

Pre-clinical evaluation of the impact of a natural mixture on cellular energy metabolism for mild cognitive impairment

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Abstract

Background: Neurological diseases are pathologies that affect the central nervous system, such as Alzheimer's disease and other forms of dementia. A correlation between cognitive decline and energy metabolism has been identified, and the potential to treat mental deterioration through the integration of natural mixtures into therapeutic strategies has gained increasing interest. **Objective:** Given the close relationship between hypometabolism and cognitive impairment, this study investigated the metabolic effects of a mixture containing medium-chain triglycerides, omega-3 fatty acids, and choline bitartrate in a human neuronal cell model, with relevance to cognitive decline. This was achieved by comparing the synergistic effects of the combined ingredients with the effects of the individual ingredients on energy metabolism (ATP) in a human neuronal model. **Methods:** Starting from the maximum non-toxic concentration common to both the mixture and individual components (1.563 mg/mL), the selected cell model (SH-SY5Y) was incubated for 24 h with decreasing concentrations of the mixture to evaluate ATP levels and to identify the optimal concentration (0.391 mg/mL). The cell viability results of the mixture were compared with the dose–response curves generated from cells treated with the individual components at equivalent concentrations to those present in the mixture. **Results:** A statistically significant increase ($p < 0.05$) was observed in ATP content following treatment with the mixture compared to the individual active ingredients. **Conclusion:** The pre-clinical results demonstrate that a mixture containing medium-chain triglycerides, omega-3 fatty acids, and choline bitartrate exerts a synergistic effect on cellular energy metabolism, enhancing ATP production in a neuronal model. This formulation supports energy metabolism and may be beneficial for patients suffering from neurodegenerative diseases.

Keywords: Adenosine triphosphate, Mitochondria, SH-SY5Y cells, Mild cognitive impairment, Medium-chain triglyceride, Omega-3, Choline

1. Introduction

Alzheimer's disease (AD) is a multifactorial condition characterized by reduced glucose uptake and utilization, insulin resistance, impaired autophagy and proteostasis, increased inflammation, oxidative stress, and mitochondrial dysfunction. Cerebral hypometabolism, which is typical of this condition, precedes the development of amyloid- β (A β) pathology, supporting the rationale for interventions focused on metabolism and mitochondrial function as potential strategies to halt disease progression.¹ A reduced number of insulin receptors has been observed in the brains of patients, resulting in cerebral insulin resistance. Furthermore, neurons have a reduced number of mitochondria, many of which display diminished activity, contributing to reduced energy production.^{2,3} Although the human brain accounts for only 2% of body mass, it consumes 20% of the body's energy for mitochondrial respiration and

ATP production. Therefore, the brain is highly dependent on mitochondrial efficiency and is particularly vulnerable to bioenergetic decline when this function is impaired.⁴

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The neuropathological processes of AD begin years before functional decline, and the identification of prodromal states is crucial for disease intervention. The most widely studied prodromal state is mild cognitive impairment (MCI), a key target population for preventive intervention.⁵ In patients with MCI, glucose hypometabolism accurately predicts future clinical progression to AD.⁴ MCI is generally described as an intermediate stage between cognitive normality and dementia. It represents a measurable cognitive deficit in at least one domain, without meeting the criteria for dementia, but conferring an increased risk of progression to the disease.

There are two primary forms: amnesic MCI and non-amnesic MCI.⁶ The accumulation of A β in senile plaques and Tau proteins in neurofibrillary tangles can begin before the age of 30 in AD. In patients with MCI, neurofibrillary tangles have been detected in the hippocampus and other medial temporal regions. As MCI progresses to AD, these neurofibrillary tangles spread to the neocortical region, particularly the parietal and frontal areas of the brain.⁷ The A β peptide is generated through the proteolytic cleavage of a type I membrane glycoprotein known as amyloid precursor protein, which plays a key role in various neuronal biological processes.⁸ This cleavage produces amyloidogenic peptide fragments—A β 1-40 and A β 1-42—which tend to aggregate into oligomers or fibrils. Notably, A β oligomers are particularly neurotoxic and contribute to neuronal cell death.^{8,9}

This pathological protein aggregation can be prevented by ATP, as it can effectively inhibit A β 40 dimerization and induce conformational changes by hindering β -sheet formation between the central hydrophobic core and secondary hydrophobic region fragments. Furthermore, ATP not only inhibits aggregation but also disintegrates previously formed dimers¹⁰—it acts as a conformational modulator of A β . Under conditions of cerebral energy deficit, common in MCI and AD, ATP depletion can promote amyloid aggregation. Using a combination of computational biology methods and biochemical approaches, it was observed that ATP interacts with tyrosine and serine residues in the A β fibril and reduces its folding and misfolding. This provides a direct and verifiable link between metabolic dysfunction and A β fibril misfolding.¹¹ Thus, it can be assumed that a mixture capable of increasing neuronal ATP levels may exert a positive and preventive effect in MCI.

