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Articles

A new model for the pathophysiology of Alzheimer's disease

Aluminium toxicity is exacerbated by hydrogen peroxide and attenuated by an amyloid protein fragment and melatonin

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Objectives. Although Alzheimer's disease (AD) is the leading cause of dementia in developed countries, there is an as yet unexplained lower prevalence of the disease in parts of Africa. AD is characterised by a catastrophic loss of neurons; free radicals (oxidative toxins) have been implicated in the destruction of the cells through the process of lipid peroxidative damage of cell membranes. Previously aluminium (Al) and a fragment of beta amyloid (Aβ 25 - 35) were shown to exacerbate free-radical damage, while melatonin reduced this effect. The aim of the present study was: (*i*) to investigate the conditions determining the toxicity of Al and Aβ 25 - 35; and (*ii*) to assess whether melatonin could attenuate the damage done by both aluminium and the amyloid fragment, thus suggesting a pathway for the aetiology of AD.

Design. An *in vitro* model system was used in which free radicals were generated, causing lipid peroxidation of platelet membranes, thus simulating the disease process found in the brain.

Results. 1. Al and Aβ 25 - 35 caused lipid peroxidation in the presence of the iron (II) ion (Fe^{2+}), Al being more toxic than Aβ 25 - 35. 2. Aβ 25 - 35 attenuated the lipid peroxidation promoted by Al. 3. Hydrogen peroxide (H_2O_2) greatly exacerbated the toxicity of Al and Aβ 25 - 35. 4. Melatonin prevented lipid peroxidation by Al and Aβ 25 - 35 in the absence of H_2O_2, but only reduced the process when H_2O_2 was present.

Conclusions. In the light of the results obtained from the present study, the following hypotheses are formulated. 1. In AD, excessive quantities of Al are taken up into the brain, where the Al exacerbates iron-induced lipid peroxidation in the lysosomes. 2. In response, the normal synthetic pathway of amyloid protein is altered to produce Aβ fragments which attenuate the toxicity of Al. In the process of sequestering the Al and iron, immature plaques are formed in the brain. 3. Microglia are activated, in an attempt to destroy the plaques by secreting reactive oxygen species such as H_2O_2. At this point in the disease process, lipid peroxidation causes a catastrophic loss of brain cells. 4. Melatonin, together with other free radical scavengers in the brain, reduces the free-radical damage caused by Al and Aβ, except in the latter stages of the disease process. Since melatonin is produced by the pineal gland only in the dark, the excess of electric light in developed countries may help explain why AD is more prevalent in these countries than in rural Africa.

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Alzheimer's disease (AD) is characterised by the loss of cells in certain brain areas, as revealed by temporal lobe-orientated X-ray computed tomography (CT) scans.[1] By the time of clinical AD diagnosis, about 90% of the hippocampus will have been destroyed. AD is the leading cause of dementia in developed countries; however, there is an as yet unexplained lower prevalence of the disease in parts of Africa,[2] even though other dementias such as vascular dementia and alcohol-related dementia are common.

An understanding of the action of certain chemically reactive molecules called free radicals has provided evidence for their involvement in the destruction of brain cells.[3] Free radicals are toxic byproducts of cellular biochemical reactions, as found for example when adenosine triphosphate (ATP) is generated in the mitochondria.[4] Free radicals do, however, serve a purpose in the body, e.g. when cells of the immune system produce free radicals in order to destroy micro-organisms, or digest unwanted cell material.[5] These reactions are usually carefully controlled for, if left to themselves, free radicals attack living cells, damaging phospholipids (by causing lipid peroxidation), proteins and even DNA.[6]

Chemically speaking, free radicals are ions and molecules which take part in electron transfer reactions.[7] An example is the iron (II) ion (Fe^{2+}), which transfers an electron to molecular oxygen, thus producing the superoxide radical (O_2^-). Superoxide can dismute to form hydrogen peroxide and, again through the intervention of Fe^{2+}, form hydroxyl radicals ($OH^{\bullet}$).[7]

Superoxide and hydroxyl radicals are extremely damaging to living cells, especially the cell membranes. Membranes play an integral part in cell function and health; besides keeping the cell contents intact, they also determine which molecules enter and leave cells, and in themselves take part in chemical reactions. If the damaged membranes cannot be repaired, the cells die. The principal constituent of membranes, the phospholipids, are particularly vulnerable to free-radical damage (lipid peroxidation) and, as such, provide a model which can be utilised for studying the abovementioned reactions *in vitro*.[3]

