

Neuroprotection Afforded by a Preventive CogniXtra Treatment Against Amyloid Beta $A\beta_{25-35}$ Peptide-induced Toxicity in Mice

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Abstract

Objective: A number of encouraging research studies have shown the importance of nutritional approach in order to protect the brain health. Here we present the efficacy of a daily administration of a unique complex combination of omega 3 fatty acids and antioxidants (cogniXtra) as a neuroprotective treatment on early symptoms of Alzheimer's disease (AD) mouse model.

Methods: Mice were treated per os once a day with various combinations of docosahexaenoic acid (DHA), glutathione (GSH), phosphatidylcholine (PC), curcumin (CUR) and resveratrol (RES) including the special combination cogniXtra (GSH + PC + CUR + RES + DHA) starting 20 days before and until 10 days after the onset of the neurotoxicity induced by a central injection of amyloid-beta 25-35 ($A\beta_{25-35}$)-oligomeric peptide. Protection against the neurotoxicity of $A\beta_{25-35}$ was assessed carrying out two behavior tests evaluating short-term memory (Y-maze) and long term memory (step through passive avoidance test-STPA) and the measurement of a key brain biomarker, lipid peroxidation (LPO).

Results: Our present research demonstrates the importance of a correct association of different substances whereas the treatment with each of them alone was unable to provide any protection from the toxic effects produced by $A\beta_{25-35}$ injection. CogniXtra formulation combining all of the components was the only one able to fully reverse all the memory deficits both in the Y-maze and in the STPA tests and also to completely protect from oxidative stress as demonstrated by the important LPO elevation measured in the hippocampus.

Conclusion: This study indicates that a combination treatment (cogniXtra) administration for thirty consecutive days produces a complete neuroprotective effect on the neurotoxic events produced by $A\beta_{25-35}$ oligomeric peptide injection. The efficacy of a preventive treatment with cogniXtra in this preclinical model is similar to what could be achieved with other pharmacological approaches. These results strongly suggest the therapeutic interest of cogniXtra for the preventive treatment of AD.

Introduction

Alzheimer's disease (AD) is the most common form of dementia, which is characterized by a progressive memory loss and the decline of other cognitive abilities affecting seriously normal daily life of older adults and representing one of the main difficult health conditions in aged people. According to the National Institute of Health, without any change, the expected number of people suffering will increase to 131 million by 2050 worldwide and the cost of care in the US, currently more than \$200 billion annually, will grow to an unsupportable \$1 trillion [1]. The cause of AD is not fully elucidated but it is widely accepted the implication of several processes including the accumulation of amyloid-beta peptide ($A\beta$), oxidative stress, inflammation and several biochemical pathways [2]. The formation of extracellular deposits of $A\beta$ leads to the formation of neurofibrillary tangles and neuritic plaques in the cortex and hippocampus [3] and $A\beta$ -induced toxicity is affected by free radicals, responsible of lipid peroxidation of neuronal cell membranes [4].

AD is a complex neurodegenerative disorder and even though no cure is still available, there are considerable research advances for treating and slow down its symptoms. In recent years, several studies have shown the importance of nutraceutical approach in order to delay the onset and to reduce the risk of AD and dementia [5,6]. Many products have been tested in patients with not always convincing outcomes such as phosphatidylcholine [7,8], vitamins, (B6, B9, B12) [9]. Antioxidants (vitamin E, vitamin C, beta-carotene, acetyl-

carnitine, coenzyme Q10, polyphenols, ginkgo biloba, long chain polyunsaturated fatty acids -PUFAs) have been devoted a lot of attention [10,11].

In this paper we investigate the neuroprotective efficacy of docosahexaenoic acid (DHA), glutathione (GSH), phosphatidylcholine (PC), curcumin (CUR) and resveratrol (RES) given alone or in combination for thirty consecutive days as a preventive treatment in a well recognized mouse model of AD. This model consists in a unique intracerebroventricular (i.c.v.) injection of oligomeric amyloid-beta peptide 25-35 ($A\beta_{25-35}$) in mice [12] which is able to mimic both cognitive impairment and associated neuronal degeneration. Though $A\beta_{1-40/42}$ peptides are considered as the major protagonists in the pathology, other small oligomeric fragments have been identified and among those, the highly toxic one is $A\beta_{25-35}$ [13,14] which is also endogenously present in AD human brain [15].