In this study, given the close connection between hypometabolism and cognitive impairment, we evaluated the effect of three ingredients on neuronal energy metabolism related to cognitive decline: Medium-chain triglycerides (MCTs), omega-3 fatty acids—docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)—in a 4:1 ratio, and choline bitartrate. This was achieved by evaluating the potential synergistic effects among the individual components on energy metabolism in a human neuronal model, SH-SY5Y

cells. There is extensive scientific evidence supporting that the ingredients included in the mixture are extensive, and each component has been shown to mitigate cognitive impairment or AD through distinct mechanisms.

Polyunsaturated fatty acids (PUFAs), such as EPA and DHA, regulate the structure and function of neurons and play an essential role in neuroplasticity.¹² A meta-analysis investigated the effectiveness of DHA and/or EPA supplementation in elderly individuals with MCI. The study reported that treatment with *n*-3 PUFAs led to a significant improvement in overall cognitive function in elderly patients with MCI. Furthermore, treatment with *n*-3 PUFAs resulted in a slight improvement in memory in patients with MCI, suggesting that *n*-3 PUFAs may reduce A β -related biomarkers and plasma inflammatory cytokines in this population.¹³ In addition, previous studies suggest that apolipoprotein E4 carriers have lower brain DHA uptake and levels and may particularly benefit from DHA intervention prior to significant neuropathology, which affects brain DHA uptake.¹⁴

Choline is an essential nutrient involved in several cognitive processes, including signal transduction, myelination, and cholinergic neurotransmission.¹⁵ Dysregulation of cholinergic neurotransmission is linked to several cognitive disorders, such as AD.¹⁶ Low levels of circulating choline are associated with increased inflammation and neuropathology, suggesting that adequate dietary choline intake and regular monitoring of circulating levels should be implemented to promote healthy aging. Therefore, choline is essential for the proper functioning of the body and brain during adulthood, serving as a preventive strategy to slow the pathological progression of AD.¹⁷

Medium-chain triglycerides are metabolized into ketone bodies, which are used by astrocytes as an alternative energy source, exerting beneficial effects on cognitive outcomes.¹⁸ The brains of AD patients exhibit glucose hypometabolism but may utilize ketones for energy production. Ketone levels can be enhanced through oral intake of MCTs. Meta-analyses of randomized controlled trials in AD and MCI have shown that oral MCT administration improves cognition on combined scales, including the AD Assessment Scale–Cognitive Subscale and the mini-mental state examination.¹⁹

Given that mitochondrial dysfunction and ATP depletion are major markers of neurodegeneration, this study developed a mixture containing three ingredients—MCT, omega-3 fatty acids, and choline bitartrate—at physiologically relevant doses, and evaluated its effect on neuronal cells by measuring ATP levels before and after treatment.

2. Materials and methods

The synergistic efficacy of the mixture on ATP synthesis was evaluated using the human neuroblastoma cell line,

SH-SY5Y, derived from a biopsy of a metastatic bone tumor and originating as a subclone of the parental line SK-N-SH. Based on its characteristics, this cellular model is widely used to study neuronal function and differentiation *in vitro*. The study was conducted by Mérieux NutriSciences at the ECSIN laboratory of EcamRicert S.r.l., under report No. 24-008586

The mixture was prepared using selected raw materials and a controlled mixing technique under specific temperature and pressure conditions, following the proportions of the mixture outlined in Table 1.

2.1. Solubilization of the mixture

Before assessing the effects of the mixture on cellular energy metabolism using SH-SY5Y cells, the mixture was subjected to dissolution tests in cell culture medium (CCM). Specifically, the mixture was suspended in CCM at a concentration of 50 mg/mL, corresponding to 27.7 mg/mL MCT, 19.4 mg/mL omega-3 fatty acids, and 2.9 mg/mL choline. The solution was sonicated for 5 min at 80 Hz to achieve complete solubilization of the formulation. Dissolution tests were also performed on the individual components while maintaining the same stoichiometric ratios as in the mixture. Once the solubility of the mixture in CCM was determined, this solution was used to treat SH-SY5Y cells to identify the maximum non-toxic concentration to be used in evaluating the effect on cellular metabolism.

