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Pomegranate seed oil nanoemulsions for the prevention and treatment of neurodegenerative diseases: the case of genetic CJD

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Abstract

Neurodegenerative diseases generate the accumulation of specific misfolded proteins, such as PrP^{Sc} prions or A-beta in Alzheimer's diseases, and share common pathological features, like neuronal death and oxidative damage. To test whether reduced oxidation alters disease manifestation, we treated TgMhu2ME199K mice, modeling for genetic prion disease, with Nano-PSO, a nanodroplet formulation of pomegranate seed oil (PSO). PSO comprises large concentrations of a unique polyunsaturated fatty acid, Punicic acid, among the strongest natural antioxidants. Nano-PSO significantly delayed disease presentation when administered to asymptomatic TgMhu2ME199K mice and postponed disease aggravation in already sick mice. Analysis of brain samples revealed that Nano-PSO treatment did not decrease PrP^{Sc} accumulation, but rather reduced lipid oxidation and neuronal loss, indicating a strong neuroprotective effect. We propose that Nano-PSO and alike formulations may be both beneficial and safe enough to be administered for long years to subjects at risk or to those already affected by neurodegenerative conditions.

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Key words: Neurodegeneration; Oxidation; Prion; PSO; Nanoparticles

Background

Neurodegenerative diseases are late onset fatal disorders that affect large numbers of individuals in our society.¹ Since clinical signs typically present after considerable irreversible loss of brain cells have occurred, therefore there is a clear unmet need not only for delaying disease progression in diagnosed patients but also for preventing disease manifestation in subjects at risk. Ongoing efforts in the search of disease modifying agents are mainly focused on screening for molecules that can dismantle or inhibit the formation of misfolded “key” disease proteins aggregates,^{2,3} which individually characterize each of these conditions, such as

Amyloid β in Alzheimer's disease (AD), α synuclein in Parkinson's disease (PD) and PrP^{Sc} in prion diseases such as Creutzfeldt Jacob disease (CJD),^{4,5} considered the hallmark of neurodegeneration.^{6,7}

A complementary concept would be to look for therapeutic targets common to all neurodegenerative diseases, as is the case for sensitivity to oxidative stress.⁸ In fact, most of the aggregated key disease proteins mentioned above are oxidized,^{9,10} and in prion diseases oxidation of Met residues in PrP helix 3 precedes the acquisition of protease resistance by this protein.¹¹ Also brain lipids are oxidized in these and other brain diseases,^{12,13} suggesting lipid oxidation may play an important role in the pathogenesis of neurodegenerative diseases.^{14–16} Indeed, oxidized phospholipids generate compounds such as 4-oxo-2-nonenal and acrolein, which are predominantly toxic to brain cells.¹⁷ Consistent with this, we propose to investigate whether safe α -oxidant reagents could be beneficial for individuals at risk of developing neurodegenerative conditions, constituting today a large fraction of the world population.^{1,18–22}

To this effect, we tested whether administration of pomegranate seed oil (PSO), either in its natural form added to food or as a water

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soluble Nano-emulsion, can delay the clinical advance and ameliorate prion and neurodegeneration pathological features in TgMHu2ME199K mice,²³ which model for genetic CJD (gCJD) linked to the E200K PrP mutation.²⁴ As of today, therapeutic intervention in all forms of human prion diseases had failed.^{25–28} PSO comprises a unique component, punicic acid (PA), a conjugated polyunsaturated fatty acid considered as one of the strongest natural antioxidants.²⁹ Unsaturated fatty acids such as linoleic acid, similar to PA, were shown to readily cross the blood brain barrier (BBB).^{30–32} PA is present only in PSO (60–80%) and in *Trichosanthes kirilowii* (40%),³³ and was effective in protecting tissue lipid profiles in inflammatory disease models.³⁴ PSO lack of toxicity and partial bioavailability was already established in humans.³³ An additional antioxidant, β -sitosterol, which was demonstrated to accumulate in the plasma membrane of brain cells,³⁵ is present in PSO at significantly higher concentrations as compared to oils from other plants,³⁶ indicating PSO may constitute a natural compound with stronger antioxidant activities than its individual components.

To increase the bioavailability and activity of PSO, we generated water soluble nanoemulsions hereby denominated Nano-PSO.³⁷ This approach, as is the case for delivery systems such as phospholipid micelles or nanoparticles,^{38–40} may change the target and distribution of the oil components between different organs, thereby enabling a longer circulation which may increase the levels of PA available to pass the BBB.

TgMHu2ME199K mice²³ express human-mouse chimeric E199K PrP on a null (for homozygous) or a wt PrP (for heterozygous) background. Mice from both lines suffer from progressive neurological symptoms as early as 5–6 months of age and deteriorate to a terminal condition several months thereafter, concomitant with the accumulation of a truncated form of PK resistant.^{23,41} TgMHu2ME199K mice exhibit typical pathological features of human CJD and of general neurodegeneration.²¹ This model therefore represents the most stringent challenge for candidate therapies in neurodegenerative diseases.

We show here that administration of PSO significantly delayed disease onset in TgMHu2ME199K mice, constituting a proof of concept that a natural antioxidant may fight neurodegeneration. Nano-PSO delayed disease onset and progression in a considerably faster mode and lower dose than natural PSO. Most important, it was only Nano-PSO and not PSO that could prevent further advance of disease when administrated to already sick TgMHu2ME199K mice. No aberrant side effect was observed in the time frame (months) and PSO doses used in these experiments. Analysis of brain samples revealed that while accumulation of PK resistant PrP was not affected by PSO formulations, brains from treated mice exhibited a strong neuroprotection effect, as seen by decreased lipid oxidation and neuronal loss, as well as increased synaptophysin expression and neurogenesis.

