Buttermilk and Krill Oil Phospholipids Improve Hippocampal Insulin Resistance and Synaptic Signaling in Aged Rats

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Abstract

Impaired glucose metabolism and mitochondrial decay greatly increase with age, when cognitive decline becomes rampant. No pharmacological or dietary intervention has proven effective, but proper diet and lifestyle do postpone the onset of neurodegeneration and some nutrients are being investigated.We studied insulin signaling,mitochondrial activity and biogenesis, and synaptic signaling in the hippocampus and cortex following dietary supplementation with bioactive phospholipid concentrates of krill oil (KOC), buttermilk fat globule membranes (BMFC), and a combination of both in aged rats. After 3 months of supplementation, although all groups of animals showed clear signs of peripheral insulin resistance, the combination of KOC and BMFC was able to improve peripheral insulin sensitivity. We also explored brain energy balance. Interestingly, the hippocampus of supplemented rats—mainly when supplemented with BMFC or the combination of KOC and BMFC—showed an increase in intracelular adenosine triphosphate (ATP) levels, whereas no difference was observed in the cerebral cortex. Moreover, we found a significant increase of brain-derived neurotrophic factor (BDNF) in the hippocampus of BMFC+KO animals. In summary, dietary supplementation with KOC and/or BMFC improves peripheral and central insulin resistance, suggesting that their administration could delay the onset of these phenomena. Moreover, n-3 fatty acids (FAs) ingested as phospholipids increase BDNF levels favoring an improvement in energy state within neurons and facilitating both mitochondrial and protein synthesis, which are necessary for synaptic plasticity. Thus, dietary supplementation with n-3 FAs could protect local protein synthesis and energy balance within dendrites, favoring neuronal health and delaying cognitive decline associated to age-related disrepair.

Introduction

It has been shown that insulin controls not only whole-body energy and glucose homeostasis in the periphery of the human body but also exerts specific effects in the brain (Ghasemi et al., 2013; Kleinridders et al., 2014). In fact, impaired glucose metabolism or mitochondrial dysfunction are among the major pathological changes observed in various neurodegenerative diseases (Bhat et al., 2015), which is in accordance with an increase in insulin resistance with age (Akintola and van Heemst, 2015).

Insulin signaling in the brain follows the same steps described for peripheral tissues, and its receptor is expressed in neurons and glial cells in different brain regions. Among them, hippocampus and temporal cortex shows the highest levels of insulin receptor expression, indicating the important role of insulin in learning and memory (Akintola and van Heemst, 2015; Unger et al., 1991). Previous works have demonstrated that the glucose flux might regulate hippocampal memory processing through an increase of Glut4 translocation in this region (Pearson-Leary and McNay, 2016). Thus, insulin resistance in brain, due to impaired insulin receptor signaling and/or decreased insulin transport through the blood-brain-barrier, could severely affect normal cognitive processes. This becomes important when considering risk factors associated with insulin resistance, such as obesity, poor diet, physical inactivity, aging and genetic predisposition, that were further related with cognitive dysfunction and dementia (Biessels and Reagan, 2015; Bartke, 2008). Most of these factors are modifiable, indicating that prevention is crucial to avoid detrimental effects of insulin resistance.

Mediterranean diet has been largely associated with lower incidence of age-related diseases, such as Alzheimer´s disease (AD). For instance, amelioration of insulin signaling was seen after treatment with hydroxytyrosol, the dominant polyphenol in extra virgin olive oil, in an astrocytic model of AD (Crespo et al., 2017b), suggesting that diet fats could play an important role at this level. Moreover, during aging, there is a constant

loss of the polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA) in the nervous system, both in neurons and glial cells, which has been associated with pathologies such as senile dementia and AD (Jicha and Markesbery, 2010). For this reason, DHA supplementation to this risk group is of special relevance, aiming to prevent or alleviate the symptoms of these and other pathologies.

FAO and WHO recommend a daily intake in adults of at least 500 mg / day EPA (eicosapentaenoic acid) + DHA (FAO et al., 2012). However, the current recommendations aim to increase the consumption of both free fatty acids (FFAs) through the intake of fatty fish, functional foods, nutraceuticals or consuming supplemented foods (Riediger et al., 2009). Regarding this, n-3 FAs associated to phospholipids are more bioavailable to different tissues, such as the brain (Wijendran et al., 2002). For this reason, krill oil is an adequate supplement, since it is rich in n-3 FAs incorporated into phosphatidylcholine (PC) and its beneficial effects have been described on blood lipids (Berge et al., 2014), inflammation (Deutsch, 2007), and even at cognitive function levels in the elderly (Konagai et al., 2013).

Other source of FFAs is milk fat. The milk fat globule consists of a core, mainly composed of triacylglycerides (TAG; 98%–99%), and different concentrations of lipid compounds such as diacylglicerides, monoacylglycerides, FFAs, and cholesterol. This core is surrounded by the milk fat globule membrane (MFGM), which contains different phospho- and sphingolipids and has potential positive effects on human health, namely in neurological pathologies (Castro-Gomez et al., 2015). Buttermilk (BM), a byproduct obtained from butter manufacturing with a high content of MFGM, accounts for a significantly increased polar lipids content that may reach up to 20% of total lipid concentration (Castro-Gomez et al., 2016), which could be further increased by using

food-grade solvents to obtain BM lipids and isolate different BM fractions (Castro-Gomez, 2016).

One possible application of these BM fractions might be as functional food rich in phospho- and sphingolipids, along with linolenic acid, DHA and EPA precursor. Particularly, phosphatidylserine (PS) and sphingomyelin (SM) are present in this fractions, playing PS an important role in cellular functions including mitochondrial membrane integrity, release of presynaptic neurotransmitters, activity of postsynaptic receptors and activation of Protein Kinasa C in memory formation (Osella, Re et al., 2008). Also, decrease of SM in myelin content in the brain has been related to the slowing in the speed of the cognitive process associated with aging (Lu, Lee et al., 2011).