2.2. Evaluation of the effect of the concentration of the mixture on SH-SY5Y cell viability

The effect of the concentration of the mixture on SH-SY5Y cell viability was assessed by establishing a dose–response curve after exposing the cells to decreasing concentrations of the mixture for 24 h.

The CCM solution was pre-diluted at a ratio of 1:4 (12.5 mg/mL, corresponding to 6.925 mg/mL MCT, 4.850 mg/mL omega-3 fatty acids, and 0.725 mg/mL choline), and serial dilutions were prepared to treat SH-SY5Y cells to identify the maximum non-toxic concentration of the mixture.

Table 1. The proportion of the mixture

Ingredients	Quantity
<i>Elaeis guineensis</i> extract (70% MCT)	14.28 g
MCT	10.00 g
Omega-3 (4:1 DHA: EPA)	10.00 g
Omega-3	1.50 g
Choline bitartrate (48% choline)	1.48 g
Choline	600 mg

Abbreviations: DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; MCT: Medium-chain triglyceride.

Untreated and staurosporine (STS)-treated cells were used as negative and positive controls for cell death, respectively. At the end of treatment, cell viability was measured using the MTS colorimetric assay (CellTiter 96 AQueous One Solution Assay, Promega, United States [USA]). The MTS assay is based on the reduction of a tetrazolium compound by viable cells to generate the colored product, which can be quantified by measuring its absorbance at 490 nm.²⁰ A formulation was considered cytotoxic if it reduced cell viability by more than 30% compared with the negative control (NT; untreated cells). Results were expressed as a percentage (%) compared to NT.

Based on the cell viability results of the mixture and the comparison with the dose–response curves of the individual active components obtained from previous experiments, the maximum non-cytotoxic concentration common to both the mixture and the individual actives was identified for subsequent analyses.

2.3. Evaluation of the effect of the concentration of the mixture on energy metabolism in the SH-SY5Y neuronal model

Starting from the highest non-toxic concentration common to the individual active components and the mixture, as determined from the previous cytotoxicity experiment, the effect of the concentration of the mixture on cellular energy metabolism was assessed. SH-SY5Y cells were treated with decreasing concentrations of the mixture for 24 h. Untreated and STS-treated cells were used as NT (basal ATP release) and positive control (cell death and ATP reduction), respectively. After incubation, the impact on metabolism was assessed using the ATPlite 1step Luminescence ATP Detection Assay System (Perkin Elmer, USA). The assay is based on the production of luminescence resulting from the reaction of ATP with luciferase and D-luciferin. The luminescence signal is proportional to the ATP content and, consequently, to the number of metabolically active cells. The results were expressed as the percentage (%) of ATP relative to the NT.

The effects of the mixture on cell metabolism were compared with those of the individual active components to evaluate a potential synergistic interaction among the components when combined in appropriate proportions within the formulation.

The maximum non-toxic concentration common to the mixture and the individual active components (1.563 mg/mL) was identified. Starting from this concentration, the selected cell model (SH-SY5Y) was incubated with decreasing concentrations of the mixture for 24 h to evaluate ATP levels. From this experiment, an optimal concentration of

the mixture (0.391 mg/mL) was determined. Subsequently, when comparing the effects on metabolism at the same dilution between the mixture and the individual components, a statistically significant increase ($p < 0.05$) in ATP content was observed following treatment with the mixture compared to the individual active ingredients.

2.4. Statistical analysis

All data are presented as mean \pm standard deviation of three independent experiments. Student's *t*-test analysis was performed to determine statistically significant differences between the different conditions. The *t*-test is a statistical method used to test differences between the means of two groups. Differences between groups were considered statistically significant at $p < 0.05$. All statistical analyses were performed using OriginLab software (OriginLab Corporation, USA).