Methods

Animal experiments

All animal experiments were conducted under the guidelines and supervision of the Hebrew University Ethical Committee,

Table 1
Score of disease signs.

Score	Disease signs
1	Initial hind limb weakness
1.5	Partial hind limb weakness
2	Significant hind limb weakness/paralysis
2.5	+Legs claspings
3	+Full paralysis in one limb
3.5	+Significant weakness at the other hind foot
4	Full paralysis in both limbs
5	Death

which approved of the methods employed in this project (Permit Number: MD-11746-5).

Treatment of TgMHu2ME199K mice

PSO and Nano-PSO were administrated to TgMHu2ME199K mice modeling for E200K CJD expressing human-mouse chimeric E199K PrP on a null (for homozygous) or a wt PrP (for heterozygous) background,²³ as described in Table 2. In the PSO experiments,^{1–3} mouse pelleted feed to which PSO was added as described below was unlimitedly administered to young and older mice as applicable for as long as described in the table. Nano-PSO was administered either by gavage 5 times a week as in experiment 4 (150 μ l/day), or by adding Nano-PSO to the mice drinking water (experiment 5). At the end of the experiments, mice were sacrificed and their brains processed for pathological and biochemical experiments.

Mice scoring for disease signs

TgMHu2ME199K mice were followed twice a week for the appearance of spontaneous neurological disease. Mice were scored for disease severity and progression according to the scale described here and in Table 1. No clinical signs: 0; Initial hind limbs weakness = 1; Partial hind limbs weakness = 1.5; Significant hind limb/s weakness or paralysis = 2; Significant hind limb/s weakness or paralysis with significant legs claspings = 2.5; Full paralysis in one limb = 3; Full paralysis in one limb, and significant weakness at the other hind foot = 3.5; Full paralysis in both limbs = 4; Death = 5.

Mice were sacrificed at designated time points when required, according to the ethical requirements of the Hebrew University Animal Authorities when they were too sick or paralyzed to reach food and water, or after losing 20% body weight.

Preparation of PSO-enriched food

1 kg of mouse pelleted food (Harlan, Taklad) was dissolved in water and subsequently supplemented with 25 ml of PSO (Flavex, Germany). Mixture was next reassembled and dehydrated as pellets.

Preparation of O/W pomegranate oil nanoemulsion by sonication

A nanoemulsion with 10.8% oil fraction was prepared as follows: 1.56 g of Pomegranate oil, 0.65 g of Tween 80, and 0.39 g of glyceryl monooleate were mixed by a magnetic stirrer for 20 min. 2.5 g from the above mixture was mixed by a magnetic stirrer with 0.277 g of glycerol, for 15 min. 2 g of the glycerol mixture was then added drop wise to 8 g of deionized

Table 2
Groups in in vivo experiments.

Treatment	Exp #	Genetic background	Gender	Number of mice experiment/control	Days of treatment
PSO	1	Tg/wt	Male	5/4	86-270
PSO	2	Tg/ko	Male	8/7	76-270
PSO	3	Tg/ko	Female	6/7	190-270
Nano-PSO	4	Tg/ko	Female	6/6	70-270
Nano-PSO	5	Tg/ko	Male	11/10	236-300

water. A crude white emulsion was obtained. At the second stage this crude emulsion was sonicated using a horn sonicator (model Vibra-Cell, Sonics & Materials Inc., USA) for 10 min at 750 W. The samples were cooled in an ice water bath during the sonication process. A bluish nano emulsion was obtained.

Dynamic light scattering

Droplets size measurements were performed with a Zetasizer Nano-S (Malvern Instruments Ltd., Worcestershire, UK). Size measurements were performed in triplicates after dilution of the emulsion in water. The coarse white emulsion droplets size was in the range of few microns. The average droplets size of the O/W nano emulsion used in these experiments was 135 ± 12 nm.

Statistical analysis

The survival curves were compared using the Kaplan–Meier analysis with log rank test calculating X squares on one degree of freedom, *P* values and medians were calculated and are stated through the manuscript.

The clinical score severity curves in mice were compared between control and experimental groups using Mann–Whitney test (two tailed) and performed on the means + SEM (number of mice in each experimental group are detailed in Table 2).

Western blot analysis

Brains from TgMHu2ME199K mice at the designated end point of the experiments were homogenized at 10% (W/V) in 10 mM Tris–HCl, pH 7.4 and 0.3 M sucrose. For Proteinase K digestions, 30 μ l of 10% brain homogenates extracted with 2% sarkosyl on ice was incubated with 40 mg/ml Proteinase K for 30 min at 37 °C. Samples were boiled in the presence of SDS, subjected to SDS PAGE and immunoblotted with α -PrP pAb RTC.¹¹

Immunocytochemistry

Four μ m thick sections of formalin fixed, paraffin embedded brains of treated and untreated TgMHu2ME199K mice were evaluated for the levels of oxidized phospholipids with EO6 mAb (Avanti) and for the levels of neuronal synapses with an α -Synaptophysin pAb (Novus).

