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DHA/EPA supplementation decreases anxiety-like behaviour, but it does not ameliorate metabolic profile in obese male rats

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Abstract

Obesity is a major public health problem that predisposes to several diseases and higher mortality in patients with COVID-19. Obesity also generates neuroinflammation, which predisposes to the development of neuropsychiatric diseases. Since there is a lack of effective treatments for obesity, the search for new strategies to reverse its consequences is urgent. In this perspective, the anti-inflammatory properties of omega-3 polyunsaturated fatty acids such as DHA/EPA might reduce the harmful effects of obesity. Here, we used the cafeteria diet (CAF) model to induce obesity in Wistar rats. Animals received ultra-processed food for 20 weeks, and DHA/EPA supplementation (500 mg/kg per d) was performed between the 16th and the 20th week. At the end of the experiment, it was evaluated: body weight, visceral fat deposition, plasma glucose, insulin and triglycerides, and it was also measured the levels of inflammatory cytokines TNF-α and IL-6 in plasma and liver, and TNF-α in the prefrontal cortex. The elevated plus maze test was performed to analyse anxiety-like behaviour. Our results demonstrated that DHA/EPA could not reverse weight and fat gain and did not modify plasma dosages. However, there was a decrease in IL-6 in the liver (DHA/EPA effect: $P = 0.023$) and TNF-α in the brain (CAF compared with CAF + DHA/EPA, $P < 0.05$). Also, there was a decrease in the anxiety index in CAF + DHA/EPA compared with the CAF group ($P < 0.01$). Thus, DHA/EPA supplementation is helpful to reverse the consequences of obesity in the brain.

Key words: **Obesity: Cafeteria diet: Neuroinflammation: Anxiety: *n*-3 PUFA**

The global prevalence of obesity has risen over the years representing a major health issue nowadays[1]. Obesity increases the risk of developing several chronic diseases such as diabetes mellitus, cardiovascular diseases, some types of cancer and non-alcoholic fatty liver disease[2,3]. More recently, increased pathogenicity to infectious diseases has also been linked to excessive body fat[4], seen by higher mortality rates in patients with obesity in the COVID-19 pandemic[5,6].

Treatments available for obesity are not efficient for many patients since they are based on (1) lifestyle interventions, which commonly result in weight regain; (2) pharmacological treatments, but most of them are not well tolerated because of side effects; and (3) bariatric surgery, which is associated with several risks and it should be carefully recommended[1]. Considering the harmful effects of obesity, the search for new therapeutic strategies is urgent. In this perspective, it has been shown that the consumption of omega-3 (*n*-3) polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) is associated with a significant reduction in plasma levels of triglycerides[7,8] and reduced fasting glucose levels[9]. In this regard, *n*-3 supplementation is recognised for reducing the risk of coronary heart

Abbreviations: CAF, cafeteria diet; CT, control diet; HFD, high-fat diet; IR-HOMA, insulin resistance-homeostasis model assessment.

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disease mortality and related events[10]. Its anti-inflammatory properties are demonstrated in several animal models of inflammatory diseases[11]. Moreover, *n*-3 may be related to anxiolytic and anti-depressant effects[12], although these results are controversial and need further investigations[13,14].

It is worth mentioning that obesity is related to a low-grade chronic inflammatory profile originating from adipose tissue's immune signals. These signals activate the NF-κB signalling pathway in the cells, followed by an increase in the transcription of proinflammatory cytokines such as interleukin 6 (IL-6) and tumour necrosis factor-alpha (TNF-*α*)[15]. The increased levels of inflammatory mediators appear not only in the periphery but also in the brain, establishing a neuroinflammatory state[16]. Pathological brain conditions may be initiated or intensified due to neuroinflammation such as cognitive decline and neurodegenerative diseases, characterised by impaired memory and attention[17,18], and psychiatric diseases, such as depression and anxiety[19]. In this regard, it is shown that obesity and metabolic dysfunction are correlated with cognitive dysfunction[20] and anxiety symptoms[21].

Among different protocols to induce obesity in animal models, the cafeteria diet (CAF) is beneficial since it mimics the human population's Western diet. CAF is composed of ultra-processed foods that are characterised by their high palatability. Therefore, it can lead to a hyperphagia state similar to the human pattern of food consumption[22]. Another advantage of animal studies using CAF is the presence of several food additives and the lack of vitamins and minerals[23]. Consequently, this diet induces metabolic changes related to obesity such as hepatic steatosis, increased visceral adiposity, glucose intolerance[24] and is also capable of inducing neuroinflammation in regions such as the hippocampus[25], which is essential for memory consolidation and neurogenesis[26].

Although DHA/EPA benefits in metabolic dysfunction are largely studied, there are still controversial findings. In addition, DHA/EPA effects on the brain are also debated. To elucidate the beneficial properties of DHA/EPA in the management of obesity and obesity-related neurological outcomes, we evaluated whether 4 weeks of DHA/EPA supplementation were able to change metabolic and neuroinflammatory parameters and anxiety-like behaviour in rats fed with CAF.

Materials and methods

Animals

Forty-eight adult male Wistar rats were obtained from the animal facility of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) and housed in plastic cages at 23 (SD 1)°C with a 12/12 h of light/dark cycle. This project was approved by the Institutional Animal Care and Use Committee of UFCSPA under the protocol Nº. 570/18. The procedures were designed to minimise the number and the suffering of animals following the international laws for laboratory animals' care. Only male rats were employed to avoid female hormonal fluctuations, which may impact the results of this study.

Table 1. Nutritional information of cafeteria diet*

Diet components	Energy (kJ)	Protein (g)	Carbohydrates (g)	Lipids (g)
Salty snacks	2142·2	9·2	56	28
Biscuits	2119·6	6·66	66·66	23·66
Wafer	2259·36	3·33	63·33	30·33
Chocolate	2292·83	3·33	60	33·33
Mortadella	1181·9	12	6	23·33
Sausage	1004·1	12	6	19
Soft drink	207·1	0	12	0

* Values obtained from food labels considering a portion of 100 g of food or 100 ml of soft drink.