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Materials and Methods

Animals

Male Swiss mice (RjOrl: SWISS, Janvier, Saint Berthevin, France) 5–6 weeks of age were used for A β_{25-35} peptide intoxication. The animals were housed in Amylgen's regulated animal facility (agreement #A 34-169-002 delivered on May 02, 2014), in plastic cages with free access to food and water, except during behavioral experiments. They were kept in a regulated environment under a 12 h light/dark cycle (lights off at 7 pm). All experiments were carried out in the Amylgen facility (Montpellier, France). Animal procedures were carried out in strict adherence to the European Community Council Directive of September 22, 2010 (2010/63/UE). All experiments and protocols were authorized and approved by the French Ministry of Research, as well as by the Regional Animal Welfare Committee. All efforts were made to minimize the number of animals used.

Products

Glutathione/Phosphatidylcholine (batch: #GLUK1616), Curcuma/Resveratrol (batch: #6091206A), Docosahexaenoic acid (DHA) (batch #6070505) were obtained from HOD (Health Optimisation Devices B.V.), Maastricht, Netherlands.

Amyloid peptides A β_{25-35} and Scramble A β (Scr.A β) were obtained from Polypeptides (Montpellier, France). Homogeneous oligomeric preparation of the A β_{25-35} peptide was performed according to AMYLGEN's owned procedure.

Treatments

Male Swiss mice were anesthetized with isoflurane 2.5% and injected i.c.v. with A β_{25-35} peptide (9 nmol/mouse) or Scramble A β (Scr. A β) peptide (9 nmol/mouse), with a final volume of 3 μ l/mouse, according to the previously described method [16]. The mice were treated 20 days before the i.c.v. injection and 10 days after that until the end of the behavioral experiments, that was also the day of their sacrifice by daily gavage by gavage, using an inox steel cannula (ref 075486, Dominique Dutscher). The daily doses of the various products were (expressed in mg/mouse): GSH: 3.01; PC: 2.07; CUR: 3.67, RES: 1.38; DHA: 5.33. Several combinations of the different products were composed. CogniXtra contained all of the compounds combined together. Each group of treatment included n= 12 animals.

Y-maze

Mice were tested for spontaneous alternation in the Y-maze, an index of spatial working memory according to the procedure already described [17,18]. The Y-mazes built according to the characteristics described previously [19]. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. An alternation was defined as entries into all three arms on consecutive occasions. The number of maximum alternations were therefore the total number of arm entries minus two and the percentage of alternation was calculated as (actual alternations / maximum alternations) x 100. Parameters included the percentage of alternation (memory index) and total number of arm entries (exploration index).

Step through passive avoidance

Passive avoidance performance, an index of contextual long-term memory, was tested using the apparatus described previously [17,18] and consisting of two compartments, one white and one black separated by a guillotine door. The black compartment is equipped with a grid floor able to deliver scrambled footshocks. Each mouse was placed into the white compartment. After 5 seconds, the door was raised. When the mouse entered the dark compartment and placed all its paws on the grid floor, the door was closed and the footshocks (0.3 mA) delivered for 3 seconds. The step-through latency, i.e., the latency spent to enter the dark compartment was recorded. The retention test was carried out 24 hours post training. Each mouse was placed again into the white compartment. After 5 seconds, the door was raised. The step-through latency was recorded up to a cut-off time of 300 seconds. Animals with a good retention hesitated a long time before entering the dark box or never entered (latency > 300 s). Escape latency was determined by measuring the time spent in the dark box when animals were forced to enter. It was very short when retention was good.

Lipid peroxidation

On day 10, animals were euthanized after the last behavioral test. The brain was removed and the hippocampus and frontal cortex were dissected. The hippocampus was used to determine lipid peroxidation (LPO) levels using a colorimetric method [20]. The absorbance was measured at 580 nm ($A_{580,2}$). The level of lipid peroxidation was determined as CHP equivalents according to: $CHPE = A_{580,1}/A_{580,2} \times [CHP \text{ (nmol)}]$ and expressed as CHP equivalents per wet weight of tissue and as percentage of control group data (V-treated Sc.A β -administered mice).