Neurogenesis

For identifying proliferating brain cells (neurogenesis), 10 months old wt, as well as Nano-PSO treated and untreated TgMHu2ME199K mice were injected intraperitoneally with Bromodeoxyuridine (BrdU, Sigma-Aldrich, 50 μ g/1 g body weight) for 7 consecutive days. Subsequently, mice were

anesthetized with a lethal dose of pentobarbital and brains were perfused via the ascending aorta with ice-cold PBS followed by cold 4% paraformaldehyde. Tissues were deep frozen in liquid nitrogen, and next serial 10 μ M coronal sections were immunostained for BrdU (rat α -BrdU, Serotec) as previously described.⁴²

Results

Delay of disease onset following administration of PSO in food to young and asymptomatic TgMHu2ME199K mice

Groups of asymptomatic TgMHu2ME199K mice (TgMHu2ME199K/wt and TgMHu2ME199K/KO),²³ as well as 7 months old TgMHu2ME199K/KO mice already presenting significant neurological signs (see Table 1 for description of disease scores, and Table 2 for details about the experimental groups) were fed either with regular rodent food or with food enriched with pomegranate seed oil (PSO) at a concentration of 25 ml oil/kg. Since mice consume about 3–4 g of food/day, we may assume treated mice received about 100 μ l of PSO per day or 700 μ l of PSO/week. Disease progression in each TgMHu2ME199K mouse was evaluated by frequent scoring of clinical signs, and by calculating twice a week the average of group scores in the treated animals as compared to untreated littermate groups generated from the same male and several of its sibling females. Administration of PSO in food to young TgMHu2ME199K mice was initiated just before they reached 3 months of age, the first time point in which PK resistant PrP can be easily detected in the still asymptomatic TgMHu2ME199K mice.^{23,41} Controls and treated mice from the same experiment were sacrificed simultaneously (at 270 days of age) and thereafter their brains processed for biochemical and pathological analysis. In the older mice, treatment commenced at 200 days (average score in the group = 2) and continued for 9 weeks before termination of the experiment. No adverse effects were observed in any of the mice following the long term administration with the oil enriched food.

Figure 1 shows the effect of PSO administration on the clinical presentation and advance of the disease in TgMHu2ME199K mice. Results were similar for treatment of heterozygous and homozygous TgMHu2ME199K mice (panels *AI* and *BI*), consistent with our recent results indicating that wt PrP does not participate in disease presentation in the genetic mice,⁴¹ and show a significant delay in disease presentation in the treated mice ($P < 0.02$ for both panels *A* and *B*, see methods for description of statistical analysis). To better understand the meaning of this result for medical practice, we evaluated the progression of disease for individual mice at two clinical time points, as represented by the percentage of mice under score 2 (*A* and *B* panels *II*), or under score 2.5 (*A* and *B* panels *III*). We found that PSO administration in food could confer a beneficial effect to most treated mice when data was estimated for the more advance stage, as depicted by the 2.5 score (difference in medians between treated and untreated groups were 60 and 80 days (panels *A* and *B*) ($P = 0.04$, $\chi^2 = 3.9$; $P = 0.02$, $\chi^2 = 4.1$)). At score 2, less than 50% of the mice reacted to the treatment, as indicated by a median difference of only 10 or 20 days respectively ($P > 0.05$). These results indicate that the beneficial effect of PSO on disease presentation (for most mice)