Experimental groups and diet

Animals (*n*=12/group) were randomly allocated into four groups: Control diet (CT); CT + DHA/EPA; CAF; and CAF + DHA/EPA. Animals received normal chow diet Nuvilab® CR-1 (Nuvital®) or normal chow diet plus CAF for 20 weeks with DHA/EPA supplementation starting at the 16th week. Groups supplemented with DHA/EPA received Mega DHA® (Vitafor®) in a high concentration of EPA (10 %) and DHA (50 %) at the dose of 500 mg/kg every day by gavage. The other groups received water (0.5 ml/rat) instead of DHA/EPA. The sample size was chosen based on previous studies using a similar methodology[27,28].

Cafeteria groups were fed with three distinct menus interchanged every 2 d to maintain novelty and stimulate consumption. Menus were composed of standard chow plus palatable human food as cookies, wafer, sausage, bologna and white chocolate. They also received orange-based soft drink besides water *ad libitum*. Every component's leftovers were weighed every 2 d, including soft-drink consumption, to determine food intake per cage.

The regular chow diet's total energy content was 14·22 kJ/g (3·4 kcal/g, 63 % carbohydrates, 26 % protein, 11 % fat). The CAF energy content was calculated based on the manufacturer's information and provided 18·8 kJ/g (4·5 kcal/g) distributed in 42 % carbohydrates, 16 % protein and 42 % lipids. Detailed nutritional information on the components of the diet is shown in Table 1. Animals were weighed weekly to determine weight gain.

Elevated plus maze test

Elevated plus maze was used to assess anxiety-like behaviour in rats. The test was conducted on the 20th week of the experiment and was applied as described by ref.[29]. The apparatus consisted of four arms 50 cm long and 10 cm wide. Two of those are open, and the other two arms are closed with walls (40 cm high) on the sides. The maze was 50 cm above the floor. The rats were placed on the central platform facing an open arm and freely explored the maze for 5 min. The entrance was defined when the four paws touched one of the arms. More time spent in the closed arm and a decrease in risk behaviour (head dipping) could indicate anxious-like behaviour[30,31].

The following parameters were analysed in the test: number of head dipping, the number of entries in the open and the

closed arms, total time spent in the open and closed arms. Subsequently, it was measured the percentage of open arm entries (100 × open arms entries/total arms entries; PEOA) and the percentage of time spent in the open arm (100 × time in the open arms/total time; PTOA)[30,31]. Also, the anxiety index was calculated using the formula: anxiety index = 1 − ((open arm time/5 min) + (open arm entry/total entry))/2[32]. Results vary from 0 to 1; values closer to 1 indicate high anxiety.

Tissue and blood collection

In the 20th week of the experiment, the rats were euthanised by decapitation after fasting for 6 h. Trunk blood was collected and, after 30 min, it was centrifuged at 3500 rpm for 10 min. Plasma obtained was aliquoted and stored at −80°C for further analysis. Brains were quickly removed. The prefrontal cortex was manually dissected and immediately frozen in liquid nitrogen. Samples were kept at −80°C until further processing.

Plasma metabolic parameters

Plasma levels of glucose and triglycerides were measured using commercial enzymatic colourimetric kits (Labtest©). Insulin levels were assessed by ELISA (Sigma©). Tests were conducted following the manufacturer's instructions.

Insulin resistance-homeostasis model assessment index

Insulin resistance-homeostasis model assessment (IR-HOMA) index was used to evaluate insulin resistance[33]. The IR-HOMA index was calculated using the formula: fasting blood glucose levels × fasting blood insulin levels/22.5[34].

Pro-inflammatory cytokines

Levels of inflammatory cytokines in plasma (IL-6 and TNF-α), liver (IL-6 and TNF-α) and prefrontal cortex (TNF-α) were quantified by ELISA (Invitrogen©), following the manufacturer's instructions.

Statistical analysis

Main effects and interactions were analysed with two-way ANOVA and differences between groups with Tukey post-hoc test. The results of two-way ANOVA are shown as the main effects (diet or DHA/EPA supplementation) and the interaction between diet and supplementation. Weight gain per week was analysed by two-way repeated-measures ANOVA. Rout test was used to remove outliers. All statistical analysis was conducted using Graphpad Prism 8 (GraphPad Software, Inc.). The results were expressed as mean values with their standard error of the mean. The results were considered statistically significant at $P < 0.05$.

Results

Weight gain, adiposity and metabolic profile

Weight gain was significantly increased in CAF groups (diet effect: $F_{(1,42)} = 114.2$, $P < 0.0001$) regardless of DHA/EPA supplementation (DHA/EPA effect: $F_{(1,42)} = 3.92$, $P = 0.054$; interaction: $F_{(1,42)} = 0.05$, $P = 0.81$), as shown in Fig. 1(a). Weight gain was significantly different between CAF and CT groups from the 8th week onwards (Fig. 1(b)). Similarly, the visceral fat deposition was higher in CAF-fed groups (diet effect: $F_{(1,42)} = 140.3$, $P < 0.0001$) with no effect of DHA/EPA (DHA/EPA effect: $F_{(1,42)} = 1.72$, $P = 0.19$; interaction: $F_{(1,42)} = 0.07$, $P = 0.78$) as demonstrated in Fig. 1(c).