Statistical analyses

All values, except passive avoidance latencies, were expressed as mean \pm S.E.M. Statistic analyses were performed on the different conditions using one-way ANOVA (*F* value), followed by the Dunnett's post-hoc multiple comparison test. Passive avoidance latencies did not follow a Gaussian distribution, since the upper cut-off times were set. They were therefore analyzed using a Kruskal-Wallis non-parametric ANOVA (*H* value), followed by a Dunn's multiple comparison test. All *p* < 0.05 was considered as statistically significant.

Results

Eight days after the i.c.v. injection, A β_{25-35} peptide induced important spatial working memory deficits by decreasing very significantly the percentage of spontaneous alternation of mice in the Y-maze as compared to Sc A β_{25-35} injected mice (Figure 1). Mice injected with the non-toxic Sc A β_{25-35} peptide display a spontaneous alternation in the successive exploration of the Y-maze arms of about 70% whereas A β_{25-35} peptide injection produced a loss of alternation as successive exploration of arms is at random (alternation of 50%). In a similar manner, A β_{25-35} peptide injection induced very significant contextual long-term memory deficits in the STPA test in terms of step-through latency (Figure 2) and escape latency (Figure 3). Mice injected with the non-toxic Sc A β_{25-35} peptide remembered the danger associated with the entrance in the dark box where an electric shock was present the day before: they displayed a long latency to enter the dark box very often > 300 s. Furthermore, when forced to enter, they came out very quickly and that was measured with a very short escape latency time.

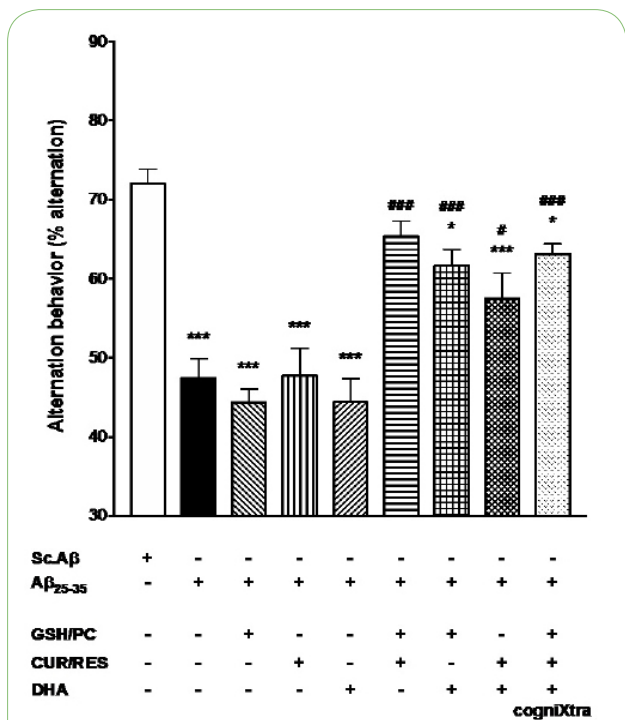


Figure 1: Effect of GSH/PC, CUR/RES, DHA treatments and various combinations on $A\beta_{25-35}$ oligomeric peptide induced impairment of spontaneous alternation in the Y-maze. n is between 9 and 12 depending on the groups; * $p < 0.05$, *** $p < 0.001$ vs. the Sc.Aβ group, # $p < 0.05$, ### $p < 0.001$, vs. the $A\beta_{25-35}$ group; Dunnett's test.