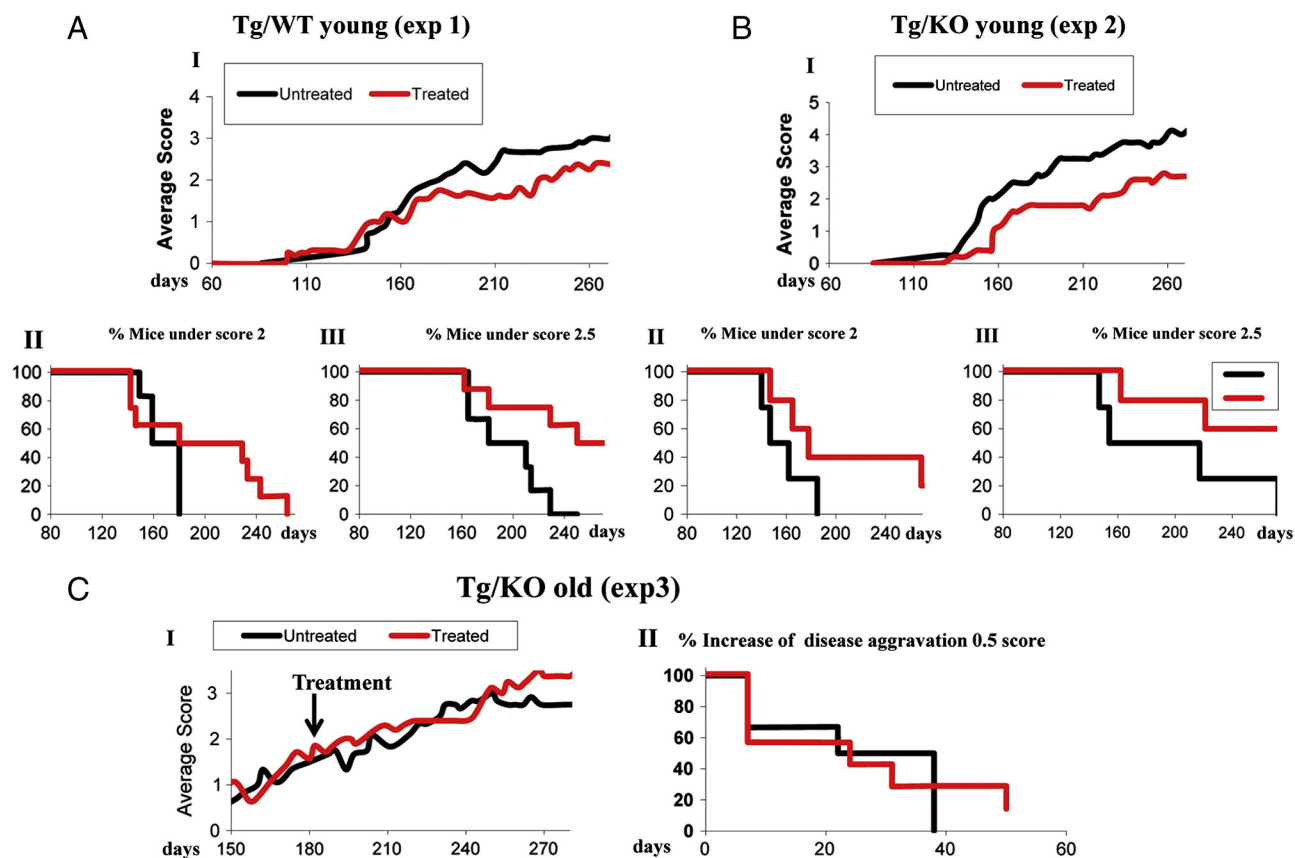


Figure 1. Natural PSO delays disease onset in TgMHu2ME199K mice. Young TgMHu2ME199K/wt and TgMHu2ME199K/ko mice or 190 days old TgMHu2ME199K/KO mice were treated with regular or with PSO enriched food for the designated time course (Table 2). Mice were scored for disease signs as described in Table 1. Figures (I) in panels (A), (B) and (C): Average group score as related to age of mice. Figures (II) in panels (A) and (B): % of mice under score 2 as related to age of mice. Figures (III) in panels (A) and (B): Percentage of mice under score 2.5 as related to age of mice. Figure (II) in panel (C): % of mice aggravated by 0.5 score.

can be observed only after long term consumption of the oil, and in many cases manifest when disease is already well advanced. As for the treatment of older and sick mice, Figure 1, C shows no difference in the average group scores or on the progression of disease for individual mice between PSO treated and untreated mice 200 days old sick mice (panels I and II).

Overall, these findings constitute the proof of concept that PSO may serve as an anti-prion/neuroprotective compound, but also present the limitations of such treatment; it requires long term administration of the anti-oxidant from the subclinical stage, while results may be apparent only when subjects are already affected.

Increased delay of disease onset and of disease progression following the administration of Nano-PSO to young and old TgMHu2ME199K mice

To generate a more effective and bioavailable formulation, we emulsified PSO to form oil nanodroplets with an average diameter of approximately 150 nm (see methods), hereby denominated Nano-PSO. As stated in the introduction, such formulations may allow for a longer circulation time of the now dispersed drug and thereby larger activity.⁴³ Figure 2, A presents

the results of an experiment comparable to those depicted in Figure 1, A and B for PSO enriched food. In this case, Nano-PSO was administrated to TgMHu2ME199K/KO mice from 70 to 270 days by gavage (15 μ l PSO/day; 5 days a week or 75 μ l/week). As in the previous experiment, treated and untreated mice were sacrificed at the same time point, to compare pathological parameters. The dose of PSO in the Nano-PSO formulation used in this experiment constitutes approximately 10% of the PSO administered as enriched food in the experiments described in Figure 1. Panel A I of Figure 2 shows a significant difference between the average group score of disease as related to the time of Nano-PSO administration ($P < 0.001$). When comparing effect on individual mice, panel A (II and III) indicate that administration of Nano-PSO resulted in a more rapid response to treatment as compared to TgMHu2ME199K mice treated with PSO enriched food. Already at score 1 (panel II), which is a very mild and early diagnosed disease condition (see Table 1) there is a 40 days difference between the median of treated and untreated mice ($P = 0.002$, $\chi^2 = 9.55$). In addition, the graph in panel III shows that while the median difference between the treated and untreated groups at score 2 was only 65 days ($P = 0.0082$, $\chi^2 = 6.98$), 50% of the treated mice never reached score 2 during the duration of the experiment. In fact, none of the mice treated