Metabolic effects of CAF and DHA/EPA were analysed by measuring plasma levels of glucose, triglycerides, insulin and HOMA-index calculation. A diet effect ($F_{(1,35)} = 26.75$, $P < 0.0001$) was found in plasma glucose, which was increased in CAF groups. Two-way ANOVA did not show a significant effect of DHA/EPA supplementation (DHA/EPA effect: $F_{(1,35)} = 2.6$, $P = 0.11$; interaction: $F_{(1,35)} = 1.8$, $P = 0.18$). In the multiple comparisons, the CAF group was significantly different from the CT and CT + DHA/EPA groups ($P < 0.001$) (Fig. 2(a)). Also, CAF increased triglycerides levels (diet effect: $F_{(1,41)} = 45.39$, $P < 0.0001$) and DHA/EPA supplementation did not mitigate it (DHA/EPA effect: $F_{(1,41)} = 2.35$, $P = 0.13$; interaction: $F_{(1,41)} = 0.35$, $P = 0.55$) (Fig. 2(b)). Following that, insulin levels were also affected by the diet ($F_{(1,38)} = 12.62$, $P = 0.001$) but not by the DHA/EPA treatment (DHA/EPA effect: $F_{(1,38)} = 1.25$, $P = 0.27$; interaction: $F_{(1,38)} = 0.10$, $P = 0.75$) (Fig. 2(c)). Insulin resistance, assessed by the HOMA-index, also showed a CAF effect ($F_{(1,38)} = 12.28$, $P = 0.0012$), although the supplementation did not impact it (DHA/EPA effect: $F_{(1,38)} = 0.49$, $P = 0.48$; interaction: $F_{(1,38)} = 0.0009$, $P = 0.97$). However, Tukey post-hoc test showed a significant difference between CAF and CT + DHA/EPA group ($P < 0.05$) for insulin levels and HOMA-index (Fig. 2(d)).

Inflammatory markers

We also evaluated the levels of inflammatory cytokines TNF-α and IL-6 in plasma and liver, and TNF-α in the prefrontal cerebral cortex. Plasma TNF-α (diet effect: $F_{(1,25)} = 0.014$, $P = 0.9$; DHA/EPA effect: $F_{(1,25)} = 1.21$, $P = 0.28$; interaction: $F_{(1,25)} = 0.14$, $P = 0.70$) and IL-6 (diet effect: $F_{(1,22)} = 0.22$, $P = 0.63$; DHA/EPA effect: $F_{(1,22)} = 0.75$, $P = 0.39$; interaction: $F_{(1,22)} = 0.69$, $P = 0.41$) were affected neither by diet nor by supplementation (Fig. 3(a) and (b), respectively). However, in the liver, TNF-α levels were increased following CAF (diet effect: $F_{(1,24)} = 31.55$, $P < 0.0001$), with no effect of DHA/EPA (DHA/EPA effect: $F_{(1,24)} = 0.19$, $P = 0.66$; interaction: $F_{(1,24)} = 0.93$, $P = 0.34$), as shown in Fig. 3(c). Interestingly, IL-6 in the liver was not affected by diet (diet effect: $F_{(1,21)} = 0.02$, $P = 0.88$; interaction: $F_{(1,21)} = 0.50$, $P = 0.48$), but DHA/EPA supplementation diminished it in both CT and CAF groups (DHA/EPA effect: $F_{(1,21)} = 6.003$, $P = 0.0231$), although post-hoc analysis did not show differences among groups (Fig. 3(d)). In the prefrontal cerebral cortex, TNF-α level showed an interaction between diet and supplementation ($F_{(1,19)} = 6.121$, $P = 0.023$; diet effect: $F_{(1,19)} = 0.74$, $P = 0.39$; DHA/EPA effect: $F_{(1,19)} = 2.56$, $P = 0.12$). Post-hoc test also evidenced that DHA/EPA supplementation decreased TNF-α in the CAF + DHA/EPA compared with CAF (Fig. 3(e)), showing the ability of *n*-3 in reducing this pro-inflammatory cytokine in the cerebral cortex of obese animals.

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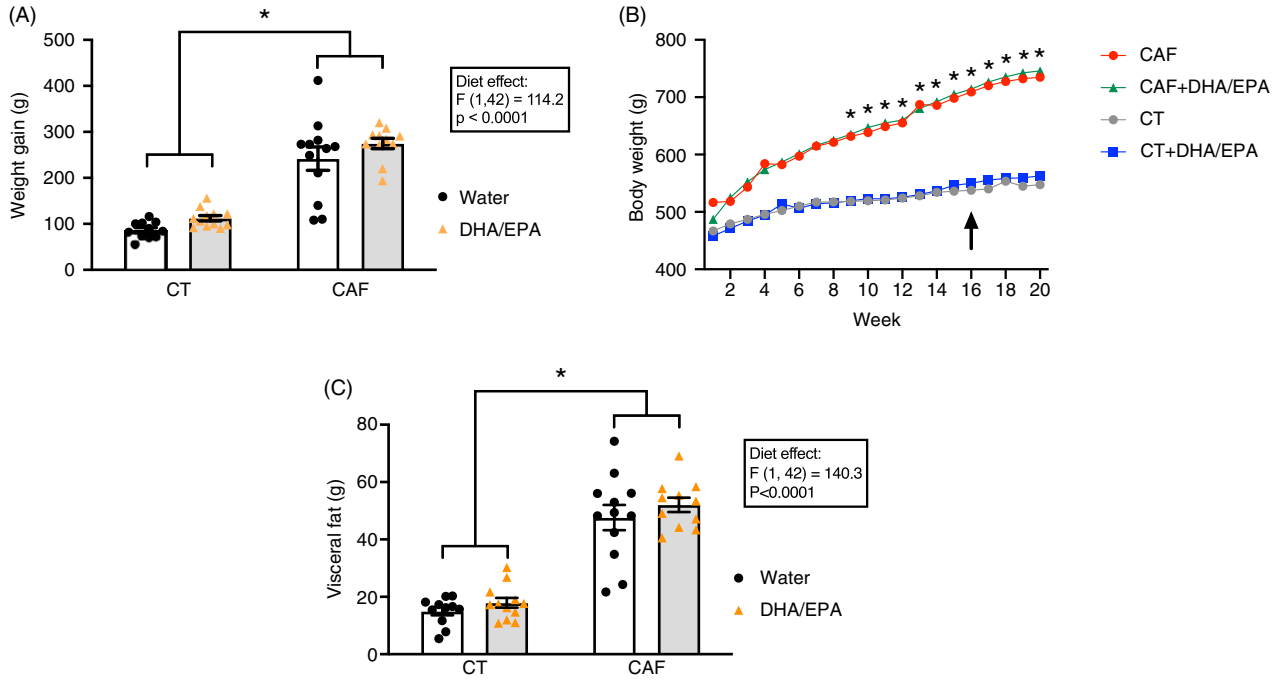

Fig. 1. DHA/EPA supplementation did not change weight gain or visceral adiposity in cafeteria diet (CAF)-fed rats. (a) Weight gain at the end of the experiment. (b) Body weight over time. (c) Visceral fat mass at the end of the experiment. Arrow indicates the beginning of DHA/EPA supplementation. Significant differences showed by two-way ANOVA regarding effects of diet (CAF and CAF + DHA/EPA *v.* CT and CT + DHA/EPA), EPA/DHA treatment (CAF and CT *v.* CAF + DHA/EPA and CT + DHA/EPA) and diet × DHA/EPA interactions are indicated in the text boxes. Multiple comparisons were performed by Tukey post-hoc test and are indicated as follows: $^{*}P < 0.0001$ comparing CAF *v.* CT or CT + DHA/EPA; CAF + DHA/EPA *v.* CT or CT + DHA/EPA. *n*=11–12 animals/group.

