules, acting as mediators or signaling molecules affecting several other functions. There is growing evidence supporting the key role of amino acids as signaling molecules in regulating protein synthesis and that dietary protein intake is essential for normal growth and development, as well as for effective therapeutic approaches for some pathophysiological circumstances (Pena-Leon et al, 2020). There is also growing evidence that high dietary protein intake contributes to obesity, insulin resistance, and type 2 diabetes (Heberle et al, 2021). Therefore, the mechanisms by which ingested excess amino acids leads to high accumulation of muscle proteins and/or the

development of metabolic disorders have important implications for optimizing the growth and development of farm animals. In the present study, the first data on mTORC1, one of the main molecular pathways, is considered as a central axis of metabolism control. On the other hand, it is worth mentioning that all the tests were done under the 3R scheme in an *in vitro* system of brain cells of *C. viridis* a species of carnivorous fish, and known to require a high supply of amino acids in the diet. These findings suggest that the mechanisms involved in the regulation of mTORC1 activity are well conserved throughout the evolution between Vertebrates, at least in the group of humans and fish, although it is worth investigating other key initiation factors that control protein synthesis.

In the present research work we proposed to study in an *in vitro* model of cell culture the modulating kinases of the upstream mTOR pathway; SestrinA as a leucine sensor, MPK

as an inhibitor of the mTORC1 pathway. When evaluating the safety of BCAAS on the cell line, we verified by the MTT assay that BCAAS administered to the brain cells of the snook are harmless and safe for this cell line no effect on proliferation and viability respectively results showed that BCAAS do not affect the proliferation or viability of treated brain cells even at high concentrations of BCAAS. these results suggest that the mechanisms involved in the regulation of mRNA translation and the mechanisms involved in the regulation of the TOR pathway are well conserved among lower and higher vertebrates. Our data based on sea bass brain cell culture show that amino acids regulate TOR signaling at least in vitro and may therefore play a key role in the effect of metabolic regulation in this species as in mammals.

Amino acids for a long time were considered only precursors of protein synthesis, now recognized to fulfill other functions significant, that is, as precursors of essential molecules, acting as mediators or signaling molecules that affect several other functions. There is increasing scientific evidence supporting the key role of amino acids as signaling molecules in the regulation of protein synthesis and that adequate dietary protein intake is essential for growth and development (Hutson et al, 2005). The present study provides the first data in a carnivorous species, known to require a high supply of amino acids in the diet, that feeding improves phosphorylation and/or activity of PKB, TOR, S6K1 and 4E-BP1 in muscle. These findings suggest that the mechanisms involved in regulating mRNA translation are well conserved among lower and higher vertebrates, although they are worthwhile.

CONCLUSIÓN

Studies conducted to evaluate the safety of compounds with therapeutic potential; They are an important part of the development of a new drug, since their objective is to evaluate the risk or potential danger that a chemical or biological agent may exert. Toxicity assessment could be defined as the scientific study of the behaviour, metabolism and mechanism of action of substances or elements causing organic or functional disturbances.

One of the tests that should be evaluated is cytotoxicity, which can be measured by various methods, including the MTT method (Mosmann, 1983). The effect on viability in the brain cell line of *C. viridis* was determined.

In the evaluation of safety in the in vitro model, it was decided to study the three BCAAs together and individually to determine if their safety is modified. Treatments were administered at different concentrations, with no cytotoxic effects observed even in treatments of high concentrations. The results obtained at 48 and 72 hours of exposure to the different concentrations of BCA treatment showed a percentage of viability like the control group in all treatments (Fig. 2 to 5). At present there are no reports of biosafety of

the BCAAs, in similar cell lines, however, we observe that at concentrations of 2500, 5000 and 10000 mg / mL, considered high concentrations, did not alter the cell viability, indicating that they are not toxic. This indicates that branched-chain amino acids can be considered safe at concentrations as high as 10000mg/mL for *Centropomus viridis* brain cells grown in vitro.