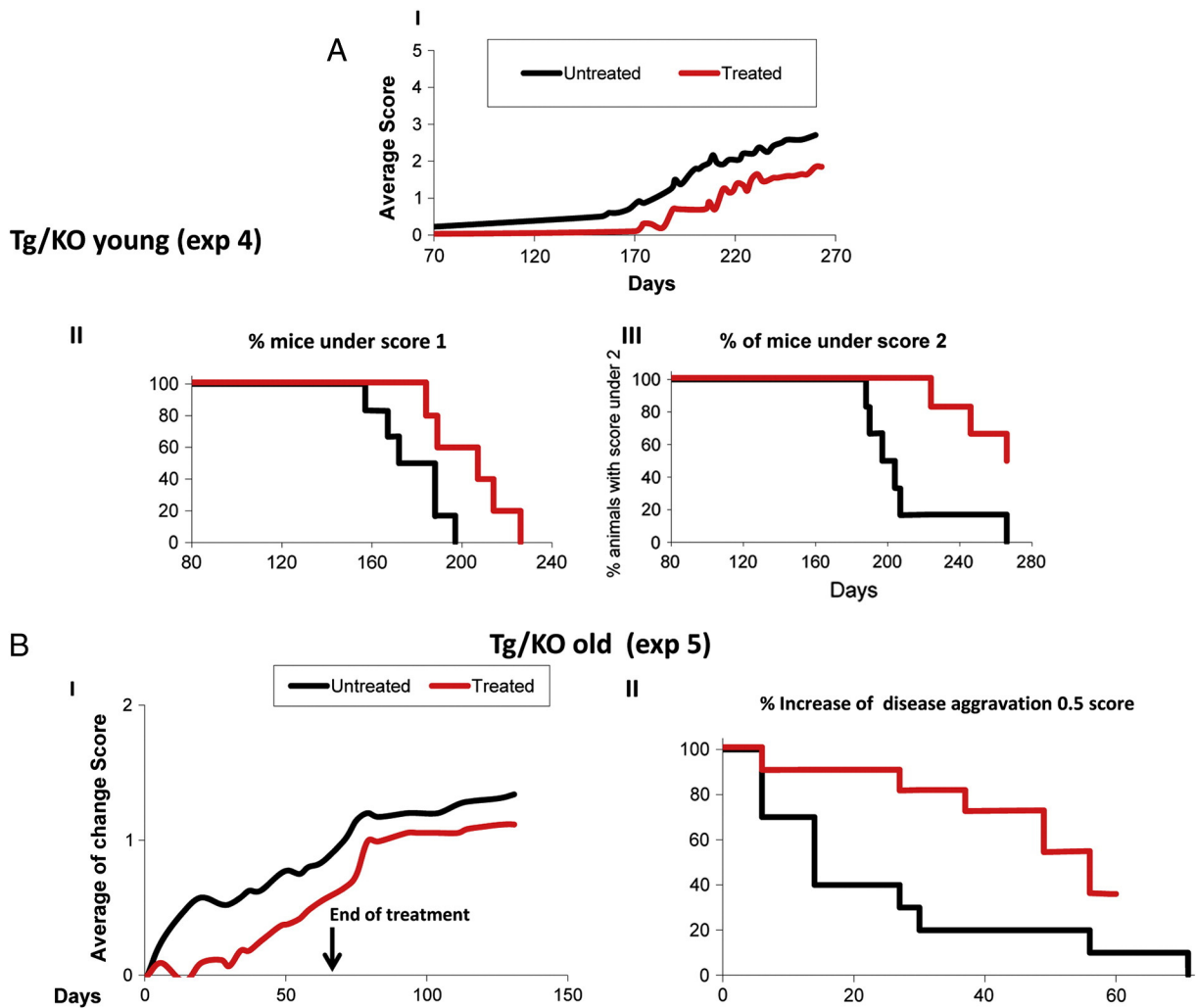


Figure 2. Administration of Nano-PSO to TgMHu2ME199K mice significantly delays prion disease onset and progression. Young and old TgMHu2ME199K/KO mice were treated by gavage with Nano-PSO (young, panel (A)) or by addition of the novel formulation to the drinking water (older mice, panel (B)). Mice were scored for disease signs as described in Table 1. Figure (I) in panel (A): Average group score as related to age of mice. Figure (II) in panel (A): % of mice under score 1 as related to age of mice. Figure (III) in panel (A): % of mice under score 2 as related to age of mice. Figure (I) in panel (B): Average group score as related to time of days of treatment. Figure (II) in panel (B): % of mice aggravated by 0.5 score as related to time of treatment.

with Nano-PSO reached score 2.5 in the frame of the experiment (see Figure 2, A), as opposed to 50 to 80% of the mice treated with PSO food. These results indicate that Nano-PSO can delay both the onset and the progression of disease at a much shorter time frame and at a much lower dose than natural PSO, indicating this and alike formulations may be suitable and effective for treatment of humans at risk, as is the case for mutation carriers of pathogenic PrP mutations.⁴⁴

Next, we administered Nano-PSO to TgMHu2ME199K mice already suffering from severe disease (individual scores of mice were between 2.5 and 3 at beginning of treatment), and then scored the mice 2-3 times a week to establish further advance of clinical signs. In this experiment, Nano-PSO was added to the mice drinking water for 5 days each week (9 weeks total). The calculated dose of PSO for each mouse was 30 μ l/day or 150 μ l/week. Figure 2, B depicts the results of these experiments. Panel I

demonstrated a significant difference ($P < 0.05$) between the average rate of disease advance between treated and untreated mice. Indeed, Nano-PSO was able to detent clinical deterioration for the duration of the treatment (63 days) and even to maintain a difference in scores between treated and untreated mice after treatment was terminated.

Panel BII presents the individual deterioration by half a score ratio of treated and untreated mice. It shows that Nano-PSO administration delayed the advance of disease for each treated mouse and that the difference in the median of both curves was again significant (42 days, $P = 0.01$, $\chi^2 = 6.635$). We therefore conclude that while natural PSO exerts a statistically significant anti-prion activity at some conditions, it is Nano-PSO that is suitable as a suitable therapeutic agent. A direct comparison of the beneficial activity of PSO versus Nano-PSO at different scores of disease is presented in Table 3.

Table 3
Comparing the clinical effect of the effect of PSO to Nano-PSO.

Experimental group	Parameter measured	PSO	Nano-PSO
Young, asymptomatic	Δ = The difference in medians between treated and untreated groups	Score 1 = no difference	Score 1 = 40 days $P < 0.002$
		Score 2 = 10 days	At score 2 \gg 65* days $P < 0.082$
		Score 2.5 = 80 days* $P < 0.04$	No score 2.5 in* treated mice
Old, symptomatic	Δ = The difference in deterioration by half a score ratio	No difference	42 days * $P < 0.01$

* Statistically significant

No reduction in disease related PrP in PSO/Nano-PSO treated TgMHu2ME199K mice

Disease related PrP forms, mostly resistant to digestion by proteinase K (PK), are the main markers of prion diseases, and are also considered the major/only components of the infectious prion agents.⁴ In our TgMHu2ME199K model, mutant PrP can be detected as a PK resistant and truncated form (PrPST) already at 3 months of age, when mice are still asymptomatic for the disease. PK resistant PrP levels increase with age, in parallel with disease progression.⁴¹ Figure 3 shows individual brain extracts of TgMHu2ME199K mice treated with PSO or Nano-PSO (from experiments 2 and 4, 270 days at end point) when immunoblotted with α PrP pAb RTC.¹¹ No difference in the total PrP levels was observed in the corresponding groups before PK digestion, indicating that administration of PSO in both forms (panel A as PSO, panel B as Nano-PSO) had no effect on PrP expression. Most important, no reduction in PK resistant PrP was observed in the treated brains in spite of the profound difference in disease score between treated and untreated mice (Figures 1 and 2). Actually, the signal for PK resistant PrP even increased slightly in some of the treated mice, as was the case for scrapie infected mice treated with Simvastatin.⁴⁵ This implies that PSO does not interfere with the pathological process or aberrant metabolic pathway which leads to the accumulation of disease related PrP in TgMHu2ME199K mice, but may rather induce neuroprotective pathways that manifest as reduced cell death. Such a mechanism may even explain an apparent increase in prion protein accumulation in the treated brains.