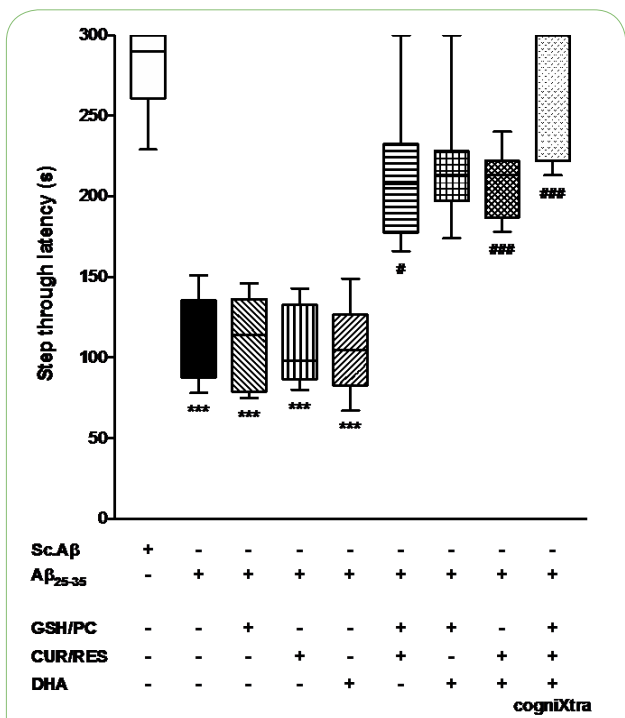


Figure 2: Effect of GSH/PC, CUR/RES, DHA treatments and various combinations on $A\beta_{25-35}$ oligomeric peptide induced impairment of entrance latency in the step through passive avoidance test. n is between 9 and 12 depending on the groups; ** $p < 0.01$, *** $p < 0.0001$ vs. the Sc.Aβ group, ### $p < 0.001$ vs. the $A\beta_{25-35}$ group; Dunn's test.

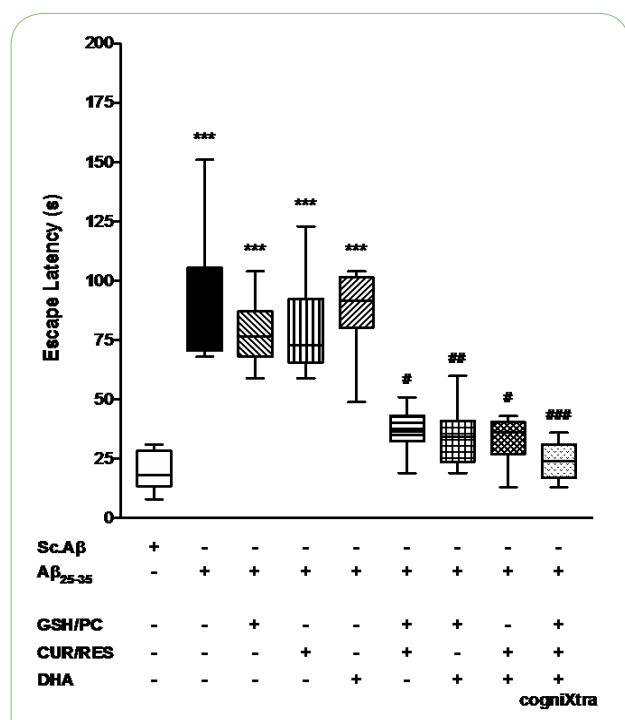


Figure 3: Effect of GSH/PC, CUR/RES, DHA treatments and various combinations on $A\beta_{25-35}$ oligomeric peptide induced impairment of escape latency in the step through passive avoidance test. n is between 9 and 12 depending on the groups; ** $p < 0.01$, *** $p < 0.0001$ vs. the Sc.Aβ group, ### $p < 0.001$ vs. the $A\beta_{25-35}$ group; Dunn's test.

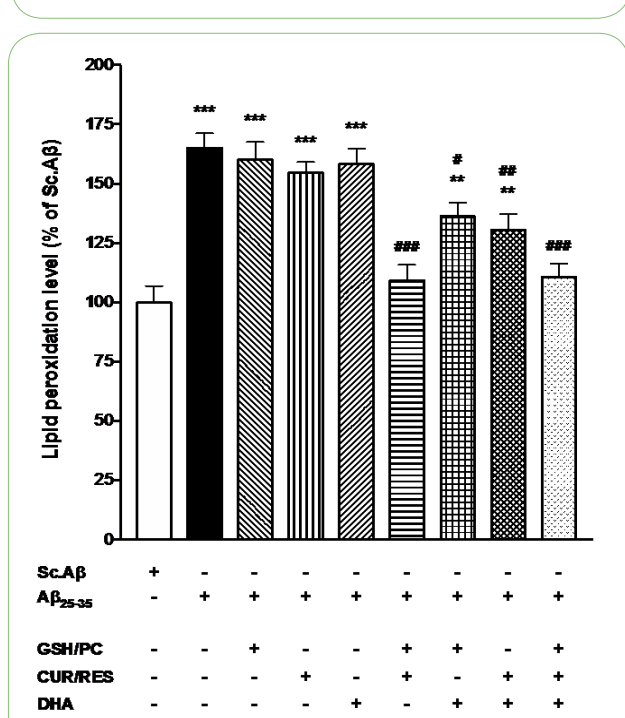


Figure 4: Effect of GSH/PC, CUR/RES, DHA treatments and various combinations on $A\beta_{25-35}$ oligomeric peptide induced elevation of LPO in the hippocampus. n=6; ** $p < 0.01$, *** $p < 0.001$ vs. the Sc.Aβ group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. the $A\beta_{25-35}$ group; Dunnett's test.

