Effects of long-term administration of a cocoa polyphenolic extract (Acticoa powder) on cognitive performances in aged rats

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Numerous studies have indicated that increased vulnerability to oxidative stress may be the main factor involved in functional declines during normal and pathological ageing, and that antioxidant agents, such as polyphenols, may improve or prevent these deficits. We examined whether 1-year administration of a cocoa polyphenolic extract (Acticoa powder), orally delivered at the dose of 24 mg/kg per d between 15 and 27 months of age, affects the onset of age-related cognitive deficits, urinary free dopamine levels and lifespan in old Wistar-Unilever rats. Acticoa powder improved cognitive performances in light extinction and water maze paradigms, increased lifespan and preserved high urinary free dopamine levels. These results suggest that Acticoa powder may be beneficial in retarding age-related brain impairments, including cognitive deficits in normal ageing and perhaps neurodegenerative diseases. Further studies are required to elucidate the mechanisms of cocoa polyphenols in neuroprotection and to explore their effects in man.

Cocoa polyphenols: Cognitive performances: Ageing: Lifespan: Rats

Because of their post-mitotic nature, neurons contend with cellular damage accumulated over many decades. The brain is more vulnerable to oxidative stress than other organs due to its low antioxidant protection system and increased exposure of target molecules to reactive oxygen species, one of the major damaging agents involved in age-associated decline (1). Increased reactive oxygen species levels produced by mitochondrial activity, inflammatory processes and excessive glutamate accelerate neurodegenerative processes (2). Some parameters related to neurotransmission also decline during normal ageing (3–5).

Numerous epidemiological studies indicate that dietary flavonoids derived from fruits, vegetables, red wine and green tea decrease the risk of death from CHD (6–7), cancer (8) and stroke (9), and may prevent neurodegenerative diseases and diabetes mellitus (10). Only recently have the beneficial effects of dietary polyphenols come to the attention of nutritionists (11). Polyphenols are present in plants in the form of conjugated molecules, including (–)-epicatechin and (–)-catechin, as well as their oligomers, also named procyanidins (12–14). Cocoa-derived products contain high levels of flavonoids (12,15,16) and show potent antioxidant effects (17). Biomarkers associated with CVD, such as oxidant defence molecules, LDL oxidation state and platelet function, have been assessed after acute consumption of chocolate and cocoa (18–20). The increase in blood epicatechin after acute procyanidin-rich chocolate consumption was associated with increased plasma antioxidant capacity and decreased plasma 2-thiobarbituric acid reactive substances (20). In addition, (–)-catechin and (–)-epicatechin delayed lipid oxidation as well as depleted α-tocopherol and β-carotene levels induced by a free radical generator in human oxidized plasma (21). It was shown that consumption of green tea prevented LDL oxidation in man (22) and that tea catechins attenuated the development of atherosclerosis in apoE-deficient mice (23). The antioxidant properties of polyphenols and their beneficial effects on cognition have been demonstrated in animal studies (24). There is a possible role for (–)-epicatechin in reducing neurodegenerative disorders such as Parkinson’s and Alzheimer’s diseases (25,26). Polyphenols may also possess other types of neuroprotective effects (27,28).

The main purpose of the present study was to investigate the effects of Acticoa powder, a cocoa polyphenolic extract, on cognitive function and lifespan in aged Wistar-Unilever rats, assessed with light extinction (29–31) and water maze tests, the latter adapted from Morris (32) in which rats were let go from the same starting position. The rats were also evaluated for urinary free dopamine levels, susceptible to age-related decreases in Wistar rats (33).

Materials and methods

Animals

Eighteen male Wistar-Unilever rats (Harlan, The Netherlands), weighing 250–275 g at reception, were housed three
per cage in 48 \times 27 \times 20 \text{ cm} polycarbonate cages (U.A.R., Epinay-Sur-Orge, France) in a regulated environment (temperature 22 \pm 2^\circ \text{C}; humidity 50 \pm 10\%\)), provided standard food (food pellets M20; Dietex, Saint Gratien, France) and tap water \textit{ad libitum}, and maintained on a 12 h light/dark cycle (lights on 20.00–08.00 hours).

After an 8-month acclimatization period in our facility, the rats were matched according to weight and randomly assigned to one of two groups (n = 9): a control-vehicle or Acticoa powder (cocoa polyphenolic extract) at the dose of 24 mg/kg per d (AP24), given by oral gavage. The rats were tested for cognitive functions at 9, 13 and 15 months, received AP24 or its vehicle (spring water), and were then retested at 17, 21 and 25 months for light extinction and water maze tests.