PSO formulations protect neurons from death

Next, we immunostained paraffin embedded brain slices from wt mice as well as from treated and untreated TgMHu2ME199K mice with an α -synaphophysin antibody. The samples used were from experiments 2 (PSO) and 4 (Nano-PSO), as in the experiments described in Figure 3. Synaphophysin is an integral membrane protein located in presynaptic vesicles.^{46,47} Decreased levels of this and other synaptic proteins are considered a general marker of neurodegeneration⁴⁸⁻⁵⁰ and were observed in the brains of CJD patients, bovine spongiform encephalopathy (BSE) infected cattle and scrapie infected mice.⁵¹⁻⁵⁴

Figure 4, A shows that while synaphophysin levels were largely reduced in the untreated TgMHu2ME199K mice, as seen by the reduce number of brown dots, as compared to age matched

wt mice, such immunostaining was significantly restored in the treated mice from the parallel groups for both experiments. This indicates that early treatment with PSO formulations may prevent not only the advance of clinical signs but also the presentation of general neurodegenerative features of prion and other neurodegenerative diseases, as is the case of synaphophysin expression.

Next, and to further establish whether PSO formulations can inhibit neuronal death, we counted in age matched wt, as well as in treated and untreated TgMHu2ME199K mice frozen sections the number of cells in the CA1 and CA3 regions of the hippocampus, as manifested by DAPI staining. Figure 4, B shows the results of such an experiments for sick TgMHu2ME199K mice treated with and without Nano-PSO (experiment 5). Indeed, while the brains from the sick TgMHu2ME199K mice presented significant neuronal loss as compared to those of wt mice (see thinner blue sections and numbers in the graph below), such reduction was partially prevented by the Nano-PSO treatment. Indeed, differences between cell numbers in treated and untreated sections, as well as between non-treated to wt mice were statistically significant ($P < 0.01$ or $P < 0007$, respectively). Death of neurons in the hippocampus was shown to correlate with dementia,⁵⁵ suggesting both that our TgMHu2ME199K mice model is a good model of neurodegeneration and that Nano-PSO exerts a strong neuroprotective affect in the most severe conditions, as represented by treatment of already sick TgMHu2ME199K mice.

Nano-PSO may restore hippocampal neurogenesis in sick TgMHu2ME199K mice

Numerous new studies demonstrate that during normal aging, certain areas of the brain retain pluripotent precursors with the capacity of self-renewal.^{56,57} This feature, also known as adult neurogenesis, may be impaired in neurodegenerative diseases such as CJD and AD.^{58,59} To evaluate levels of neurogenesis in treated and untreated TgMHu2ME199K mice, we injected Bromodeoxyuridine (BrdU), a synthetic analog of thymidine commonly used in the detection of proliferating cells in living tissues⁶⁰ to wt and TgMHu2ME199K mice of several ages as well as to Nano-PSO treated TgMHu2ME199K mice (experiment 5). Mice were sacrificed a week after the first injection and new neurons in the granular zone of the dentate gyrus were detected in frozen sections with an α -BrdU antibody. Figure 4, C shows that at 4 months of age, when the TgMHu2ME199K mice were still asymptomatic, there was no significant difference in the number of new cells between their brains and those of wt mice. Contrarily, in

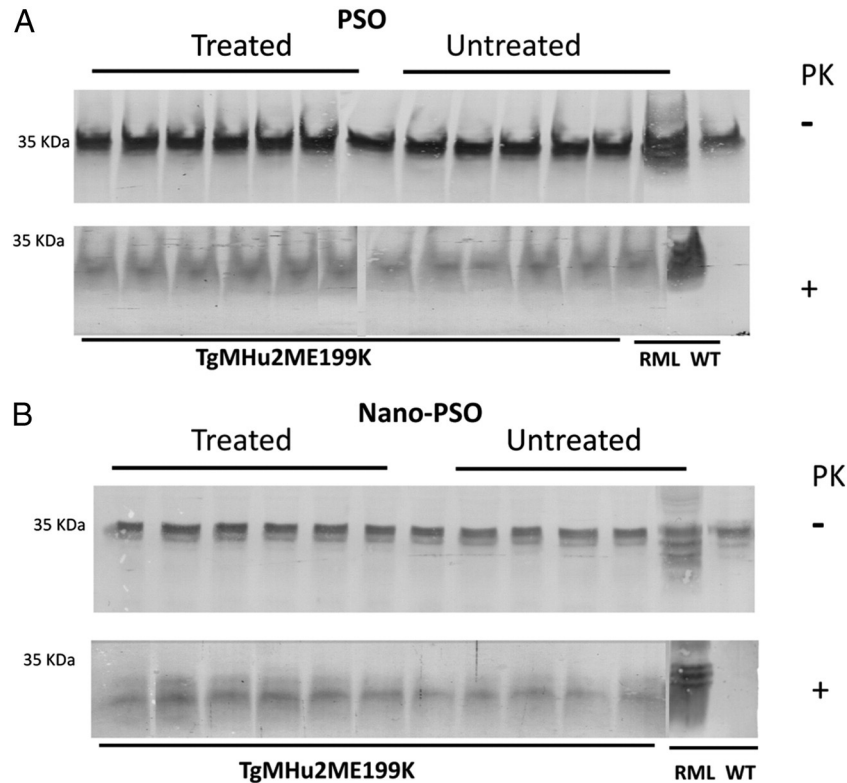


Figure 3. No reduction in disease related PrP in PSO/Nano-PSO treated Tg mice: Brain extracts of mice sacrificed at the end point of experiments 2 and 4 were digested in the presence and absence of PK, as described in the methods, and immunoblotted with α PrP pAb RTC.¹¹ Panel (A): PSO treated mice, last 2 lanes represent scrapie infected brains (RML, and wt mice). Panel (B): Nano-PSO treated mice, last 2 lanes represent scrapie infected brains (RML, and wt mice).