On the same animals, compared to Sc A β injected mice, A β_{25-35} peptide induced a very significant elevation of LPO (Figure 4).

When animals were submitted to a treatment with a combination of GSH/PC alone, a combination of CUR/RES or DHA alone during 30 days, no improvement was observed as compared to the effects produced by A β_{25-35} peptide injection.

However, only cogniXtra combination was able to fully reverse all memory deficits and fully normalize the levels of LPO elevation in the hippocampus. More particularly, the superiority of cogniXtra was the most obvious in the STPA test evaluating long term memory.

Discussion

In this study, our results clearly show the protective synergistic actions of a long treatment of GSH/PC, CUR/RES and DHA, on the cognitive impairments and oxidative stress induced by A β_{25-35} i.c.v. injection in mice when they were given in various combinations with each other and the lack of effect of these substances when they were given alone. CogniXtra was the best effective combination of compounds for significantly improving oxidative stress and the performance in memory tests related to short term memory and long term memory as measured by the Y maze and the step through passive avoidance tests, respectively.

Although this rodent model is far away from reproducing the complexity of the physiopathological characteristics of dementia in human, it has proven very useful to screen compounds for their neuroprotective effects. The toxicity induced by the A β_{25-35} peptide in oligomeric form has been repeatedly shown to result seven days after in profound biochemical and behavioral changes: neuroinflammation and reactive gliosis [21,22], pro-apoptotic caspases activity enhancement [21], oxidative stress [23], endogenously produced amyloid protein deposition [21], tau protein hyper phosphorylation [21] and increase of kinases [24,25], reduction in the number of neurons measured in hippocampal pyramidal cell layers [12,26], loss of cholinergic neurons [27], and memory deficits [12,18,21,26,28,29]. All together these characteristics correspond to the main hallmarks of AD making this model a quite severe model of the pathology and explaining why synergistic combinations of products are needed to overcome the profound dysfunctions produced by A β_{25-35} oligomeric peptide injection.

The efficacy found for the preventive treatment with cogniXtra, the combination displaying the highest efficacy on memory and oxidative stress, can be compared to what has been found in the same model with pharmacological therapeutic approaches such as the daily treatment with donepezil [30], one of the few compounds used in humans, but also ibuprofen [29], a γ -secretase inhibitor (BMS299,897) [17], a DYRK1A inhibitor (Leucettine L41) [31], synthetic neurosteroids (ent-pregnenolone sulphate/ ent-DHEA) [32] or sigma1 receptor agonists (Anavex 2-73) [18] that are currently evaluated in clinical studies.

These results are quite unexpected given the lack of efficacy of each of the components alone and demonstrate that a clear synergy happens when the following combinations are performed: GSH/PC + CUR/RES; GSH/PC + DHA; CUR/RES + DHA. The highest synergistic effect is observed with the combination of all the products obtained in cogniXtra composition. This composition is combining several products with properties corresponding to several therapeutic

strategies that are believed to be relevant for the treatment of AD. The combination produces a pleiotropic treatment that is targeting at the same time the prevention from vulnerability to oxidative stress by GSH [33-35] the modulation of pro-inflammatory cytokines and apoptotic proteins by CUR [36], preservation of the neuronal function by DHA [35]. RES is a polyphenol who exhibits neuroprotective effects (Huang). PC has an important role in maintaining the normal integrity of cell membranes and synaptic function and it is also a precursor of acetylcholine, a critical neurotransmitter involved in memory process.

All these products are included in cogniXtra in liposomal formulation that allows a better bioavailability and efficacy.

It is important to emphasize again that results reported in this study were generated using an animal model far away from reproducing the complexity of the physiopathological characteristics of dementia in human and translation to human needs a lot of caution and further experiments are needed to characterize more completely the neuroprotective effects of cogniXtra.

Conclusion

CogniXtra may be considered as a very interesting food supplement for the prevention of neurodegenerative diseases such as Alzheimer's disease.

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Competing Interests

The authors declare that they have no competing interests.

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