Due to the loss of PUFA during of aging, dietary supplementation with Butter milk fat concentrate (BMFC) and Krill oil Concentrate (KOC) could be an approach to overcome this deficit. For this reason, we aimed to study insulin signaling in hippocampus and cortex in response to dietary supplementation with bioactive phospholipids concentrates of krill oil (rich on 3-n FA and PC), BMFC (rich on linolenic acid, PS and SM) or a mix of both in aged rats. Mitochondrial activity and biogenesis, and synaptic signaling were also assessed.

Materials and methods

Materials

Buttermilk and krill oil concentrates (BMFC and KOC, respectively) and the elaboration of daily doses in the form of a jelly lollipop were produced at the Institute of Food Science Research (CIAL, Madrid, Spain). Briefly,BM fat was extracted by pressurized liquid extraction (PLE) using an accelerated solid ASE-200 extractor (Dionex Corp. Sunnyvale, CA). Fifteen grams of powdered BMwere mixed with sand (1:1, by weight) and loaded into a stainless steel extraction cell. To obtain the maximum BMfat

yield, the extraction procedure was based on the optimized PLE method of Castro-Gomez et al. 2014. The lipid extracts were capped under nitrogen and stored at − 35 °C.

Animals

This research project followed the Guide for the Care and Use of Laboratory Animals, published by the US National Research Council (Eight Edition, 2010). Animal care was according to the standards proposed by the European Community (86/609/EEC). Animal experiments were approved by the Animal Experimentation Committee of the National University of Distance Education. A total of 41 male Wistar rats (9 months of age) were purchased from Charles River Laboratories (Barcelona, Spain). Rats were distributed randomly in groups of two per box and maintained in a 12:12 light-dark cycle (8 a.m. to 8 p.m.), with constant temperature and humidity conditions (22 ± 2° C and 50% RH), during the following 9 months. Food and water were given ad libitum.

Experimental design and diets

Since 18-months' rats are considered old and present a variety of cognitive deficiency (CD) symptoms associated with aging, when animals reached this age they were randomly assigned to four experimental groups (**Table 1**). All groups were given a standard diet EURodent (LabDiet, San Luis, Misuri) plus a group-specific supplement intake in form of frozen strawberry jellies: 1) control group - refined olive oil, 2) BMFC group – concentrate of phospholipids from dairy fat globule membrane, 3) KOC group - concentrate of krill oil (KO) omega-3 fatty acids (eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)) enriched phospholipids, and 4) BMFC + KOC group – one identical jelly containing the same treatment from group 2 and group 3 given simultaneously. Detailed composition of different lipid classes is described in **Table 2**. Full intake of supplements in form of frozen strawberry jellies was visually verified every day.

The nutritional composition of the daily diet of the four experimental groups is shown in **Table 3**. Rats were sacrificed by decapitation after three months of supplementation, following a 12-hour fast. Hippocampus and temporal cortex were quickly extracted, washed in PBS, snap-frozen in liquid nitrogen, and stored at -80 ˚C. Blood samples were collected with heparin (100UI/mL), centrifuged for plasma collection at 1500 xg for 15 minutes and stored at -80 ºC until used.

Determination of circulating biochemical parameters.

Concentration of plasma glucose was measured with commercial kits (WAKO, Neuss, Germany) and insulin levels were determined by an ELISA kit (Rat Insulin, 96-well plate assay, Millipore, Madrid, Spain), according to the manufacturer's instructions. The index of insulin resistance, a.k.a. homeostasis model assessment ratio (HOMA-R), was calculated using the following formula:

HOMA= fasting glucose (mmol/L)×fasting insulin (μIU/mL)/22.5.

Measurement of ATP levels in tissue samples

To evaluate the energy status of the cells in the hippocampus and temporal cortex, a bioluminescent assay was used to assess intracellular adenosine triphosphate (ATP) levels (Sigma- Aldrich, St Lois MO, USA). The homogenized samples were previously filtered through 0.22 pm filters (Millipore Corp., Bedford, Mass.). The kit was performed according to the manufacturer's protocol and luminescence was measured using a microplate reader at 570 nm (Biochrom Asys UVM 340, Cambridge, UK).

RNA isolation and qRT-PCR

Total RNA extraction from both tissues were performed according to the manufacturer's instructions for the miRNeasy Mini kit (Qiagen, Madrid, Spain), including DNA digestion. RNA quantity and purity were analysed using a NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Spain) and RNA

integrity using an Agilent's 2100 bioanalyser. Total RNA was converted into first strand cDNA using miScript®II Reverse Transcription kit, Qiagen (Izasa, Barcelona, Spain) according to the manufacturer's guidelines.

Electron transport chain array

A Mitochondria energy metabolism (SAB Target List) H384 Predesigned 384-well panel (Bio-Rad, Madrid, Spain) was carried out. Five samples of each experimental group with the highest RINs were selected to perform the assay. The pre-designed plates were formed by 87 genes, within these 10 are reference genes, 27 genes belong to complex I; 4 to complex II, 6 to complex III, 15 to complex IV, 18 to complex V, and 7 are associated with the respiratory chain, although they are not included in any complex.

qRT-PCR reactions were performed using miScript SYBR Green PCR Kit from Qiagen (Izasa, Madrid, Spain) using a 7900HT Real-Time PCR System (Life Technologies, Alcobendas, Spain). Cycling conditions were a first step of activation at 95 °C for 15 minutes; 40 cycles of denaturation at 94 °C for 15 seconds, annealing at 58 °C for 30 seconds and a final step for dissociation curve. PPA1 was identified by NormFinder as the most stable reference gene among 10 other candidates.

Determination of synaptoproteins gene expression.

Gene expression analysis of genes encoding proteins involved in the nerve synapses were performed by qRT-PCR (see above) in a 384-well plate using ABI PRISM 7900HT real-time PCR system. Cycling conditions were the same as describe above. Specific primers for each gene were designed using Primer3 software (**Supplementary Table 1**). Quantification of relative gene expression was performed using the comparative delta Ct method using GAPDH as reference genes for normalization.