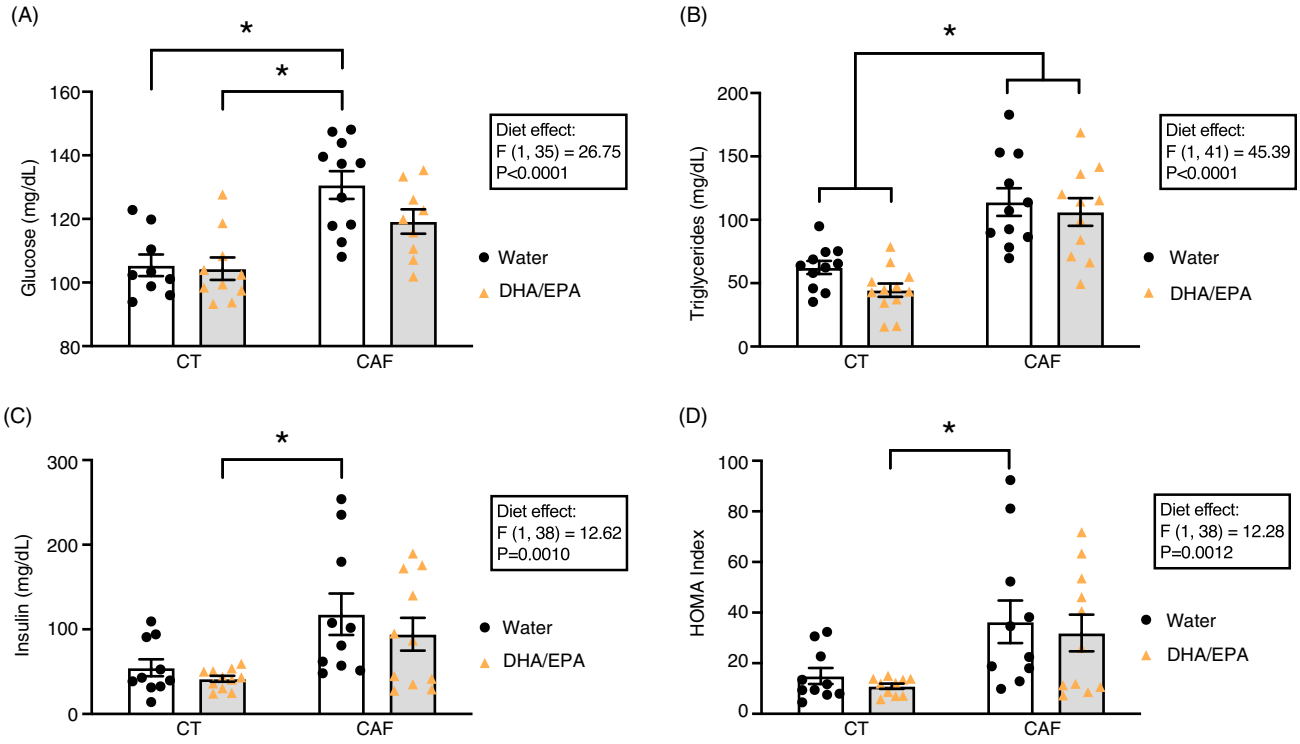

Fig. 2. DHA/EPA supplementation did not change glycaemic control and triglycerides levels following cafeteria diet (CAF). (a) Fasting plasma glucose. (b) Triglycerides levels. (c) Plasma insulin levels. (d) HOMA-Index. Significant differences showed by two-way ANOVA regarding effects of diet (CAF and CAF + DHA/EPA *v.* CT and CT + DHA/EPA), EPA/DHA treatment (CAF and CT *v.* CAF + DHA/EPA & CT + DHA/EPA) and diet × DHA/EPA interactions are indicated in the text boxes. Multiple comparisons were performed by Tukey post-hoc test and are indicated as follows: glucose: $^{*}P < 0.001$ comparing CAF *v.* CT or CT + DHA/EPA; triglycerides: $^{*}P < 0.01$ comparing CAF *v.* CT or CT + DHA/EPA; CAF + DHA/EPA *v.* CT or CT + DHA/EPA; insulin, HOMA: # $P < 0.05$ comparing CAF *v.* CT + DHA/EPA. *n*=9–12 animals/group.