In living cells, any free radicals formed are normally neutralised by anti-radical defence systems, e.g. free-radical scavengers (anti-oxidants), enzymes such as glutathione peroxidase and superoxide dismutase, and iron-binding proteins such as transferrin.[8] However, if these defence systems are breached, for example, when transferrin does not bind the iron completely, the iron becomes available for free-radical production. A genetic isoform of transferrin (Tf C2), which has a decreased iron-binding capacity, is more frequently present in AD patients than controls and would therefore abet this process.[9] But, in order to combat increased concentrations of free radicals, other levels of defence may exist, for example in the form of the hormone, melatonin, which protects membranes against lipid peroxidation.[10]

Aluminium (Al)[11] and beta amyloid (Aβ)[12] have both been shown to exacerbate lipid peroxidation and are implicated in the aetiology of AD, although the mechanistic pathway has not been established. In the present study, an *in vitro* system was used: (*i*) to determine the conditions under which Al and a fragment of the Aβ protein, Aβ 25 - 35, cause lipid peroxidation; and (*ii*) to determine whether melatonin could attenuate the damage done by either the Al or the amyloid fragment.

Methods and materials

Chemicals. Diphenylhexatriene (DPH), 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT) and Aβ 25 - 35 were obtained from Sigma Chemical Co; $Al_2(SO_4)_3.18H_2O$ was obtained from Merck.

Preparation of membranes. Platelets suspended in citrate-dextrose buffer, isolated as described previously,[3] were obtained from healthy donors. Erythrocytes were removed by centrifugation at 800 g for 5 minutes. Platelets were then collected by centrifugation at 3 500 g for 15 minutes. The pellets were resuspended in 0.01M phosphate-buffered saline (PBS), pH 7.4. Cell counts were done on a Technicon H2 Blood Analyser, which showed that there were no erythrocytes or white blood cells present. Membranes were prepared by sonication of the suspension with an MSE sonicator for 10 seconds at an amplitude of 5 microns. The fragmented membranes were not washed, in order to maintain their integrity. The protein concentration of the suspension was 0.07 mg/ml, as determined by the method of Lowry *et al.*[13]

Determination of lipid peroxidation. Of the membrane suspension, 100 µl aliquots were pipetted into silicon glass test tubes (Kimble). The following reagents were then added in sequence, to give final concentrations as indicated: ascorbate (160 µM), EDTA (10 µM), $Al_2(SO_4)_3.18H_2O$ (0.1 - 100 µM), Aβ 25 - 35 (0.01 - 100 µM) or melatonin (1 mM); $FeSO_4$ (40 µM) and hydrogen peroxide (H_2O_2; 0.5 - 500 µM). The total volume per tube was adjusted to 1 000 µl with PBS (pH 5.5, to keep the Al in solution).

The tubes were incubated at 37°C for 30 minutes. The following were then added: 1 ml of a 1% (w/v) TBA solution plus 1 ml of 2.8% (w/v) trichloro-acetic acid. BHT (0.01%) was also included to neutralise metal-catalysed auto-oxidation of lipids during heating of the mixtures at 100°C for 10 minutes. After cooling, the formation of thiobarbituric reactive substances (TBARS) was determined by reading the chromogen at 535 nm in a Beckman DU 640 spectrophotometer. Appropriate corrections to the absorbance readings were made for the presence of $FeSO_4$-EDTA and ascorbate in the reaction mixtures. Absorbance readings at 535 nm are proportional to the TBARS formed, and are therefore relative values reflecting lipid peroxidation.

Statistical analysis. The statistical significance of the results was determined by the Mann-Whitney U-test. Results are the mean ± SEM of 6 observations.

Results

1. **Al caused lipid peroxidation only in the presence of Fe^{2+}.** In Fig. 1 (column A), 100 µM $Al_2(SO_4)_3.18H_2O$ (i.e. 8.1 µM Al) did not use cause lipid peroxidation on its own when iron was absent, but when 40 µM iron sulphate and Al were present simultaneously (Fig. 1, column B), a significant increase in lipid peroxidation ($P < 0.05$) was observed.