Western blot assays

Temporal cortex and hippocampus samples were homogenized with RIPA lysis buffer containing protease and phosphatase inhibitors (Sigma-Aldrich, Madrid, Spain). Homogenates were kept at -80°C for 24 h, centrifuged (4 °C, 12000 xg, 30 min) and supernatants were collected. Total protein concentrations were determined using BCA protein assay reagent (ThermoFisher Scientific, Madrid, Spain). Equal amounts of protein (30 μg) were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad, Madrid, Spain). Membranes were blocked with 5% non-fat milk powder in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 2 h at room temperature, and then incubated overnight at 4 °C with various primary antibodies regarding key components of the insulin signaling pathway, components of the neurotransmissions and neurodegenerations (**Supplementary Table 2**) and involved in respiratory mithochondrial chain (abcam ab110413). Following incubation with appropriate secondary antibodies protein bands were detected by an enhanced chemiluminescence method using the ECL kit (Bio-Rad, Madrid, Spain). Normalization of total protein expression was carried out using GAPDH or with their corresponding total form in the case of phosphorylated proteins.

Statistical analysis

Statistical analyses were carried out using GradPad Prism 7.02 software (GraphPad Software, Inc., La Jolla, CA, USA). For the gene expression analysis, data was quantified using the ΔΔCt method and fold-change values were reported as $2^{-(\Delta\Delta Ct)}$. Data from independent samples from all experiments was compared by one-way ANOVA using Tuckey test to compare all study groups. Values of $p < 0.05$ was considered significant. Results are presented as means ± SEM.

Results

Combination of BMFC and KOC improves peripheral insulin sensitivity

Aged rats fed with isocaloric diets (**Table 1**) during 3 months did not show any apparent changes in body weight (data not shown). Since insulin resistance is tightly associated to aging and cognitive dysfunction (Umegaki et al., 2017), we wondered whether different dietary supplements affected glycemic profiles. No significant differences in glucose levels in any of the study groups were observed (**Figure 1A**). However, glucose levels in all groups indicated that there is an insulin resistance process, since the normal physiological glucose range is around 75.4 ± 5.5 mg/ml in adults rats (from 4 months) (Burgos-Ramos et al., 2011). Indeed, the studied aged rats presented values closed to 150 mg/dl. Along with this, serum insulin concentrations were higher than in adult rats. Compared to the control group, the combination of BMFC with KOC reached lower insulin levels (**Figure 1B**). This observation was further confirmed by the HOMA ratio (**Figure 1C**), suggesting that the mixture improves peripheral insulin sensitivity.

Hippocampal and cortical insulin signaling is increased in high phospholipid concentrate diets

Dysregulation of brain insulin signaling has been linked to aging and neurodegenerative disorders (Biessels and Reagan, 2015; Ghasemi et al., 2013; Kleinridders et al., 2014). To determine whether the different supplements influence central insulin signaling, we next evaluated key insulin pathway molecules in hippocampus and cerebral cortex. Protein analysis from hippocampal samples showed a significant increase in the insulin receptor subunit IRβ expression levels both in KOC and BMFC+KOC (**Figure 2A**), whereas the activated insulin substrate IRS1 was augmented in those groups supplement with BMFC. This activation mediated by insulin receptor was not observed in the IRS2 protein. Since

cytoplasmic insulin signaling is mediated through PI3K, we also explored the expression levels of the regulatory subunit p85 from this kinase. As seen for IRS1, BMFC supplementation rose PI3K expression levels. Since the last step of the insulin pathway, Akt activation, was detected in hippocampal samples of the three experimental groups, it seems possible that a high phospholipid diet improves hippocampal insulin signaling. Moreover, cerebral cortex insulin signal analysis showed that this route was also activated in this area (**Figure 2B**), suggesting that this effect is not only restricted to one brain region.

Activation of the insulin intracellular signaling stimulates the translocation of the Glut4 transporter facilitating glucose uptake (Biessels and Reagan, 2015). Thus, we examined transporter levels in our samples (**Figure 2A and B**). However, we did not find significant differences in Glut4 levels in any of the supplemented groups.

BMFC+KOC Ameliorate Cellular Energy States and Increase Mitochondrial Biogenesis in Hippocampus

Energetic state levels in brain tissue are influenced by mitochondrial biogenesis, which can be dramatically damaged during aging. Thus, we first evaluated the intracellular levels of adenosine triphosphate (ATP) in hippocampal samples. We found that all experimental groups showed an increase in ATP levels, being significant in BMFC animals (**Figure 3A**). Interestingly, this improvement was not detected in cerebral cortex samples (**Figure 3B**), suggesting that supplementation with bioactive phospholipids differently affects brain areas at this level. Thus, we wanted to figure out whether this increased energetic state in hippocampus could be due to an augmented mitochondrial biogenesis. To achieve this, we carried out protein analysis expression of the five complexes (**Figure 3C**). Animals fed with both BMFC and KOC supplement increased the expression of proteins from all the complexes studied, suggesting that amelioration of

cell energy state is mediated by an increase in mitochondrial biogenesis. We also evaluated the expression of 70 genes corresponding to the five mitochondrial complexes and seven associated with the respiratory chain (not included in any complex) in the hippocampus. An increase in the expression of key genes involved in the complexes I, IV, and V (Fig. 3d) was detected by qRT-PCR, confirming the observed changes in protein levels.

Mitochondrial biogenesis in BMFC+KOC animals is mediated through BDNF

In order to determine whether the increased levels in mitochondrial complex proteins were mediated by the regulator of mitochondrial biogenesis, the peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), we explored the levels of this protein in hippocampal and cortical samples (**Figure 4A and 4B**). As seen is Figure 4A and 4B, PGC1-α was increased in BMFC+KOC rats in both brain areas. Since, this coactivator is regulated by the master metabolic regulator 5' AMP-activated protein kinase (AMPK), we further explored the levels of this kinase along with the NAD^+-dependent type III deacetylase SIRT1 (Canto et al., 2009). Strikingly, although phosphorylated levels of AMPK were only increased in the KOC group both in hippocampus and cerebral cortex, SIRT1 levels were decreased in all experimental groups in the hippocampus and a trend (not significant) was observed in the cortex. As it has been described that PGC-1α activation requires both AMPK and SIRT1 (Canto et al., 2009), our results suggest that AMPK-SIRT1-mediated PGC1-α regulation is not taking place in our model.