3. Results

3.1. The effect of the concentration of the mixture on SH-SY5Y cell viability

The effect of the concentration of the mixture on cell viability was evaluated using an *in vitro* neuronal model, following the protocols outlined in Section 2. The dose-response curve of the mixture in CCM is shown in Figure 1 and detailed in Table 2. As expected, the positive cell death control resulted in an almost complete reduction in cell viability compared to the NT. The dose-response curve showed adverse effects of the mixture on cell viability at concentrations ≥ 3.125 mg/mL (viability $< 70\%$ compared with the NT).

The cell viability results of the mixture were compared with the dose-response curves generated from SH-SY5Y

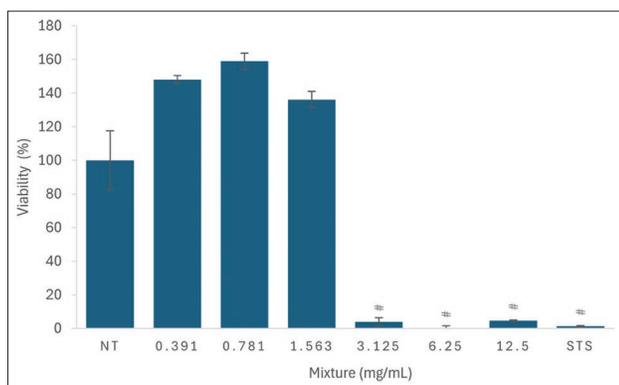


Figure 1. The effect of the concentration of the mixture on SH-SY5Y cell viability after 24 h of treatment. Cell viability is expressed as a percentage (%) relative to the NT. Data were analyzed using the Student's *t*-test. Results are reported as mean \pm standard deviation. Hash-symbol (#) indicates cell viability $< 70\%$ relative to NT.

Abbreviations: NT: Negative control; STS: Staurosporine.

cells treated with the individual components at equivalent concentrations to those present in the mixture. The effect of the concentration of individual active components on cell viability is shown in Figures 2-4 and Tables 3-5.

While omega-3 fatty acids reduced cell viability by more than 30% at the highest concentration tested (4.85 mg/mL; Figure 3), no reduction in cell viability was observed following treatment of SH-SY5Y cells with MCT and choline

Table 2. The effect of the concentration of the mixture on cell viability after 24 h of treatment

Mixture (mg/mL)	Cell viability (%)
NT	100.00 \pm 17.47
0.391	148.08 \pm 2.30
0.781	159.00 \pm 4.87
1.563	136.18 \pm 4.77
3.125	3.93 \pm 2.37
6.25	0.00 \pm 1.48
12.5	4.62 \pm 0.34
STS	1.38 \pm 0.45

Notes: Cell viability is expressed as a percentage (%) relative to the NT. Data were analyzed using the Student's *t*-test. Results are reported as mean \pm standard deviation. Abbreviations: NT: Negative control. STS: Staurosporine.

Table 3. The effect of the concentration of medium-chain triglycerides on SH-SY5Y cell viability after 24 h of treatment

MCT (mg/mL)	Viability (%)
NT	100.00 \pm 6.40
0.216	136.18 \pm 1.58
0.433	136.93 \pm 29.97
0.866	125.77 \pm 13.67
1.731	117.97 \pm 1.58
3.463	110.35 \pm 4.47
6.925	97.34 \pm 21.82
STS	0.25 \pm 0.57

Notes: Cell viability is expressed as a percentage (%) relative to the NT. Data were analyzed using the Student's *t*-test. Results are reported as mean \pm standard deviation. Abbreviations: MCT: Medium-chain triglyceride; NT: Negative control. STS: Staurosporine.

Table 4. The effect of the concentration of omega-3 fatty acids on SH-SY5Y cell viability after 24 h of treatment

Omega-3 (mg/mL)	Viability (%)
NT	100.00 \pm 6.40
0.152	122.61 \pm 8.67
0.303	110.35 \pm 11.30
0.606	116.85 \pm 6.83
1.213	111.65 \pm 1.05
2.425	131.91 \pm 0.26
4.850	4.58 \pm 2.63
STS	0.25 \pm 0.57

Notes: Cell viability is expressed as a percentage (%) relative to the NT. Data were analyzed using the Student's *t*-test. Results are reported as mean \pm standard deviation.

Abbreviations: NT: Negative control. STS: Staurosporine.