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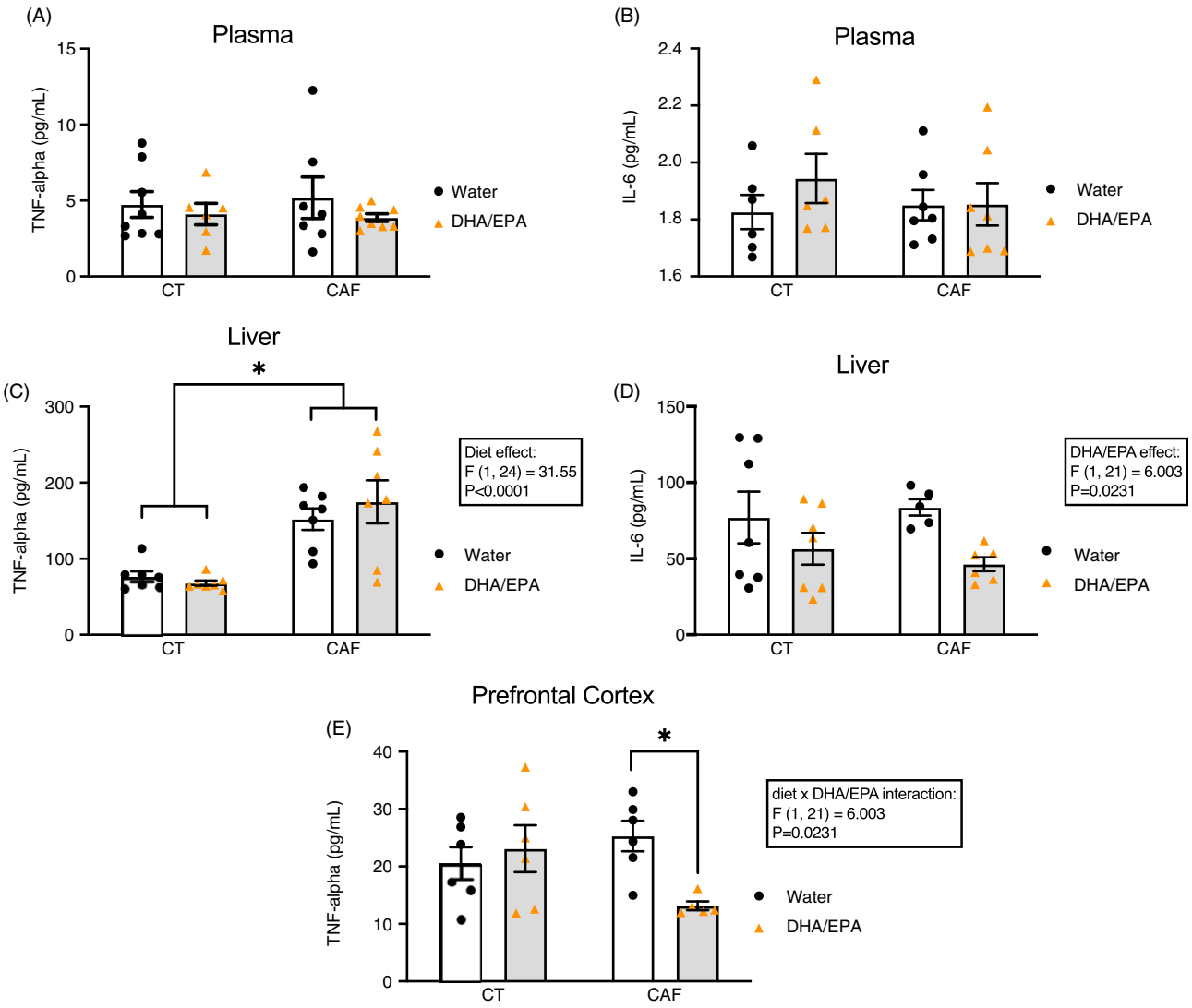

Fig. 3. DHA/EPA supplementation did not modify TNF-α and IL-6 levels in the plasma and liver, but it reduced TNF-α in the prefrontal cortex of cafeteria diet (CAF)-fed rats. (a) Plasma levels of TNF-α. (b) Plasma levels of IL-6. (c) TNF-α levels in the liver. (d) IL-6 levels in the liver. (e) TNF-α levels in the prefrontal cerebral cortex. Significant differences showed by two-way ANOVA regarding effects of diet (CAF and CAF + DHA/EPA *v.* CT and CT + DHA/EPA), DHA/EPA treatment (CAF and CT *v.* CAF + DHA/EPA and CT + DHA/EPA) and diet × DHA/EPA interactions are indicated in the text boxes. Multiple comparisons were performed by Tukey post-hoc test and are indicated as follows: TNF-α liver: *$P < 0.0001$ comparing CAF *v.* CT or CT + DHA/EPA; CAF + DHA/EPA *v.* CT or CT + DHA/EPA. TNF-α cortex: *$P < 0.05$ comparing CAF *v.* CAF + DHA/EPA. n=5–8 animals/group.

Evaluation of anxiety-like behaviour

Figure 4 shows results regarding the elevated plus maze task, which was chosen to evaluate anxiety-like behaviour. The number of head dipping (NHD), which assesses risk behaviour, was measured every time the rats inclined the head towards the lower arm; its increase is related to an anxiolytic manifestation[31]. Regarding NHD, we found a significant interaction between diet and supplementation (interaction: $F_{(1,33)} = 5.544$, $P = 0.024$; diet effect: $F_{(1,33)} = 0.2620$, $P = 0.61$; DHA/EPA effect: $F_{(1,33)} = 1.316$, $P = 0.25$), although Tukey post-hoc test did not show any difference among groups (Fig. 4(a)). Considering the number of entries in the open arms (NEOA, Fig. 4(b)) and the number of entries in the closed arms (NECA, Fig. 4(c)), there was a diet effect in both parameters, showing that rats fed with CAF had a reduced number of entries (NEOA – diet effect: $F_{(1,29)} = 6.96$, $P = 0.013$; interaction: $F_{(1,29)} = 0.83$, $P = 0.36$; DHA/EPA effect: $F_{(1,29)} = 0.83$, $P = 0.36$; NECA – diet effect: $F_{(1,32)} = 6.13$, $P = 0.018$; interaction: $F_{(1,32)} = 0.32$, $P = 0.57$; DHA/EPA effect: $F_{(1,32)} = 0.32$, $P = 0.57$). Furthermore, considering the percentage of entries in the open arms (PEOA, Fig. 4(d)), we found a significant interaction between diet and supplementation (interaction: $F_{(1,32)} = 6.27$, $P = 0.017$; diet effect: $F_{(1,32)} = 0.63$, $P = 0.43$; DHA/EPA effect: $F_{(1,32)} = 0.004$, $P = 0.94$), although Tukey post-hoc did not show differences. Another analysis we conducted was the time spent in the open arms (TSOA, Fig. 4(e)) and time spent in the closed arms (TSCA, Fig. 4(f)), which were altered by DHA/EPA supplementation (TSOA – DHA/EPA effect: $F_{(1,33)} = 6.51$, $P = 0.015$; interaction: $F_{(1,33)} = 3.89$, $P = 0.056$; diet effect: $F_{(1,33)} = 0.15$, $P = 0.69$; TSCA – DHA/EPA effect: $F_{(1,33)} = 6.51$, $P = 0.015$; interaction: $F_{(1,33)} = 3.89$, $P = 0.056$; diet effect: $F_{(1,33)} = 0.15$, $P = 0.69$). Also, Tukey post-hoc test indicated that CAF-fed animals showed