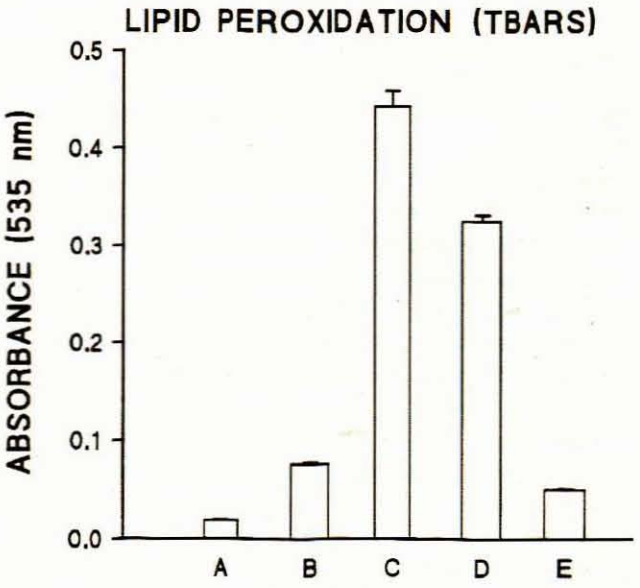

Fig. 1. Lipid peroxidation recorded in the presence of: A — 100 µM Al sulphate, without iron, B — 100 µM Al sulphate plus 40 µM iron sulphate, C — 100 µM Al sulphate, 40 µM iron sulphate plus 0.5 mM H_2O_2, D — 100 µM Al sulphate, 40 µM iron sulphate, 0.5 mM H_2O_2, plus 1 mM melatonin, E — 100 µM Al sulphate, 40 µM iron sulphate, plus 1 mM melatonin. B was significantly higher than A ($P < 0.05$); C was significantly higher than B ($P < 0.005$); D was significantly lower than C ($P < 0.005$); E was significantly lower than B ($P < 0.005$) (Mann-Whitney U-test).

2. **Aβ 25 - 35 similarly promoted lipid peroxidation when iron was present.** In the presence of Fe^{2+}, 0.01 - 1.0 µM Aβ 25 - 35 increased the lipid peroxidation significantly ($P < 0.005$) compared with Aβ 25 - 35 at the same concentrations without iron (Fig. 2).

3. **Al was more toxic than the amyloid protein fraction.** In Fig. 3, the relative toxicity of 0.1 - 100 µM Aβ 25 - 35 and 0.1 - 100 µM $Al_2(SO_4)_3.18H_2O$ (i.e. 0.008 - 8 µM Al) was compared. The Al produced significantly ($P < 0.005$) more

lipid peroxidation than Aβ 25 - 35. The amount of Al used in the present study (0.216 - 216 µg/l) was compatible with the Al concentration recorded previously in the blood of AD patients (8 - 104 µg/l).[14]

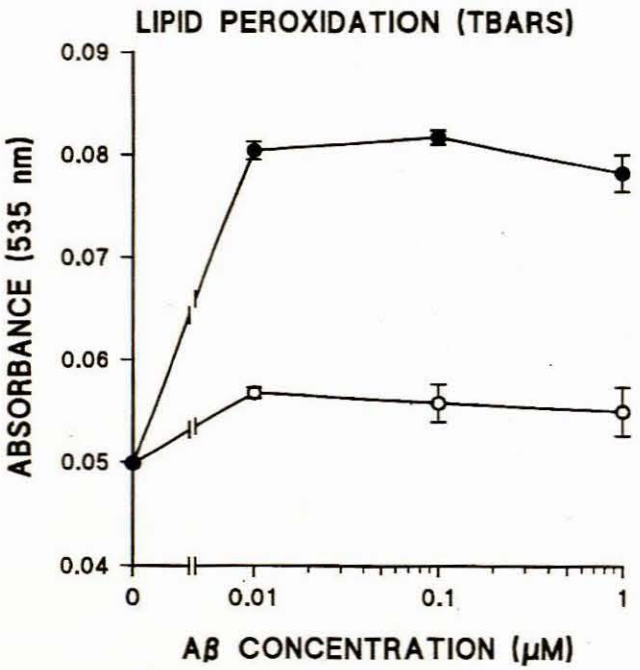

Fig. 2. Lipid peroxidation recorded in the presence of increasing concentrations of Aβ 25 - 35, (●) with and (○) without 40 µM iron sulphate. Aβ 25 - 35 with iron produced significantly more lipid peroxidation than Aβ 25 - 35 without iron ($P < 0.005$).