The rats used in the present study were treated according to rules provided by the ASAB Ethical Committee (1993) and the Canadian Council on Animal Care (1993). All standard operating procedures were in compliance with the European Communities Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for scientific purposes (Official Journal L 358, 18 December 1986, pp. 0001–0028).

**Tested product**

The solvent-free Acticoa powder was provided by Barry Callebaut France (Louviers, France). It was isolated from non-roasted beans using the patented Acticoa process recently developed by Barry Callebaut France. The general composition of AP24 was as follows (g/100 g): 3.5 g moisture, 29.4 g proteins, 13.2 g fat, 12.8 g carbohydrates, 3.8 g fibres, 2.4 g minerals and 34.9 g total polyphenols. The percentages related to the polyphenols in AP24 determined by HPLC were 88.5\% procyanidins including 0.21\% anthocyanins, 10\% epicatechin, 1\% epicatechin gallate and 0.5\% catechin. The product was freshly prepared every day; it was dissolved in spring water and administered at a dose of 24 mg/5 ml per kg body weight. AP24 and the vehicle were orally administered for 12 months from the age of 15–27 months. After daily administration, the rats were allowed free access to standard food pellets and were weighed three times per week.

Only a single dosage of AP24 was investigated on the basis of results from a chronic experiment which showed its beneficial effects on prostate cancer prevention in rats\(^{34}\).

**Procedures**

**Weight change and food and water consumption.** The body weight of each rat was recorded three times per week during the whole experimental period. Food and water consumption were recorded every week from Monday to Friday, for each cage, in order to estimate the quantity of food and water ingested by rats. Then the mean quantities of food and water were calculated in g/100 g body weight for rats of each cage in the two groups.

**Cognitive testing.** All cognitive tests were recorded by experimenters unaware of administered treatments.

Light extinction: light extinction was tested before treatment at 9, 13 and 15 months of age and after treatment at 17, 21 and 25 months of age. Learning performance was assessed by means of an operant conditioning test based on the natural aversion of rats to bright light\(^{30–31}\). The rat was placed in a strongly illuminated (1200 lux) cage (50 \times 40 \times 37 \text{ cm}) equipped with two levers. By pressing the active lever the rat switched off the light allowing access to a 30 s period of darkness, whereas the inactive lever had no effect on the environment. The test lasted 20 min and the number of active and inactive lever presses were recorded.

Water maze: at 8, 12 and 15 months of age, the rats were trained to find a hidden platform (1 cm beneath water surface at 23^\circ \text{C}) in a tank (150 cm diameter) filled with water from the same starting position for five trials per test session with a maximal duration of 180 s for each trial. The platform was placed away from the wall in a constant quadrant position during the trials. On each trial, the rats were allowed to stay on the platform for 30 s and then were immediately placed back in the water for the next trial. When unable to find the platform, the rats were placed on it for the same amount of time. At 17, 21 and 25 months of age, the rats were retested under treatment or placebo with the same procedure. In our simplified procedure, the rat is proficient in learning to locate more quickly the hidden platform by memorizing its position during a single five-trial session.

**Urinary dopamine levels.** Rats were individually placed in metabolic cages for 7 h from 09.00 to 16.00 hours for the collection of urine samples. Urinary dopamine levels were assayed at 12, 15, 18, 21, 24 and 27 months of age by HPLC with electrochemical detection. Aliquots of 1 mL acidified urine were placed in 5 mL conical-base glass vials with 50 mg alumina, and the samples were adjusted to pH 8.6 by adding Tris buffer. The adsorbed dopamine was then eluted from the alumina with 200 \mu L of 0.2 mol/L HClO₄ on Costar Spin-X microfilters. In the final phase, 50 \mu L of the eluate were injected into an HPLC apparatus (Gilson Medical Electronics, Villiers-le-Bel, France) with a minimal limit of detection of 4 \mu g/L.

**Lifespan**

Throughout the experiment, the date of death of each rat was noted in order to determine the mean lifespan in each group.