Proliferation is another of the tests carried out in the bioevaluation of in vitro safety, because many organic synthesis reactions generate impurities, intermediates, solvents, related substances; these can be carcinogenic and promote cell proliferation. (Miller & Strömmland, 1999; Ehrich & Sharova, 2000). When determining the effect of BCAAs on cell proliferation of the cell line of *Centropomus viridis*, proliferation kinetics like that of the control group was observed. This indicates that BCAAs do not have a carcinogenic effect on this cell line (Fig. 6-9). The results of the bioevaluation in the in vitro system that we used suggest so far that the branched chain amino acids at concentrations evaluated for the exposure time of 48 and 72 hours did not modify the safety of the molecules, having zero cytotoxic and carcinogenic activity. On the other hand. As in previous studies, our results show a similar behavior in that they do not cause cell damage even at high concentrations. This is evidenced by correlating it with the results of the MTT assay, considered the gold standard cytotoxicity assay. In colorimetric titrations, the number of viable cells is analyzed by excision of tetrazolium salts added to the culture medium. the entire assay, from microculture to analysis of the data on an ELISA reader, is performed on the same microplate. it is a tetrazolium salt which is hydrolyzed to formazan by enzymes of the endoplasmic reticulum. This reduction occurs only in viable cells, and is related to the production of NAD(P)H through glycolysis. Consequently, the amount of formazan dye formed shows a direct correlation with the number of metabolically active cells in the culture. Because BCAAs are especially L-leucine, amino acids that increase mitochondrial biogenesis, this was evident in the results of the trial. BCAAS treatments appear to increase mitochondrial biogenesis in *C. viridis* brain cells. which adapted to the conditions subjected and even adopted a morphology different from the initial one. This can be seen in Fig. 10 It's clear that BCAAs play an essential role in nitrogen metabolism throughout the body. First, they provide an important transport system to move nitrogen throughout the body for the synthesis of expendable amino acids. Second, they provide hormone-like signals to tissues, for example, skeletal muscle, indicating overall nutrient sufficiency. The behavior of *C. viridis* brain cells when supplemented with treatments of different concentrations of BCAAS, contrary to what was expected, did not present cellular damage and instead favored proliferation and increased mitochondrial metabolism. Future experiments in in vitro brain cell systems obtained from the snook brain will provide relevant information that will increase the understanding of the role played by BCAAs in the brain and its metabolism.

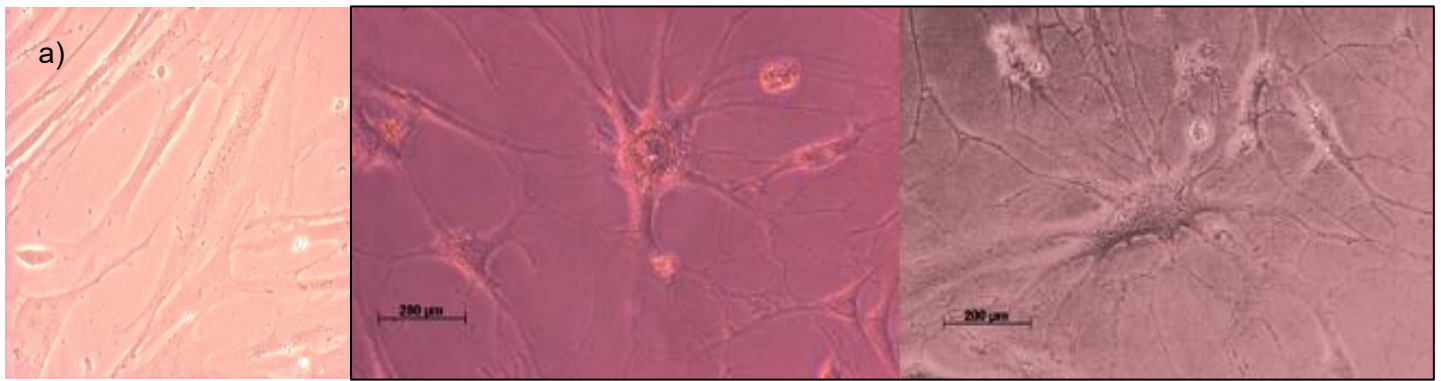


Figure 10: Effect of branched-chain amino acid treatments on the brain morphology of *Centropomus viridis*. Photograph of cells treated with 10000µg/mL of BCAAs, for a period of a) 0 hours; (b) 48 hours; c) 72 hours.

DECLARATIONS OF CONFLICTING INTERES

The author(s) declared that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CONTRIBUTION OF AN AUTHOR

AAS, MSN conducted the experiments and contributed to the analysis of the data, CH, JM contributed to study design and tutoring and technical support to students, CH, JM provided essential research materials for this study. AAS, MSN and NB wrote the article. All authors do not have competing interest information to disclose. Data supporting the findings of this study are available upon request.

DATA AVAILABILITY STATEMENT

I confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. I further confirm that the order of authors listed in the manuscript has been approved by all authors.

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I further confirm that any aspect of the work covered in this manuscript that has involved experimental animals has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript. I understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager

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