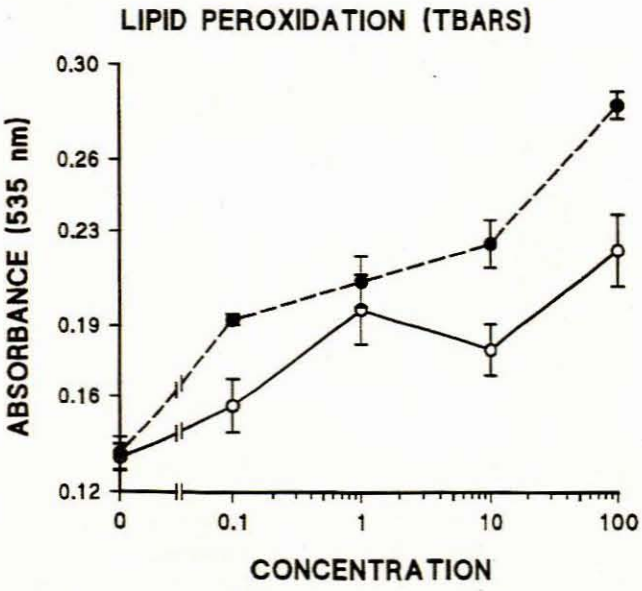

Fig. 3. Lipid peroxidation recorded in the presence of increasing concentrations of (●) Al sulphate and (○) Aβ 25 - 35. Al sulphate produced significantly more lipid peroxidation than Aβ 25 - 35 ($P < 0.005$).

4. **The amyloid protein fraction attenuated Al toxicity.** When Al and Aβ 25 - 35 were present simultaneously (Fig. 4), the lipid peroxidation produced was significantly ($P = 0.005$) less than with Al on its own. The results from two separate experiments were combined. However, when lipid peroxidation was high, as in the presence of H_2O_2 (Fig. 5), this effect was steadily lost.

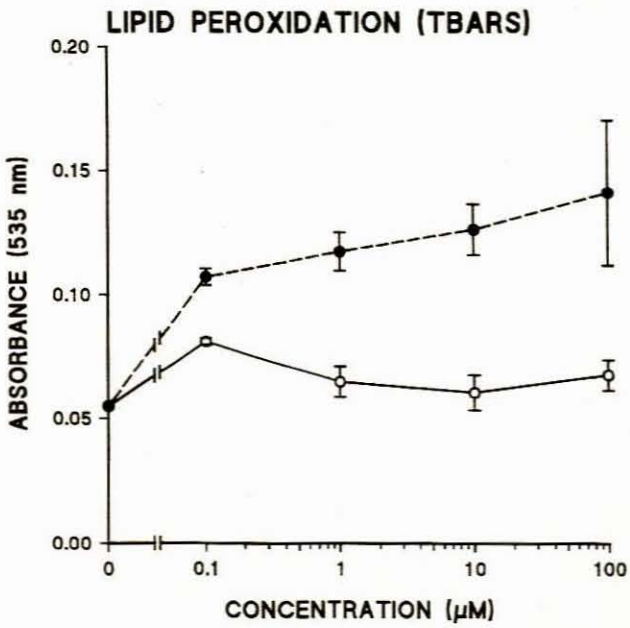

Fig. 4. Lipid peroxidation recorded in the presence of: (●) increasing concentrations of Al sulphate; and (○) 100 µM Al sulphate plus increasing concentrations of Aβ 25 - 35. Aβ 25 - 35 attenuated the toxicity of Al significantly ($P < 0.005$; Mann-Whitney U-test).