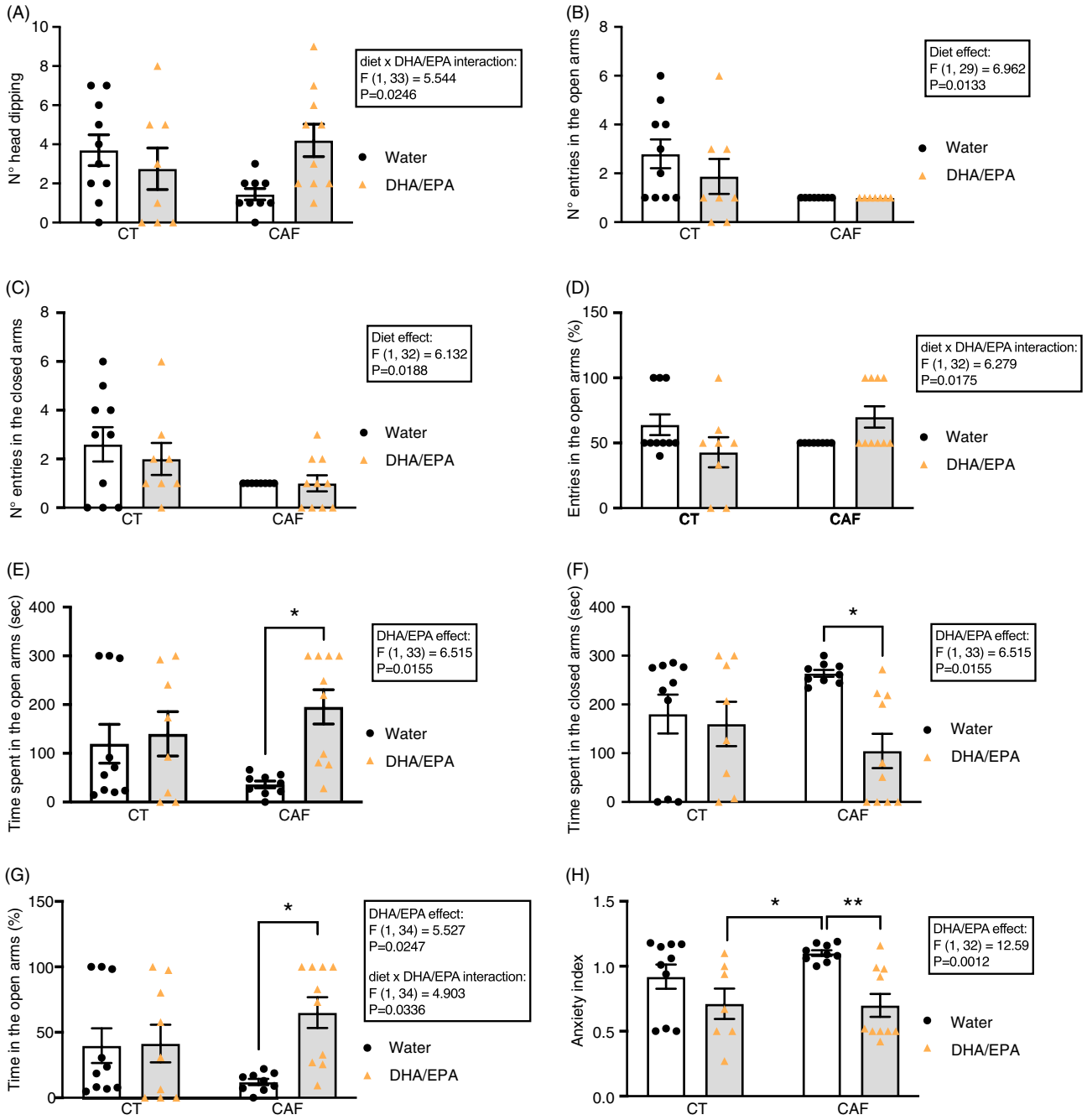

Fig. 4. DHA/EPA decreased anxiety-like behaviour in cafeteria diet (CAF)-fed rats. (a) Number of head dipping. (b) Number of entries in the open arms (NEOA). (c) Number of entries in the closed arms (NECA). (d) Percentage of entries in the open arms (PEOA). (e) Time spent in the open arms (TSOA). (f) Time spent in the closed arms (TSCA). (g) Percentage of time in the open arms (PTOA). (h) Anxiety index. Significant differences showed by two-way ANOVA regarding effects of diet (CAF and CAF + DHA/EPA *v.* CT and CT + DHA/EPA), DHA/EPA treatment (CAF and CT *v.* CAF + DHA/EPA and CT + DHA/EPA) and diet × DHA/EPA interactions are indicated in the text boxes. Multiple comparisons were performed by Tukey post-hoc test and are indicated as follows: CT, control group. TSOA, TSCA, PTOA: $^{*}P < 0.05$ comparing CAF *v.* CAF + DHA/EPA; anxiety index: $^{*}P < 0.05$ comparing CAF *v.* CT + DHA/EPA, $^{**}P < 0.01$ comparing CAF *v.* CAF + DHA/EPA. *n*=7–10 animals/group.