(Figures 2 and 4). The findings demonstrate that a 1:32 dilution of the 1.563 mg/mL mixture (corresponding to

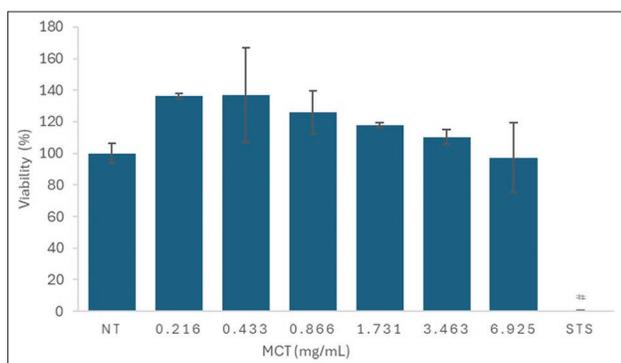


Figure 2. The effect of the concentration of MCT on SH-SY5Y cell viability after 24 h of treatment. Cell viability is expressed as a percentage (%) relative to the NT. Data were analyzed using the Student's *t*-test. Results are reported as mean \pm standard deviation. Hash-symbol (#) indicates cell viability <70% relative to NT.

Abbreviations: MCT: Medium-chain triglycerides; NT: Negative control; STS: Staurosporine.

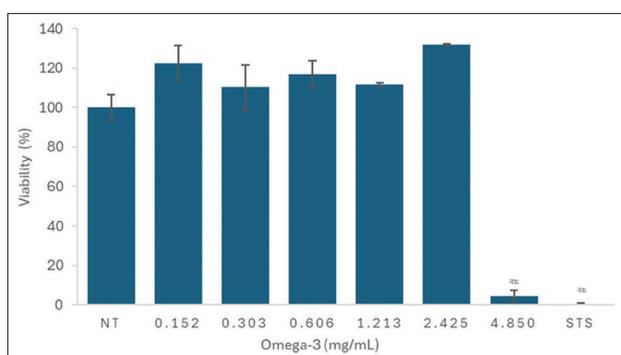


Figure 3. The effect of the concentration of omega-3 fatty acids on SH-SY5Y cell viability after 24 h of treatment. Cell viability is expressed as a percentage (%) relative to the NT. Data were analyzed using the Student's *t*-test. Results are reported as mean \pm standard deviation. Hash-symbol (#) indicates cell viability <70% relative to NT.

Abbreviations: NT: Negative control; STS: Staurosporine.

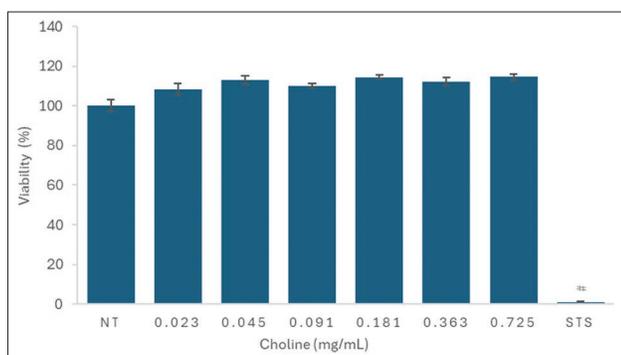


Figure 4. The effect of the concentration of choline on SH-SY5Y cell viability after 24 h of treatment. Cell viability is expressed as a percentage (%) relative to the NT. Data were analyzed using the Student's *t*-test. Results are reported as mean \pm standard deviation. Hash-symbol (#) indicates cell viability <70% relative to NT.

Abbreviations: NT: Negative control; STS: Staurosporine.

0.866 mg/mL MCT, 0.606 mg/mL omega-3 fatty acids, and 0.091 mg/mL choline) was identified as the maximum non-toxic concentration common to the mixture and individual actives.

3.2. The effect of the concentration of the mixture on energy metabolism in the SH-SY5Y neuronal model

The effect of the concentration of the mixture on cellular energy metabolism was assessed using the ATP assay. Starting from the highest non-toxic concentration common to the three active ingredients and the mixture, SH-SY5Y cells were incubated with decreasing concentrations of the mixture (from 1.563 mg/mL to 0.196 mg/mL) for 24 h. As shown in Figure 5 and Table 6, treatment of SH-SY5Y cells with the mixture at concentrations of 0.196 mg/mL and 0.391 mg/mL resulted in significantly higher ATP levels than those observed in NT ($p=0.01$ and $p=0.001$ for 0.196 mg/mL and 0.391 mg/mL, respectively).

Figure 6 shows the results of the effect of the concentration of individual active components—MCT, omega-3 fatty acids, and choline—on cellular energy metabolism in the SH-SY5Y neuronal model.