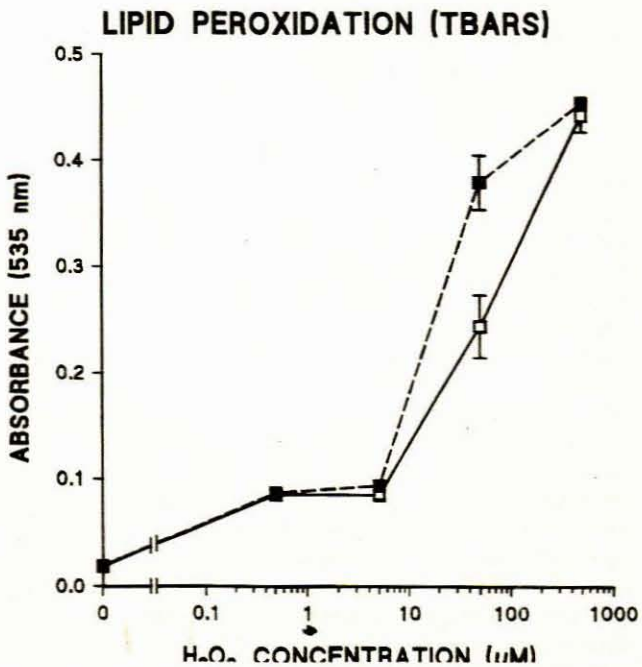

Fig. 5. Lipid peroxidation in the presence of increasing concentrations of H_2O_2: (□) with 100 µM Al sulphate; and (■) with 100 µM Al sulphate plus 100 µM Aβ 25 - 35.

5. **H_2O_2 greatly exacerbated the toxicity of Al and Aβ 25 - 35.** Fig. 5 shows that there is a significant increase in lipid peroxidation with increasing H_2O_2 concentrations ($P < 0.005$). In addition, the attenuating effect of Aβ 25 - 35 in the presence of Al is not seen in the presence of H_2O_2, although the difference between the effects of Al on its own and the combination of Al and Aβ 25 - 35 was not significant (Fig. 5). Fig. 1 (column C) demonstrates significantly higher lipid peroxidation in the presence of 100 μM Al sulphate, iron sulphate, and 0.5 mM H_2O_2, compared with iron on its own (Fig. 1, column B).

6. **Melatonin eliminated lipid peroxidation caused by Al and Aβ 25 - 35 in the absence of H_2O_2.** In Fig. 1 (column E) melatonin (1 mM) eliminates lipid peroxidation caused by Al and Aβ 25 - 35, but in the presence of H_2O_2 (0.5 mM; Fig. 1, column D), the toxicity was only reduced, not eliminated, even though the concentration of melatonin was twice that of H_2O_2.

Discussion

In the current *in vitro* system, both Al and Aβ 25 - 35 exacerbated lipid peroxidation in the presence of iron, Al being more toxic than Aβ 25 - 35. Furthermore, Aβ 25 - 35 attenuated the toxicity of Al. H_2O_2 greatly increased lipid peroxidation in the presence of both Al and Aβ 25 - 35, except in the absence of iron. Furthermore, melatonin eliminated lipid peroxidation when H_2O_2 was absent, and decreased lipid peroxidation in the presence of H_2O_2.

The above results, in conjunction with previously published data, suggest the following sequence of events in the aetiology of AD. Firstly, it has previously been established that there is an increased flow of Al into the brain in AD. Taylor *et al.*[14] demonstrated an increase in blood Al after an Al citrate drink was ingested by AD patients. In this group the mean blood Al concentrations increased from the baseline value of 8.6 μg/l to 104 μg/l within 60 minutes, whereas in age-matched controls, the blood Al level rose from 7.7 μg/l to only 37.9 μg/l over the same time period. The threshold allowing Al entry into the brain may also be lower in AD patients, given that Ward and Mason demonstrated that the concentration of Al in both the hippocampus and cortex of post-mortem brain samples from AD patients was 10 times higher than that of non-demented controls.[15] This suggests that the increased absorption of Al from the gut also results in more Al being deposited in the brains of these AD patients.

Although Al crosses the blood-brain barrier attached to molecules such as citrate, the principal carrier is transferrin (Tf).[16] In the brain Al is freed from Tf in the lysosomes[8] where the ambient pH is low,[17] which keeps Al in solution, i.e. in a more neurotoxic form. The Al ions then attach themselves to the headgroups of the phospholipid molecules of cellular membranes where, on account of their positive charge, they create artificial openings.[8] Normally, the headgroups would form a barrier against attack by free radicals, protecting the fatty acid side-chains inside the lipid bilayer, but the 'pores' allow hydroxyl radicals to reach, attack and damage the lipids (lipid peroxidation), thus changing the fluidity of the membranes.[18] It is recognised that abnormalities of the lysosomal system precede the appearance of tangles and plaques in the neurons of AD patients.[19]