a significant reduction in TSOA ($P = 0.013$) and, consequently, increased TSCA ($P = 0.013$) in comparison with the CAF + DHA/EPA group. These findings point to a decrease in anxiety-like behaviour in obese rats supplemented with DHA/EPA since higher time spent in the open arms indicates reduced anxiety-like behaviour. These results were also seen in the percentage of time in the open arms (PTOA, Fig. 4(g)), which, besides the effect of DHA/EPA, we also found an interaction between diet and supplementation (DHA/EPA effect: $F_{(1,34)} = 5.52$, $P = 0.024$; interaction: $F_{(1,34)} = 4.90$, $P = 0.033$; diet effect: $F_{(1,34)} = 0.031$, $P = 0.86$). These findings were corroborated by the post-hoc test, which demonstrated a significant reduction of PTOA in the CAF group compared with the CAF + DHA/EPA group ($P = 0.014$). In addition, the anxiety

index was calculated (Fig. 4(h)). Two-way ANOVA showed an effect of DHA/EPA supplementation (DHA/EPA effect: $F_{(1,32)} = 12.59$, $P = 0.0012$; interaction: $F_{(1,32)} = 1.26$, $P = 0.26$; diet effect: $F_{(1,32)} = 0.96$, $P = 0.33$), and post-hoc test showed a higher score in CAF compared with the CAF + DHA/EPA ($P = 0.0088$) and CT + DHA/EPA ($P = 0.0238$) groups, showing a higher anxiety level in obese rats. Taken together, these results corroborate the effect of DHA/EPA in reducing anxiety-like behaviour.

Discussion

The effects of *n*-3 PUFA in brain function have been extensively studied[35,36]; however, the results are controversial. Here, we showed an anti-inflammatory effect of DHA/EPA supplementation in the brain in an experimental model of obesity, the CAF. In addition, we showed the ability of DHA/EPA in decreasing anxiety-like behaviour in obese rats. Otherwise, despite the evidence showing the role of *n*-3 in the improvement of metabolic profile[37,38], we found that DHA/EPA was not able to revert weight gain, adiposity, lipidic profile and hepatic levels of TNF-*α* in CAF-fed rats. Thus, using CAF, a highly obesogenic diet, we are showing for the first time that DHA/EPA beneficial effects might depend on the severity of the obesity. On the other hand, DHA/EPA still provides neuroprotection, irrespective of the metabolic dysfunction.

DHA/EPA does not ameliorate metabolic dysfunction and inflammation in severe obesity

A CAF leads to a more pronounced obesity phenotype in rodents than other diet protocols such as a high-fat diet (HFD)[27,39,40]. It has been shown that CAF is more efficient in inducing hyperglycaemia, glucose intolerance and insulin resistance compared with HFD[27,41]. We tested whether DHA/EPA would abrogate the metabolic disruption triggered by CAF. Our study demonstrated that 20 weeks of CAF increased weight gain, adiposity, blood glucose, triglycerides and insulin levels, and insulin resistance, providing an efficient model to mimic Western diet-associated obesity in Wistar rats. However, 4 weeks of DHA/EPA administration (500 mg/kg) did not ameliorate any of these parameters. In a previous study, 4 weeks of *n*-3 supplementation (400 mg/kg) also failed to decrease weight gain after 6 weeks of HFD in mice, but it reduced adipose tissue storage[42]. Although it was an interesting finding, 6 weeks of HFD may be a short period to investigate chronic manifestations of obesity. In another study, 16 weeks of HFD lead to increased weight and adipose tissue deposition with no effect of fish oil supplementation at a dose of 0.7 mg/kg for 5 weeks[28]. Thus, results about the effects of DHA/EPA supplementation are controversial. However, we can suppose that in cases of more severe obesity, it may not be sufficient to revert metabolic dysregulation, as shown in the present study.

Most of the studies about the effects of PUFA in metabolism are conducted in males. When sexual differences were investigated following CAF and a HFD, no differences were found in fatty acid metabolism regarding sex, including depletion of EPA content in male and female rats[43]. In addition, the effects of DHA/EPA supplementation have already been evidenced in female rats in different experimental models. In female rats with polycystic ovary syndrome, DHA/EPA reduced levels of triglycerides, insulin, blood glucose and weight gain[44]. The present study used only male rats, but it would be important to include females to evaluate differential responses to DHA/EPA in obesity.

In obesity, the accumulation and expansion of adipocytes lead to increased inflammatory cytokine secretion, promoting a sustained inflammatory state related to the onset of other chronic diseases[45]. Here, we assessed the systemic inflammation caused by CAF by measuring IL-6 and TNF-*α* levels in the plasma and liver. Although we did not find diet or supplementation effects on circulating levels of IL-6 and TNF-*α*, evidence has shown that DHA/EPA may play an anti-inflammatory role in the periphery. Souza *et al.* showed that 8 weeks of *n*-3 supplementation decreased IL-6 and TNF-*α* levels after HFD[46]. Also, Candido *et al.* showed a protective effect of *n*-3 in decreasing IL-6 in rats fed with HFD after 2 months of supplementation[47]. In the liver, obesity increases inflammatory markers, driving hepatic dysfunction such as non-alcoholic fatty liver disease and hepatocellular carcinoma[48]. In the present study, we found increased hepatic levels of TNF-*α* after CAF, which was already expected. However, we did not find the same increase in IL-6 levels. Nonetheless, in obesity, the increase in TNF-*α* leads to the production of IL-6 in the liver[48], suggesting that this could be a subsequent event as the disease develops. Importantly, we found a statistically significant effect of DHA/EPA in reducing IL-6 hepatic basal levels, although it could not mitigate the TNF-*α* increase. Worthwhile to note, we started supplementation after 16 weeks of CAF, while most studies start this intervention simultaneously with the diet protocol. Thus, we may speculate that the time point for the beginning of the supplementation may be a determining factor in the protective effect of *n*-3, at least in the liver. This idea is corroborated by a study in which supplementation of *n*-3 decreased TNF-*α* levels, but, in this case, the supplementation started concomitantly with the high-fat and high-sucrose diet[49]. Lionetti *et al.* also found lower levels of TNF-*α* after 6 weeks of HFD rich in fish oil, demonstrating a protective effect of *n*-3 in the liver[50]. Although previous studies demonstrate a protective role of *n*-3 on hepatic TNF-*α* levels, it seems that the beneficial effect may not be seen under chronic conditions. Regarding the effects of *n*-3 in reducing hepatic IL-6, Schmöcker *et al.* found a significant decrease in IL-6 mRNA in mice treated with *n*-3 in a model of hepatitis, contributing to the reduction of inflammation[51]. It was already demonstrated that EPA and DHA interact with peroxisome proliferator-activated receptor alpha (PPAR-*α*), preventing NF-κB activation[52]. Nevertheless, it is suggested that the anti-inflammatory properties of *n*-3 may depend on the pathogenesis and the strength of the inflammation[53]. Thus, our protocol of CAF may have elicited such intense liver inflammation that DHA/EPA could not completely reverse.