Brain-derived neurotrophic factor (BDNF) has been implicated in neural ATP enhancement and in PGC-1α activation (Marosi and Mattson, 2014a). Thus, we next analyzed BDNF levels in hippocampal samples (Figure 4A). Only BMFC+KOC supplemented animals showed an increase in BDNF levels, in accordance with the results

for PGC-1α. However, although cortical BDNF was increased in all treated group, in this brain area it did not reach significant levels. This suggest that the incremented of mitochondrial biogenesis observed in hippocampus of BMFC+KOC rats could be mediated by BDNF. It has been proposed that BDNF exerts its actions through the mammalian target of rapamycin (mTOR), favoring local protein synthesis in dendrites (Takei et al., 2004). Thus, we examined mTOR levels in hippocampal and cortical samples. Increased levels of mTOR activity were detected in rats supplemented with BMFC+KOC, indicating that improvement in hippocampal energy state was mediated by mTOR through BDNF.

Synaptic proteins are differently regulated in distinct brain areas

Since mitochondrial biogenesis is necessary for BDNF to stimulate the formation of new synapses and to maintain the existing ones (Marosi and Mattson, 2014a), we wonder whether the observed changes in energy status after supplement treatment can be linked to synaptic signaling. Thus, we first analyzed the expression levels of genes encoding pre- and postsynaptic proteins in hippocampal samples (**Figure 5A**). Interestingly, syntaxin 1A gene levels (*Stx1A*) were highly augmented in BMFC+KOC animals, whereas synapsin I (*Syn1*) and synaptotagmin 1 (*Syt1*) were not changed after the different treatments. We further examined postsynaptic structural proteins PDS95, PDS93 and SAP-102 levels (*Dlg4*, *Dlg2* and *Dlg3*, respectively) and found that, surprisingly, while *Dlg4* levels were increased in KOC rats, expression of the three genes were decreased in the BMFC+KOC group. In order to confirm these results, we developed western blots for the plasma membrane protein Stx1A and two other presynaptic proteins associated with synaptic vesicles, synaptobrevin 2 (Vamp2) and the chaperone α-synuclein (α-Syn) (**Figure 5B**). Although *Stx1A* mRNA levels was increased in the BMFC+KOC animals,

protein levels were not changed in any of the experimental groups. Only Vamp-2 was increased in all supplemented animals.

In the case of cerebral cortex samples, gene expression of the pre- and postsynaptic proteins showed increased levels in some of them (**Figure 5C**). *Stx1A* levels were significantly increased in BMFC+KOC rats, whereas *Syt1* mRNA was upregulated in all studied groups compared to that of control animals. At protein level (**Figure 5D**), Stx1a and α-Syn was icreased in KOC group and only α-Syn was augmented in BMFC group. These results highlight different synaptic regulation in distinct brain areas.

Discussion

There is growing evidence that cognitive decline associated to aging can be partially prevented through the change of certain lifestyle factors, such as diet and regular exercise (Fontana et al., 2014; Daviglus et al., 2011). In fact, Mediterranean diet has been related with lower incidence of age-related diseases, such as AD and cardiovascular disease (Jackson et al., 2016; Estruch et al., 2013). Thus, dietary fats, such as those from olive oil, could act as key players in aging effects, ameliorating, for instance, insulin signalling in brain (Crespo et al., 2017a). Moreover, diet supplementation with PUFA, such as DHA, or the more available n-3 FAs associated to phospholipids, such as krill oil, could be a good approach in order to prevent aging (Wijendran et al., 2002; Konagai et al., 2013). Although milk fat globule membranes supplementation in infant and children seems to have certain neurodevelopment benefits (Hernell et al., 2016), their effects on the elderly is poorly characterized (Kim et al., 2015; Minegishi et al., 2016).

As insulin resistance has been largely related with age and its associated diseases (Akintola and van Heemst, 2015), we aimed to study brain insulin signaling in response

to dietary supplementation with bioactive phospholipids concentrates of krill oil and butter milk in aged rats. After three months of supplementation, although all groups of animals showed clear signs of peripheral insulin resistance, only the combination of KOC and BMFC was able to improve peripheral insulin sensitivity. In fact, both krill oil and MFGM have been previously shown to reduce insulin levels in type 2 diabetes and obese individuals, respectively (Lobraico et al., 2015; Demmer et al., 2016), suggesting that dietary FFA/phospholipids could help at this level. In order to know whether this amelioration in peripheral insulin resistance could also be detected at central nervous system level, we further explored insulin signaling in hippocampus and cerebral cortex, both brain areas implicated in cognitive processes. Interestingly, insulin signaling was improved in both structures, since the cascade of events was active when animals were supplemented with BMFC, KOC or both. These results highlight the importance of dietary fats in the compensation of disturbed insulin pathway observed in aging.

Considering that the energetic state level in brain is influenced by its mitochondrial content, we further explored brain energy balance. Interestingly, the hippocampus of supplemented rats—mainly with BMFC or the combination of KOC and BMFC—showed an increase in ATP levels, whereas no difference was observed in the cerebral cortex, suggesting that dietary fats induce different energy states in distinct brain areas, which is coherent with the observation that n-3 fatty acids incorporate into the various brain structures in a non-random, selected fashion (Lamaziere et al, 2011). The increased energy status of the hippocampus might be due to an increase in mitochondrial biogenesis, because both mitochondrial complexproteins and PGC-1α, the key regulator of mitochondrial biogenesis, were augmented in BMFC+KOC-supplemented animals.

Remarkably, although we did not record higher ATP levels in the cerebral cortex of supplemented animals, BMFC+KOC rats presented increased levels of PGC-1α, suggesting that the combination of the two types of fat favors the increase of this transcription factor, in turn indicating an increase in mitochondrial biogenesis signaling in this brain area. Since all treated animals presented increased levels of the neurotrophin BDNF, upregulation of PGC-1α appears to be mediated through it rather than AMPK-SIRT1. However, we only found a significant increase of BDNF in the hippocampus of BMFC+KOC animals. It is noteworthy that cerebral BDNF levels are quite low (Marosi and Mattson, 2014) and perhaps even a slight rise in BDNF levels could promote the action of PGC-1α in the cerebral cortex.