Comparison of the results between the mixture and the individual components (MCT, omega-3 fatty acids, and choline) revealed a statistically significant increase ($p<0.05$) in ATP content following treatment with the mixture compared to the individual active ingredients under the following conditions:

- Mixture vs. MCT at a 1:128 dilution, corresponding to 0.391 mg/mL of the mixture and 0.216 mg/mL of MCT ($p=0.0017$)
- Mixture vs. omega-3 fatty acids at a 1:128 dilution, corresponding to 0.391 mg/mL of the mixture and 0.152 mg/mL of omega-3 ($p=0.00017$)

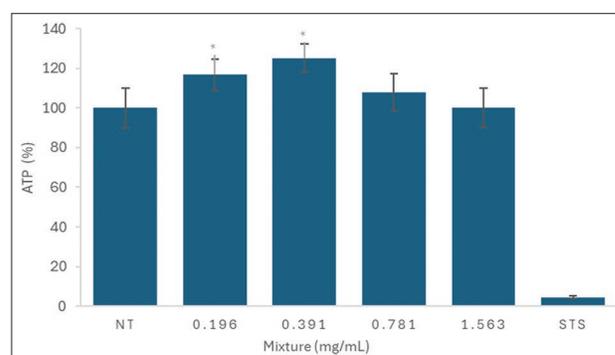


Figure 5. The effect of the concentration of the mixture on the cellular energy metabolism of SH-SY5Y cells after 24 h of treatment. Results are expressed as a percentage (%) of ATP relative to the NT and reported as mean \pm standard deviation. Data were analyzed using the Student's *t*-test. Asterisk (*) indicates statistical significance at $p<0.05$.

Abbreviations: NT: Negative control; STS: Staurosporine.

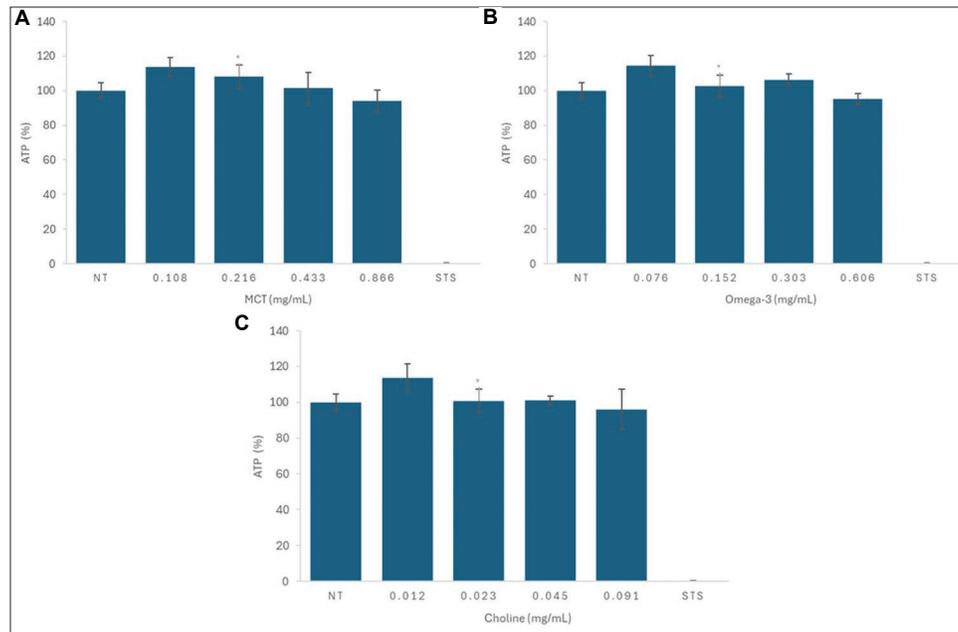


Figure 6. The effect of the concentration of (A) MCT, (B) omega-3 fatty acids, and (C) choline on cellular energy metabolism of SH-SY5Y cells after 24 h of treatment. Results are expressed as a percentage (%) of ATP relative to the NT and reported as mean \pm standard deviation. Data were analyzed using the Student's *t*-test. Asterisk (*) indicates statistical significance at $p < 0.05$ relative to the mixture. Abbreviations: MCT: Medium-chain triglycerides; NT: Negative control; STS: Staurosporine.