There is a close relationship between systemic inflammation triggered by obesity and neuroinflammation[54,55]. In the neuroinflammatory process, microglia and astrocytes carry out an inflammatory response that can result in psychiatric

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manifestations[56,57]. Here, we showed a decrease in TNF-α levels in the prefrontal cortex of CAF-fed rats supplemented with DHA/EPA compared with the CAF group with no supplementation, suggesting a neuroprotective effect of DHA/EPA. In a previous study, we demonstrated that DHA/EPA supplementation decreased IL-6 and TNF-α in the prefrontal cortex of obese rats after HFD[58]. The anti-inflammatory effect of DHA/EPA in the central nervous system may be mediated by inhibiting the activation of intracellular phospholipase A2, an enzyme that cleaves plasma membrane phospholipids to become available for metabolism to lipid mediators[59]. In this context, these PUFA can also compete with arachidonate for the enzymes 5-LOX and COX2, blocking the formation of pro-inflammatory products[60,61]. Moreover, in an *in vitro* study, DHA administration to hypothalamic neurons reduced NF-κB pathway activation and TNF-α production upon inflammatory challenge in a G protein-coupled receptor 120 (GPR120)-dependent way[62].

DHA/EPA influences brain function in obesity by decreasing anxiety-like behaviours

Obesity is considered a risk factor for anxiety disorders, but the pathophysiological mechanisms linking these conditions are still unclear. There is evidence showing a stronger association between severe obesity (defined as a BMI ≥ 35) and anxiety disorders compared with moderate obesity (BMI 30–35)[63,64]. Besides, chronic conditions related to obesity can increase anxiety risk[65,66]. On the other hand, anxiety disorders may lead to weight gain by deregulation of the hypothalamic–pituitary–adrenal axis that alters the appetite and leads to subsequent weight gain in stressed individuals[67,68]. The symptoms of anxiety disorders can increase appetite and stimulate the desire for foods rich in sugar and fat[67,69,70]. To better clarify the relationship between obesity and anxiety, Gariepy *et al.* performed a systematic review with meta-analysis, including sixteen studies with a total of 346289 individuals, suggesting (moderate evidence) that obesity is positively associated with anxiety disorders in adults[71].

Since neuroinflammatory processes are directly linked to the aetiology of behavioural disorders, *n*-3 supplementation benefits emotional states through its anti-inflammatory actions in the central nervous system[72,73]. It was shown elsewhere that CAF drives an anxiety-like behaviour[74], while *n*-3 may be efficient to improve mood[75] and attenuate anxiety symptoms in humans[76]. Studies also demonstrated that an adequate supply of *n*-3 could protect against cognitive decline and neurodegenerative diseases development by supporting adequate synaptic function and plasticity[77,78]. These findings are in agreement with our results, which also show a neuroprotective DHA/EPA effect in CAF-fed rats. In female rats, *n*-3 fatty acids also showed an anxiolytic effect[79,80]. However, to our knowledge, no study addressed the behavioural effects of DHA/EPA in obese female animals, which would be essential to investigate.

Here, we assessed anxiety-like behaviour using the elevated plus maze test. The consumption of a CAF for 20 weeks was sufficient to trigger anxiety-like behaviour in our study. CAF-fed rats supplemented with DHA/EPA showed a consistent reduction of anxiety parameters evaluated in the elevated plus maze test. It was shown elsewhere that chronic activation of GPR120 through an intracerebroventricular infusion of its agonist had an anxiolytic effect but failed to affect energy balance in HFD mice[81]. *n*-3 fatty acids are also GPR120 ligands and therefore were demonstrated to inhibit cytokine production in cultured neurons upon inflammation, evidencing its anti-inflammatory properties regarding central nervous system cells[62]. In the HFD model, a study found that fish oil enriched with DHA/EPA (0.7 mg/kg) could protect against behaviour abnormalities, glial activation and increased neuroinflammation compared with the non-treated obese counterparts[28]. In agreement with our current data, Demers *et al.* also demonstrated that *n*-3 could improve behaviour even without changing body weight[28]. However, we could speculate that although supplemented rats in our study did not benefit from significant metabolic improvements, DHA/EPA was efficient to protect obese rats against the anxiogenic manifestations observed in the obese non-treated group. This is a relevant finding considering the potent effect of CAF in inducing obesity, and even in this condition, DHA/EPA was able to improve the behaviour. Since psychiatric symptoms such as anxiety are prevalent in patients with obesity, *n*-3 may be indicated as a strategy for this population. It is worth mentioning that *n*-3 supplementation is a low-cost treatment, and it does not have any side effects, reinforcing the importance of its usage.

Limitations and conclusions

Here we showed that DHA/EPA supplementation decreased anxiety-like behaviour in a preclinical model of obesity-induced after a CAF. It is worth mentioning that the dose of 500 mg/d used in rats in the present study is higher than *n*-3 consumed by humans, even by supplementation. In human studies, the dose of DHA/EPA ranges from 1 to 4 g/d[82]. Thus, it can be a limitation to translate our findings to the human population. However, our findings are important to highlight the potential of these PUFA in exerting neuroprotection. Based on these beneficial results, more studies should be encouraged to develop new formulations of DHA/EPA with alternative routes to facilitate brain delivery, such as intranasal. On the other hand, the absence of robust metabolic effects indicates that DHA/EPA supplementation might not be a useful treatment in severe obesity, which was induced in the present study by CAF. Thus, DHA/EPA supplementation may exert an important neuroprotective effect in obesity, but further investigations are needed to elucidate effective doses of DHA/EPA.

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Authors Contributions

JN and JJ, investigation, formal analysis, writing - original draft; SO, MFB, LFSC and BFD, investigation; JCFM, MG and MP, formal anlaysis, writing - review & editing; RPG, conceptualization,

funding acquisition, supervision, writing - review & editing. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

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