BDNF stimulates mitochondrial biogenesis to form new synapses and to maintain the existing ones (Marosi and Mattson, 2014b). In fact, local protein synthesis at dendrite level is crucial in order to sustain synaptic plasticity, being BDNF the main modulator (Takei et al., 2004). Thus, BDNF facilitates synaptogenesis inducing mTOR pathway, which mediates signals for local protein synthesis (Takei et al., 2004). According to our previous results, in hippocampus, combined supplementation with both KOC and BMFC favored mTOR activation. Thus, animals fed with both fats presented all the necessary steps for synapse formation (Takei et al., 2004).

Although our results point to an improvement in synaptogenesis in hippocampal samples of BMFC+KOC-supplemented animals, when we explored synaptic genes and proteins, we did not find significant increases in this brain area. Surprisingly, cerebral cortices presented an induction of synaptic protein synthesis in all supplemented groups, although the highest levels were detected in the BMFC+KOC-supplemented group. As mentioned above, although BDNF and mTOR levels increased, statistical significance was not seen regarding controls, but it may be possible that such increase is enough to

improve synaptic protein synthesis at the dendrite level.

In summary, dietary supplementation with KOC and/or BMFC has improved peripheral and central insulin resistance, suggesting that they could be a good approach to delay this process. Moreover, n-3 fatty acids ingested as phospholipids ameliorate BDNF brain levels, as described before (Wu et al., 2004), favoring an improvement in energy state within neurons and facilitating both mitochondrial and protein synthesis, which are necessary for synaptic plasticity. Thus, dietary supplementation with n-3 FAs could protect local protein synthesis and energy balance within dendrites, favoring neuronal health and delaying cognitive decline associated to age-related diseases.

Author contributions

JF, CV and FV designed the study. JTC, MCC, EBM, IPP, SB and AV performed experiments. MCC, JTC, EBR, CTZ, AD and FV wrote the manuscript. All authors approved the submission of the final version of the manuscript.

Conflict of interest

The authors declare that no conflict of interest exist.

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Figure Legend

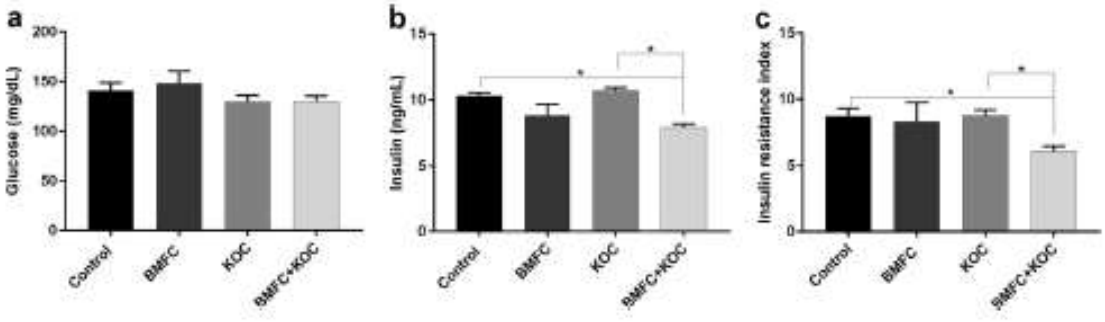

Figure 1. Determination of insulin, glucose and insulin resistance index in rat serum. Control: control group; BMFC: Concentrate of bioactive phospholipids from dairy fat globule membrane; KOC: concentrate of omega-3 fatty acids (Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) enriched in phospholipids from krill oil; BMFC+KOC: Mixing of the two bioactive phospholipid concentrates. A) Glucose levels expressed in mg/mL. B) Insulin levels expressed in mg/mL. C) homeostasis model assessment ratio (HOMA-R), which is an index of insulin resistance. Values are expressed as mean ± SEM of the mean. *Statistically significant difference regarding all study groups at p <0.05.