older mice already presenting signs of disease (6 and 10 months of age) the number of new cells in TgMHu2ME199K brains was significantly lower than the corresponding new cells in age matched wt mice ($P < 0.002$), indicating reduced neurogenesis, at least at this age, is a feature of neurodegeneration more than a property of aging. While only marginally significant ($P < 0.12$), the number of new cells in Nano-PSO treated mice was higher than in the non-treated mice, indicating a possible additional neuroprotective property of Nano-PSO treatment.

PSO protects cell lipids from oxidative insults

As reviewed above, lipid and protein oxidation are important features of all neurodegenerative diseases.^{13,12,61} To establish whether Nano-PSO can reduce lipid oxidation, we immunostained paraffin embedded brain slices brain from wt and form control and treated TgMHu2ME199K mice with EO6, an anti-oxidized phospholipids mAb⁶² which was shown to detect oxidized lipids in MS plaques.¹³ In Figure 5, we show that while we could not detect any α -EO6 immunostaining in wt mice of different ages (no dispersed brown color or specific features), TgMHu2ME199K mice brain sections from experiment 4 presented strong EO6 immunostaining in all cerebellar layers (dispersed brown in general and in cerebellum's Purkinje cells). These results are consistent with our previous findings, indicating a large sensitivity of TgMHu2ME199K mice Purkinje cells to disease aggravation.⁶³ Our results also show that Nano-PSO treated sections present considerable lower levels of EO6 staining,

both general and cell specific, as compared to the parallel untreated brains. In samples from experiment 5, mice treated when already sick, we show that EO6 may also recognize star like plaques in the brains of the untreated, but not in the treated TgMHu2ME199K mice. These results demonstrate that lipid oxidation may be an important feature of prion diseases and that Nano-PSO formulations can reduce/prevent such oxidation, concomitantly with inhibition of cell death and disease aggravation.

Discussion

A significant number of natural anti-oxidants are ubiquitously present in a healthy human diet. Many of them, such as Sulforaphane from Broccoli, Curcumin and EGCG from green tea were recognized for their neuroprotective properties in cells and tested in appropriate animal models.⁶⁴⁻⁶⁶ However, their in vivo activity was limited by the sub-pharmacological doses presented in food, their poor bioavailability to humans, rapid chemical degradation and reduced distribution to different organs in the body, in particular the CNS. In this work, and after PSO by itself was found to be clinically active in its natural oil form, we made an effort to overcome such limitations by tailoring a more active formulation in the form of Nano-PSO.

We show here that administration of PSO in food may delay the onset of spontaneous genetic prion disease in the TgMHu2ME199K mouse line, constituting a proof of concept that natural antioxidants