**Statistical analysis**

Due to the relatively small number of rats per group, which decreased during the course of the study, and due to unequal variances, non-parametric tests\(^{35}\) were used. The Mann–Whitney U test was used to compare cognitive performances of the two groups. For each group, the Friedman test was employed to compare the repeated cognitive performances throughout the test sessions; whenever significant, the Wilcoxon test was used to compare the performances of two consecutive water maze tests and to evaluate lever discrimination in the light extinction test by comparing the numbers of active and inactive lever presses. Survival data were analysed using the Kaplan–Meier method\(^{36}\). The difference between survival in the two groups was tested using the log rank test. All statistical analyses were carried out with StatView\textsuperscript{\textregistered} 5 software (SAS Inc., Cary, NC, USA). The results are expressed as means and their standard errors. Differences were considered to be significant at P<0.05.
Results

Weight change and food and water consumption

No significant differences were observed between the body weight and food and water consumption of the two groups throughout the study period (data not shown).

Cognitive testing

Light extinction. Total lever presses: as shown in Fig. 1, no significant group differences were apparent for total number of lever presses prior to Acticoa powder or vehicle administration at 9, 13 and 15 months of age (Mann–Whitney U test: U 24.00, NS; U 24.00, NS; U 31.50, NS, respectively). But at 17, 21 and 25 months of age, total lever pressing activity was significantly more elevated in AP24-treated rats (Mann–Whitney U test: U 13.0, P < 0.03; U 4.50, P < 0.004; U 5.00, P < 0.04, respectively).

Lever discrimination: as shown in Fig. 2, control rats (treated with the vehicle) failed to show a significant discrimination between the two levers during baseline at 9, 13 and 15 months (Wilcoxon test: z 0.57, NS; z 0.43, NS; z 0.48, NS, respectively) and during the treatment period at 17, 21 and 25 months (Wilcoxon test: z 0.43, NS; z 0.65, NS; z 0.38, NS, respectively). Although AP24-treated rats did not discriminate between the two levers at baseline (Wilcoxon test: z 0.34, NS; z 0.57, NS; z 1.02, NS, respectively), lever discrimination was observed at 17 and 21 months (Wilcoxon test: z 2.32, P < 0.02; z 2.68, P < 0.008, respectively). But at 25 months, presses on the active lever only tended to be higher than those on the inactive one (Wilcoxon test: z 1.70, P < 0.09).

Water maze. Global performances: during baseline at 8, 12 and 15, and during treatment at 17 months of age, no inter-group differences were found for escape latencies (Mann–Whitney U test: U 34.50, NS; U 32.50, NS; U 35.0, NS; U 20.5, NS, respectively; Fig. 3). But at 21 and 25 months, escape latencies of AP24-treated rats were significantly lower than those of control rats (Mann–Whitney U test: U 6.00, P < 0.007; U 0.00, P < 0.006, respectively).

Long-term memory: long-term memory was evaluated by comparing group performances over months using the Wilcoxon test (Fig. 3). Both groups improved their performances from month 8 to 12 (z 2.67, P < 0.008 for AP24-treated rats; z 2.52, P < 0.02 for controls) and from month 8 to 15 (z 2.67, P < 0.008 for AP24-treated rats; z 2.52, P < 0.02 for controls). The performance of control rats was still stable from month 15 to 17 (z 0.154, NS), but significantly deteriorated at 21 and 25 months (z 2.37, P = 0.018; z 2.02, P = 0.043, respectively). In contrast, the performance of AP24-treated rats was stable from 15 to 17 (z 0.48, NS), from 15 to 21 (z 0.42, NS) and from 15 to 25 (z 0.31, NS) months.

Short-term memory: short-term memory was assessed by comparing group performances over daily trials (Fig. 4). During the first water maze test at baseline, both groups improved their escape latencies from trial 1 to trial 5 (Friedman test: χ²(4 df) 10.58, P = 0.03 for controls). During 12- and 15-month test sessions (baseline), escape latencies of both groups remained low throughout every trial to the extent that short-term memory assessment became meaningless (month 12: χ²(4 df) 5.7, NS for AP24-treated rats; χ²(4 df) 10.80, P = 0.03 for controls; month 15: χ²(4 df) 14.92, P = 0.005 for AP24-treated rats; χ²(4 df) 7.52, NS for controls) (Figs. 4 (A), (B) and (C)).
At the 17-month test session, AP24-treated and control rats improved their latency to find the platform from trial 1 to trial 2 ($z=2.67, P=0.008$; $z=2.37, P=0.018$, respectively). Between trial 2 and trial 5, latencies remained low and stable in the two groups (Friedman test: $\chi^2(3 \text{ df })=2.87$, NS; $\chi^2(3 \text{ df })=0.79$, NS, respectively). However, the performance of AP24-treated rats was better than that of controls for trials 2, 3 and 4 (Fig. 4 (D)). At the 21- and 25-month test sessions, AP24-treated rats improved their latencies on the first two trials (Wilcoxon test: $z=2.08, P=0.04$; $z=2.07, P=0.03$, respectively) and between trial 2 and trial 5, their latencies remained low and stable (Friedman test: $\chi^2(3 \text{ df })=4.01$, NS; $\chi^2(3 \text{ df })=2.50$, NS, respectively). In contrast, latencies of control rats showed no improvement between trial 1 and trial 5 (Friedman test: $\chi^2(4 \text{ df })=2.84$, NS; $\chi^2(4 \text{ df })=2.26$, NS, respectively). Over the five trials of these sessions, the performance of AP24-treated rats was significantly better than that of control rats (Figs. 4 (E) and (F)).