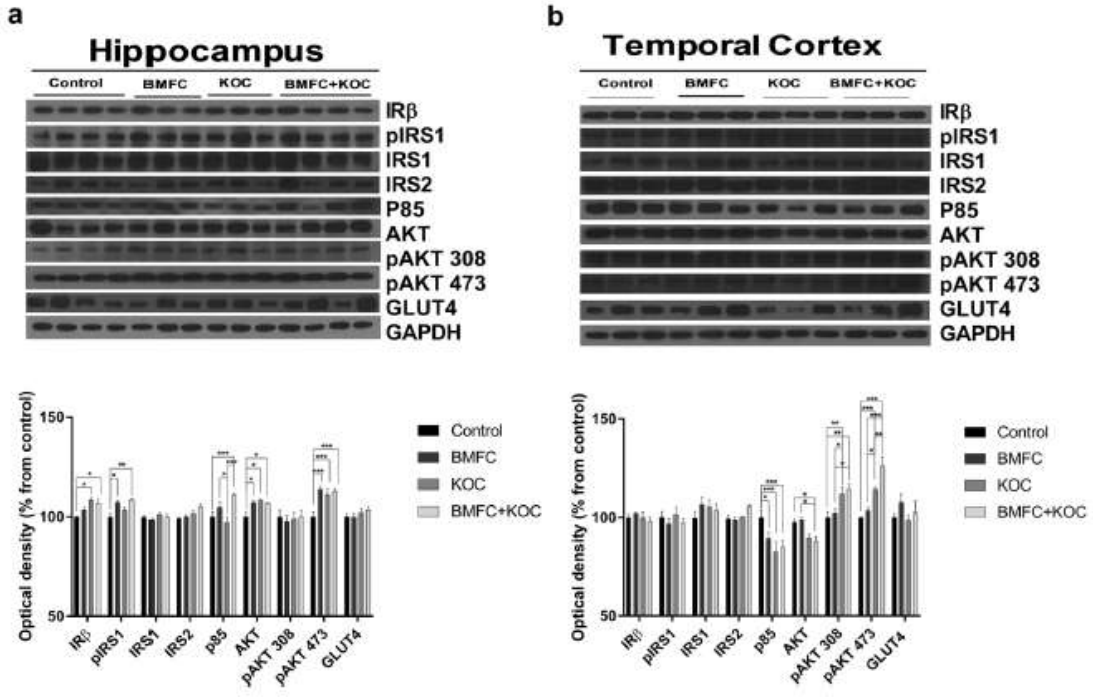

Figure 2. Western blot of proteins involved in the insulin-signaling pathway. A) Hippocampus samples B) Temporal cortex samples. IRβ: Insulin receptor; pIRS1: Phosphorylated insulin receptor substrate 1; IRS1: Insulin receptor substrate 1; IRS2: Insulin receptor substrate 2; p85: Subunit of phosphatidylinositol 3-kinase(PI3K); AKT: Serine/threonine protein kinase; pAKT 308: Phosphorylated AKT threonine 308; pAKT 473: Phosphorylated AKT serine 473; GLUT4: Glucose transporter 4; Control: Control group; BMFC: Concentrate of bioactive phospholipids from dairy fat globule membrane; KOC: concentrate of omega-3 fatty acids (Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) enriched in phospholipids from krill oil; BMFC+KOC: Mixing of the two bioactive phospholipid concentrates. Values are expressed as mean ± SEM of the mean. Statistically significant difference regarding all study groups at * p <0.05-0.005;** p <0.005-0,0005; *** p <0.0005.

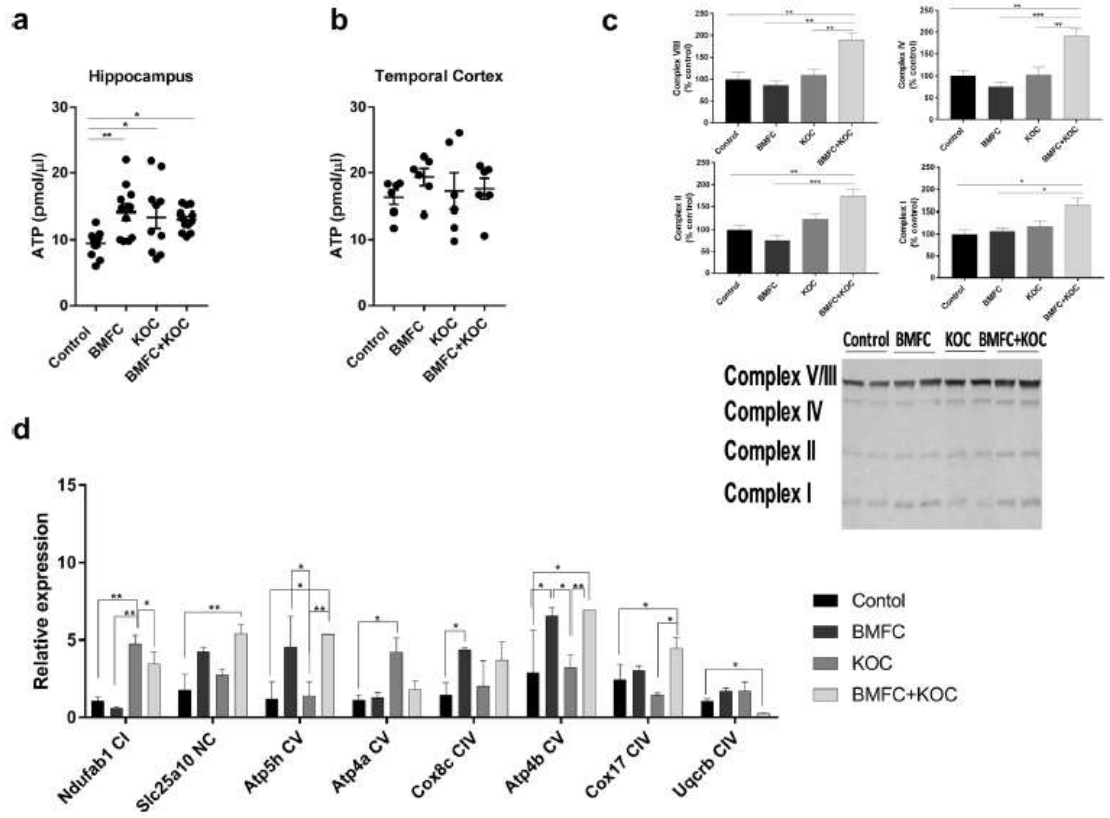

Figure 3. Measurement of cellular energy status and mitochondrial biogenesis. A & B) ATP levels expressed in pmol/µl. C) Western blots analysis involved in respiratory mitochondrial chain in hippocampus. D) Gene expression involved in mitochondrial respiratory chain pathway performed on hippocampus samples. ATP: adenosine triphosphate; Ndufab1:NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1; Slc25a10: solute carrier family 25 (mitochondrial carrier, dicarboxylate transporter), member 10; Atp5h:ATP synthase, H+ transporting, mitochondrial F0 complex, subunit D; Atp4a: ATPase H+/K+ transporting alpha subunit; Cox8c: cytochrome c oxidase subunit 8C; Atp4b:ATPase H+/K+ transporting beta subunit ; Cox17:cytochrome c oxidase copper chaperone; Uqcrb: ubiquinol-cytochrome c reductase binding protein; CI: Complex I; NC: does not belong to any complex; CIV: Complex IV; CV: Complex V; Control: control group; BMFC: Concentrate of bioactive phospholipids from dairy fat globule membrane; KOC: concentrate of omega-3 fatty acids (Eicosapentaenoic acid

(EPA) and docosahexaenoic acid (DHA) enriched in phospholipids from krill oil; BMFC+KOC: Mixing of the two bioactive phospholipid concentrates. Values are expressed as mean ± SEM of the mean. Statistically significant difference regarding all study groups at * p <0.05-0.005;** p <0.005-0,0005; *** p <0.0005.