The role of amyloid in AD has been a subject of debate. The functions of the amyloid precursor protein (APP) include tissue repair in the brain, cell adhesion, and survival and growth of neurons *in vitro*.[20] Shigematsu and McGeer found that APP accumulated in neuronal processes and microglia following intracerebral administration of Al salts in rat brain.[21] It has also been shown that amyloid fragments are generated in lysosomes.[22]

The abovementioned lipid peroxidation causes a 4 Angstrom units reduction in the lipid bilayer width,[23] exposing the transmembrane portion of the APP to proteolytic secretase enzymes which cleave the APP at a site which is normally not accessible to these enzymes,[24] producing insoluble Aβ fragments. Increased production (i.e. not only an accumulation[21]) of APP may well occur in response to increased Al levels in neurons. We hypothesise that APP, being a 'sticky protein', is meant to bind Al and sequester it. Owing to the membrane damage and faulty cleavage of the APP, large amounts of insoluble Aβ are produced instead, although Aβ also binds to metals such as Al and iron.[25] Sequestering of these metals by amyloid would result in a temporary decrease in lipid peroxidation. Increasing levels of Al result in the formation of immature amyloid plaques, which 'hold' the Al, because the brain cannot destroy a metal. At this stage AD progresses slowly.

The plaques represent foreign material in the brain. We hypothesise that this activates the microglia (brain phagocytes), which start to secrete reactive oxygen species such as H_2O_2.[5] Microglia, whose presence in the centre of mature plaques has been demonstrated, are capable of destroying nervous system tissue for scavenging purposes.[26] However, since the plaques consist of insoluble amyloid in the β-sheet conformation, they will be resistant to degradation by the microglia, which continue to produce more H_2O_2. As is evident from the results of the present study, H_2O_2 greatly exacerbates lipid peroxidation, and in these circumstances, Aβ does not have a protective effect against Al-initiated lipid peroxidation (Fig. 5). This may be the cause of the catastrophic phase of cell loss seen in the brains of AD patients (15.1% per year, v. 1.5% per year in healthy controls).[27]

Finally, it was demonstrated that melatonin eliminates lipid peroxidation by Al and Aβ 25 - 35, but in the absence of H_2O_2. When 0.5 mM H_2O_2 is present, melatonin attenuates but does not neutralise lipid peroxidation.

Melatonin, a hormone secreted by the pineal gland, is of interest in diseases associated with ageing, such as AD, since it is known to decrease in concentration with age.[28] The functions of melatonin include regulation of secretion of other hormones (i.e. regulation of the body's biological clock),[28] and it has recently been implicated as a further level of defence against lipid peroxidation.[10] Melatonin is secreted only in the dark. Provided the person is in total darkness, melatonin levels start to rise at 21h00, peaking between 02h00 and 04h00, whereafter they decline. In the presence of electric light of sufficient intensity at night time, no melatonin is produced;[29] hence the availability of melatonin as free-radical scavenger may be compromised in Western societies, where the use of electric light is excessive. This may help explain the finding of a low prevalence of AD in rural communities in Africa.

Conclusions

In the light of the above hypothesis, a few suggestions may be offered as to how we should combat AD.

1. It could be suggested that Al be removed from the diet. Al in solution has been found to be more toxic than Al in food,[30] and drinking tea ('rooibos' herbal tea excluded) has been shown to increase Al levels![31] However, it may be more profitable in the end to find the reason why the natural barriers against Al uptake in the gut and the blood-brain barrier are lifted in AD. In AD, more than anything else, prevention will be better than cure.

2. During the phase where AD progresses slowly, it will be of use to ensure that all protective systems against free-radical damage are functioning optimally. This would include taking a wide range of anti-oxidant supplements along with cognition-enhancing drugs. In addition, the damaged phospholipids would have to be replaced by supplementation with essential fatty acids.[32]

3. In the catastrophic phase, where the brain is in an inflammatory state, anti-inflammatory drugs may be of use.[33]

4. Melatonin, together with other free-radical scavengers in the brain, reduces free radical damage. Since melatonin is produced by the pineal gland only in the dark, we may have to reconsider the effects late-night living may have on our health in old age. In addition, supplementation with melatonin could be considered.[10]

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