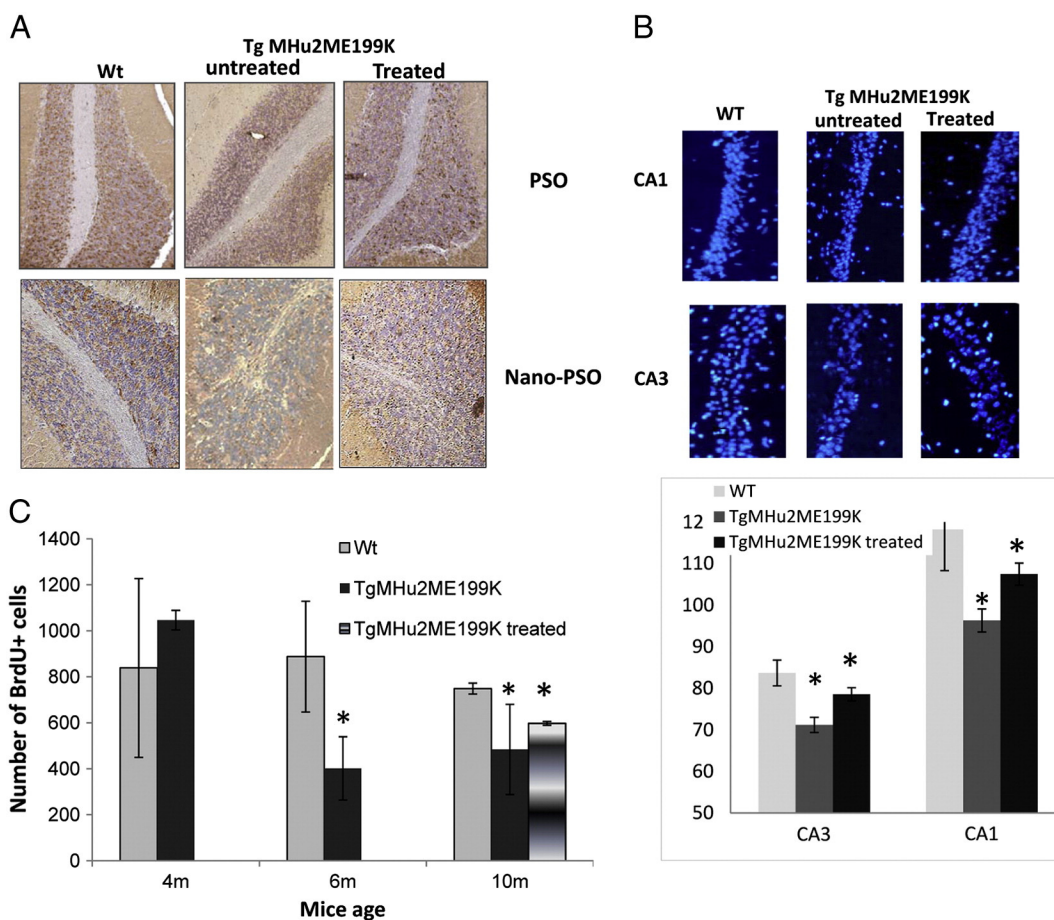


Figure 4. Neuroprotection of PSO formulations (A) PSO and Nano-PSO restore synaptophysin expression in sick mice: Paraffin embedded brain slices of wt, as well as PSO and Nano-PSO treated and untreated TgMHu2ME199K/KO mice (experiments 2 and 4), were immunostained with an α -synaptophysin antibody. Magnification in all pictures is $\times 20$. (B) Nano-PSO protects hippocampal cells from death: Frozen sections the number of cells in the CA1 and CA3 regions of the hippocampus, as manifested by DAPI staining. Statistical analysis of average from 4 mice in each group was performed by T-test. (C) Neurogenesis in treated and untreated TgMHu2ME199K mice: Slides of mice treated with BrdU and subsequently stained with an α -BrdU antibody were counted for reacting cells. Average number of cells in slides of 4 mice was plotted in the graph. Statistical significance was evaluated by T-test.

may exert a beneficial neuroprotective effect. However, it was only Nano-PSO, a novel formulation in which natural PSO was converted into soluble nanodroplets, which generated an impressive clinical effect at a much lower PSO dose for both prevention and treatment of progressive prion disease in the TgMHu2ME199K mice. Our results also show that the mechanism of activity of PSO formulations was not prion specific, or at least not PrP specific, since the long term administration of the oil did not affect the expression of mutant PrP or its accumulation as a PK resistant PrP form. Rather than a specific anti-prion effect, these PSO formulations, concomitant with an impressive clinical outcome, presented a wide neuroprotective effect, in the form of reduced neuronal death and lipid oxidation and increased neurogenesis, which may be valuable also for the treatment of an array of neurodegenerative conditions. Indeed the combination of an unsaturated fatty acid that can most probably cross the BBB,³⁰ and the increased bioavailability conferred by the nanoparticle formulation,³⁷ may explain the impressive neuroprotective activity of Nano-PSO.

The search for disease modifiers for the treatment of neurodegenerative diseases is focused mostly on screening for

reagents such as antibodies or small molecules that may dismantle or inhibit the aggregation of misfolded specific key proteins, as is the case for PrP^{Sc} in prion diseases,⁶⁷ A-beta in AD and synuclein in Parkinson's disease,⁶⁸⁻⁷¹ or reduce the expression of the normal key proteins, in the form of siRNAs⁷² or other molecules.⁷³ While these pathways may well lead to effective drugs, we propose an alternative approach in the form of brain protection from common neurodegeneration features, as is the case for neuroinflammation and oxidative stress. Indeed, Nano-PSO generated a strong clinical effect with an excellent safety profile which may allow its prolonged administration to individuals at risk. The formulation described here, oil nanodroplets of a specific size, may be further improved by optimizing particle size, Nanoemulsion ingredients, stability features and physicochemical principles of preparation. Pharmacokinetics experiments in progress in our laboratory may help to fine-tune the most active formulations, before we engage in clinical trials.

Indeed, since Nano-PSO can most probably be classified as a "safe" reagent, even in the levels of safety of a food supplement, such trials may come about soon enough. A comprehensive clinical

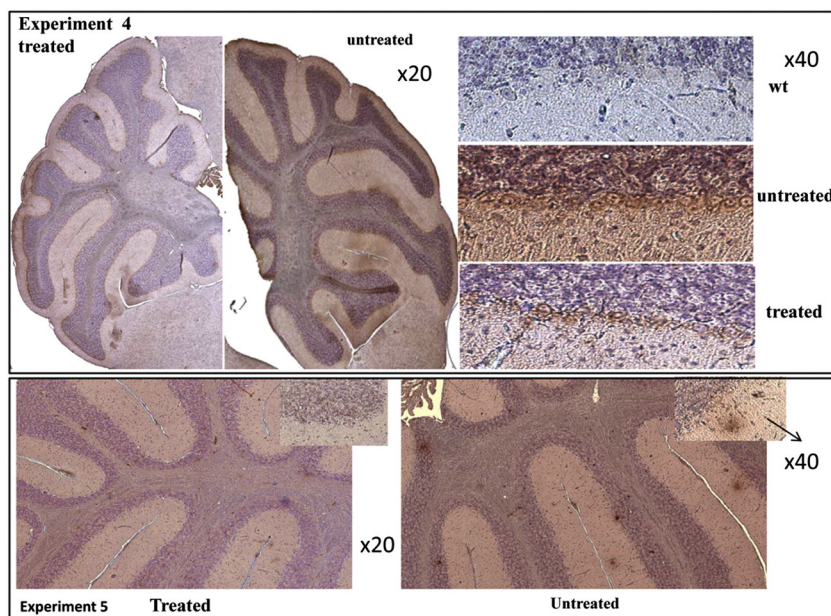


Figure 5. PSO protects brain lipids from oxidation: Paraffin embedded slides from Nano-PSO treated and untreated mice (experiments 4 and 5) were immunostained with the EO6 antibody. Initial magnification in all pictures was $\times 20$.

trial for CJD patients was recently described.²⁸ While the results for doxycycline were negative, the experimental design may be useful in our and other cases. In addition, we speculate that once sophisticated disease specific reagents become available, they may well be combined with general neuroprotective agents such as Nano-PSO for the best possible outcome in patients and at risk individuals.

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