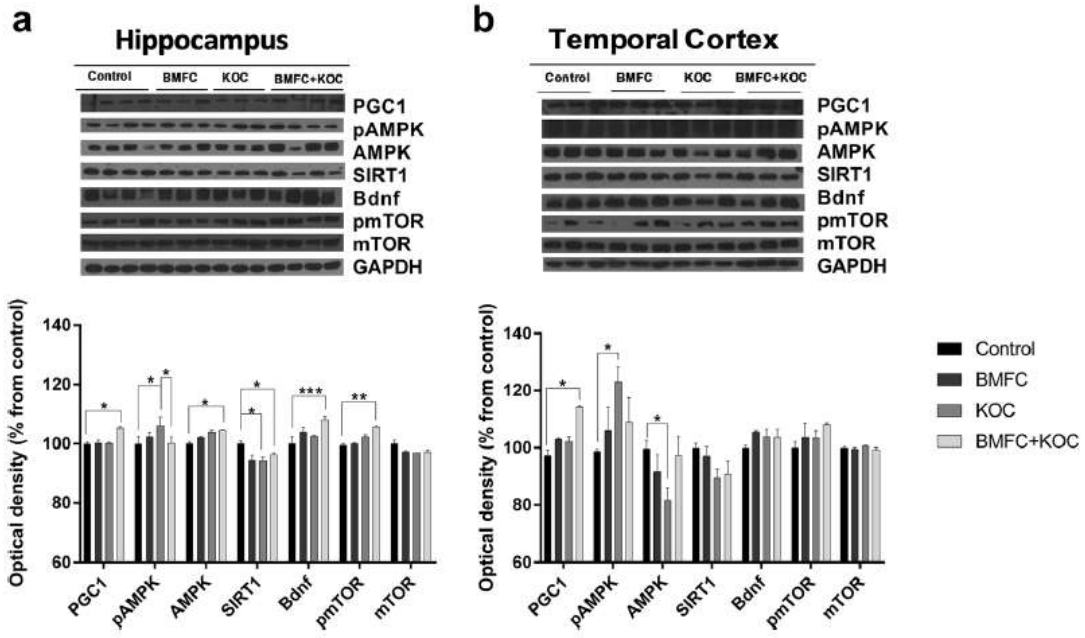

Figure 4. Western blot of proteins involved in mitochondrial biogenesis. A) Hippocampus samples B) Temporal cortex samples. PGC1: Peroxisome proliferative activated receptor, gamma, coactivador 1 alpha; pAMPK: phosphorylated AMPK; AMPK: AMP-activated protein kinase; SIRT1: Sirtuin 1; BDNF: brain derived neurotrophic factor; pmTOR: phosphorylated mechanistic target of rapamycin kinase; mTOR: mechanistic target of rapamycin kinase; Control: control group; BMFC: Concentrate of bioactive phospholipids from dairy fat globule membrane; KOC: concentrate of omega-3 fatty acids (Eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA)) enriched in phospholipids from krill oil; BMFC+KOC: Mixing of the two bioactive phospholipid concentrates. Values are expressed as mean ± SEM of the mean. Statistically significant difference regarding all study groups at * p <0.05-0.005; ** p <0.005-0,0005; *** p <0.0005.

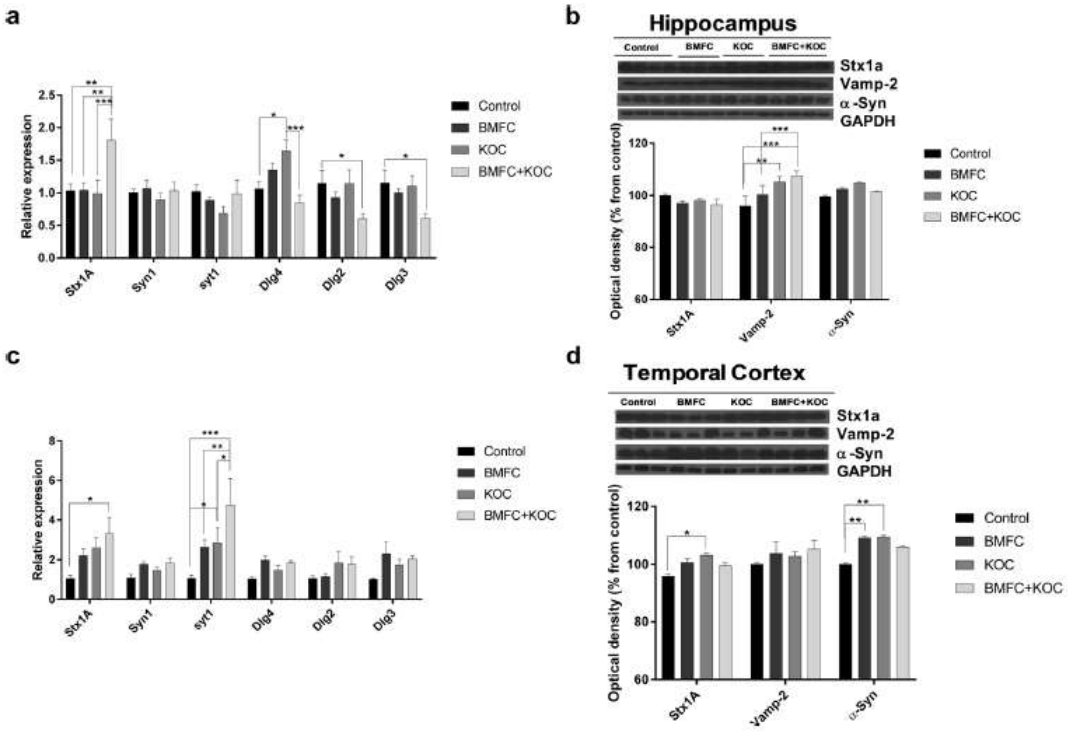

Figure 5. Synaptic-proteins expression in both tissues. A) Expression analysis of genes encoding proteins involved in the nerve synapse in hippocampus. B) Hippocampus Western blot of proteins involved in the neurotransmission signaling. C) Expression analysis of genes encoding proteins involved in the nerve synapse in Temporal cortex samples. D) Temporal Western blot of proteins involved in the neurotransmission signaling. STX1A: syntaxin 1A; SYN: synapsin I; SYT1: synaptotagmin 1; Dlg4: discs large MAGUK scaffold protein 4 (PSD95); Dlg2: discs large MAGUK scaffold protein 2 (PSD93); Dlg3: discs large MAGUK scaffold protein 3 (SAP-102); Vamp-2: vesicle-associated membrane protein 2; α-Syn: synuclein alpha; Control: control group; BMFC: Concentrate of bioactive phospholipids from dairy fat globule membrane; KOC: concentrate of omega-3 fatty acids (Eicosapantaenoic acid (EPA) and docosahexaenoic (DHA)) enriched in phospholipids from krill oil; BMFC+KOC: Mixing of the two bioactive phospholipid concentrates. Values are expressed as mean ± SEM of the mean. Statistically significant difference regarding all study groups at * p <0.05-0.005; ** p <0.005-0,0005; *** p <0.0005.