Table 5. The effect of the concentration of choline on SH-SY5Y cell viability after 24 h of treatment

Choline (mg/mL)	Viability (%)
NT	100.00 \pm 2.97
0.023	108.27 \pm 2.97
0.045	113.04 \pm 2.18
0.091	110.23 \pm 0.99
0.181	114.44 \pm 0.99
0.363	112.20 \pm 2.18
0.725	114.58 \pm 1.59
STS	0.75 \pm 0.49

Notes: Cell viability is expressed as a percentage (%) relative to the NT. Data were analyzed using the Student's *t*-test. Results are reported as mean \pm standard deviation.

Abbreviations: NT: Negative control. STS: Staurosporine.

(iii) Mixture vs. choline at a 1:128 dilution, corresponding to 0.391 mg/mL of the mixture and 0.023 mg/mL of choline ($p=0.00012$).

4. Discussion

Mental deterioration is one of the clinical conditions that most interferes with patients' quality of life, and the brain is the most metabolically active organ in the body and is particularly vulnerable to disruptions in energy resources.²¹ Mitochondrial dysfunction and ATP depletion are major

markers of neurodegeneration. Thus, the effect of the mixture of MCT, omega-3 fatty acids, and choline was preliminarily evaluated in neuronal cells by measuring ATP levels before and after treatment. This study identified a natural mixture capable of increasing cellular energy metabolism through ATP production. The mixture, based on MCTs, omega-3 fatty acids, and choline, prepared at a concentration of 0.391 mg/mL (0.216 mg/mL MCT, 0.152 mg/mL omega-3 fatty acids, and 0.023 mg/mL choline) using appropriate preparation techniques, enhanced ATP production compared to single ingredients. This effect on cellular energy metabolism was evaluated in an SH-SY5Y neuronal model.

ATP, universally present in all living organisms, is recognized as a fundamental energy molecule essential for numerous cellular processes. In addition to its well-known chemical actions, this molecule also exhibits important biophysical roles, including the stabilization of soluble proteins and the solubilization of insoluble proteins. ATP's ability to drive conformational changes¹⁰ together with our preliminary results showing an increase in ATP following treatment with this natural mixture, may be the key to developing new products aimed at mitigating mental decline and slowing the progression of major neurodegenerative diseases through ATP modulation.

Table 6. The effect of the concentration of the mixture and individual ingredients on the cellular energy metabolism of SH-SY5Y cells after 24 h of treatment

Ingredient	Concentration (mg/mL)	ATP (%)
Mixture	NT	100.00±10.03
	0.196	116.74±7.84*
	0.391	125.15±7.02*
	0.781	107.88±9.59
	1.563	100.22±9.74
	STS	4.48±0.69
MCT	NT	100.00±4.73
	0.108	113.52±5.70
	0.216	107.99±6.97 [#]
	0.433	101.28±9.18
	0.866	93.93±6.36
	STS	0.28±0.03
Omega-3	NT	100.00±4.73
	0.076	114.23±5.97
	0.152	102.64±6.28 [#]
	0.303	106.15±3.67
	0.606	95.11±3.15
	STS	0.28±0.03
Choline	NT	100.00±4.73
	0.012	113.53±7.88
	0.023	100.65±6.89 [#]
	0.045	100.99±2.23
	0.091	95.90±11.43
	STS	0.28±0.03

Notes: Results are expressed as a percentage (%) of ATP relative to the NT and reported as mean±standard deviation. Data were analyzed using the Student's *t*-test. Asterisk (*) indicates statistical significance at $P<0.05$ relative to the NT. Hash symbol (#) indicates statistical significance at $P<0.05$ for comparisons between the mixture and the individual ingredients.

Abbreviations: MCT: Medium-chain triglyceride; NT: Negative control; STS: Staurosporine.

5. Conclusion

These preliminary findings support further investigation of this mixture in both *in vitro* and *in vivo* systems, including animal models and clinical studies, to evaluate its efficacy in the real world.

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Conflict of interest

Marta Scquizzato is affiliated with Global Pharmacies Partner, whereas Marianna Colasante and Giulio Torello are affiliated with TL Pharma Consulting; however, these entities had no

role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. Other authors declare they have no competing interests.

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Validation: Giulio Torello, Marianna Colasante

Writing—original draft: Marta Scquizzato

Writing—review & editing: Marta Scquizzato

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data availability statement

Data will be made available upon reasonable request to the corresponding author.

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