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Table 1. Experimental design of the study.

Experimental Group	*Control diet*	*Daily supplement (jelly)*
Control	*ad libitum*	70 mg refined olive oil
BMFC	*ad libitum*	70 mg of BMFC
KOC	*ad libitum*	70 mg of KOC
BMFC+KOC	*ad libitum*	70 mg of BMFC + 70 mg of KOC

Control: Control group; BMFC: Concentrate of bioactive phospholipids from dairy fat globule membrane; KOC: concentrate of omega-3 fatty acids (Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) enriched in phospholipids from krill oil; BMFC+KOC: Mixing of the two bioactive phospholipid concentrates.

Table 2. Composition of lipidic classes of concentrates used to formulate the different experimental diets.

	Control	***BMFC***	***KOC***	***BMFC + KOC***
PE (%)	-	0.55	15.66	5.16
PI (%)	-	-	1.92	0.77
PS (%)	-	-	16.92	6.79
PC (%)	-	99.45	38.74	72.48
SM (%)	-	-	26.76	14.79

Control: control group; BMFC: Concentrate of bioactive phospholipids from dairy fat globule membrane; KOC: concentrate of omega-3 fatty acids (Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) enriched in phospholipids from krill oil; BMFC+KOC: Mixing of the two bioactive phospholipid concentrates. %: Percentage of different phospholipids; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; SM: sphingomyelin.

Table 3. Nutritional composition of the daily diet of the five experimental groups.

	Control	***BMFC***	***KOC***	***BMFC + KOC***
Energy (Kcal)	201,07	201,11	201,07	201,75
Lipids (g)	1,82	1,82	1,82	1,89
Carbohydrates (g)	32,10	32,11	32,10	32,11
Fiber (g)	2,05	2,05	2,05	2,05
Proteins (g)	11,01	11,01	11,01	11,01

Control: control group; BMFC: Concentrate of bioactive phospholipids from dairy fat globule membrane; KOC: concentrate of omega-3 fatty acids (Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) enriched in phospholipids from krill oil; BMFC+KOC: Mixing of the two bioactive phospholipid concentrates.

Supplemental information (SI)

Supplemental Table S1. Sequence of synapto-proteins primers analyzed

Gene		Primers sequences
Presynaptic	Stx1A	F: 5′- GCCCTGTTTGATTACGACAA-3′
		R: 5′- ACTCGGTTCTGAGCTATGAG
	Syn1	F: 5′- TGTCCCGAAAGTTTGTG-3′
		R: 5′- GCGTTCTCGGTAGTCT
	Syt1	F: 5′- CTTCTCCAAGCACGACATCA-3′
		R: 5′- CCACCCACATCCATCTTCTT -3′
Postsynaptic	Dlg4	F: 5′- TAGGGCCCTGTTTGATTACG-3′
		R: 5′- TGGCCTTTAACCTTGACCAC -3′
	Dlg3	F: 5′- GAGTTCCCGCATAAGTTTGG-3
		R: 5′- CGGACACGTCTAAGATGCAG -3′
	Dlg2	F: 5′- GTCGGAGGTTTCCCACAGTA-3′
		R: 5′- CTGTGCAGCTCCACCATCTA -3′

Stx1A: syntaxin 1A; Syn: synapsin I; Syt1: synaptotagmin 1; Dlg4: discs large MAGUK scaffold protein 4; Dlg2: discs large MAGUK scaffold protein 2; Dlg3: discs large MAGUK scaffold protein 3; F: Forward; R: Reverse.

Supplemental Table 2. List of primary antibodies used to analyze the insulin signaling pathway, neurotransmission and neurodegeneration.

Antibodies	*Molecular Weight (kDa)*	*Host*	*Company*
p-thr308-AKT	60	Rabbit	Cell Signaling *
p-thr473-AKT	60	Rabbit	Cell Signaling *
AKT	60	Rabbit	Cell Signaling *
p-mTOR	289	Rabbit	Cell Signaling *
mTOR	289	Rabbit	Cell Signaling *
p-IRS1	90	Rabbit	Santa Cruz Biotechnology **
IRS1	90	Rabbit	Santa Cruz Biotechnology **
IRS2	185	Mouse	Millipore *
IRβ	75-100	Mouse	Santa Cruz Biotechnology **
p85 PI3K	75-85	Mouse	Santa Cruz Biotechnology **
p-AMPK α1/2	60	Rabbit	Santa Cruz Biotechnology **
AMPK α1/2	63	Mouse	Santa Cruz Biotechnology **
SIRT1	120	Rabbit	Santa Cruz Biotechnology **
PGC1	90	Rabbit	Santa Cruz Biotechnology **
GLUT2	55-60	Rabbit	AD Internacional ***
GLUT4	50-63	Mouse	Santa Cruz Biotechnology **
VAMP-2	18	Mouse	Santa Cruz Biotechnology **
Syntaxin 1	35	Mouse	Santa Cruz Biotechnology **
p-TAU	50-80	Mouse	Cell Signaling *

TAU-5	46-80	Mouse	Santa Cruz Biotechnology **
α-synuclein	19	Mouse	Santa Cruz Biotechnology **
BDNF	14	Mouse	Novusbio ****
UBB	9	Mouse	Millipore *

*(Madrid, Spain); **(Heidelberg, Germany); ***(San Antonio, USA); ****(Abingdon, United Kingdom) Leyenda anticuerpos