**Urinary free dopamine**

As seen in Fig. 5, urinary free dopamine concentrations remained stable in the two groups from 12 to 15 months of...
age and no group difference was discerned (Mann–Whitney U test: U 10, NS; U 6, NS, respectively). Urinary free dopamine concentrations of AP24-treated rats were significantly lower than those of control rats at 18 months of age (U 2, \( P < 0.02 \)), but significantly higher at 24 and 27 months of age (Mann–Whitney U test: U 0, \( P < 0.002 \); U 0, \( P < 0.008 \), respectively). While dopamine levels remained stable in AP24-treated rats from 18 to 27 months (Friedman test: \( \chi^2(3 \text{ df}) \ 0.80, \text{ NS} \)), values of control rats tended to decline (Friedman test: \( \chi^2(3 \text{ df}) \ 7.46, P < 0.06 \)).

Lifespan

The log-rank statistical test showed a significant increase of survival times in the AP24-treated group in comparison to control rats (\( \chi^2(1 \text{ df}) \ 5.169, P = 0.025 \)), corresponding to a longer lifespan of about 11% over the 27-month period (Fig. 6).

Discussion

Cognitive testing

The main purpose of the present work was to determine whether a daily dose of Acticoa powder ingested by aged rats over a 12-month period retards the onset of age-related cognitive deficits. In the light extinction test, AP24-treated rats had more total lever pressing activity than control rats. Moreover, unlike placebo-treated controls, AP24-treated rats exhibited significant discrimination between active and inactive levers at 17 and 21 months of age. The reason why control rats failed to discriminate is uncertain. We previously observed significant lever discrimination in 3-month-old male Wistar rats(29–31). We suspect that age level is critical and it should be interesting to evaluate younger Wistar-Unilever rats in the same paradigm. It remains to be determined whether the elevated lever activity of AP24-treated rats is generalizable to exploratory activity in the open-field and hole-board, susceptible to decline as a result of advanced ageing in rats and mice(37). It also remains to be determined why the AP24 group discriminates. One possibility is facilitation of cognitive processes. A second possibility is that light is more aversive to them. There is a need to assess the effects of this substance on photophobia and other anxiety tests.

While water maze performance declined in control rats at 21 and 25 months of age, it remained stable in AP24-treated rats. At 21 and 25 months, but not at 17 months, escape latencies of AP24-treated rats were lower than those of control rats. Both long- and short-term memory processes were improved by the cocoa polyphenolic extract, as evaluated by month-to-month or trial-to-trial performances. The present results demonstrate beneficial effects of Acticoa powder on spatial learning.

Many studies indicate that age-associated neurobehavioural deficits are attenuated by dietary supplementation with potent antioxidant activity, such as Ginkgo biloba flavonoids, including memory(38), attention(39) and calcium-induced increases in oxidative metabolism(40). It may be possible to reduce the deleterious effects of Alzheimer’s disease with such supplements(41) or at least delay the physiological impairment associated with normal ageing(42). In particular, Moriguchi et al. (43) reported that a garlic extract prevented brain atrophy, as well as learning and memory impairments in the senescence accelerated mouse(44).

The present results are in accordance with those of Joseph et al. (24) concerning the beneficial action of dietary supplementation with spinach, strawberry and blueberry extracts high in flavonoid levels as well as antioxidant activity(45) on neuronal and cognitive functions in aged rats. Moreover, age-related declines in spatial memory tasks and hippocampal plasticity parameters were improved by antioxidant-rich diets containing blueberries(46,47). Likewise, tea polyphenols immediately after ischaemia improved memory impairment and reduced hippocampal damage in mice(48) and in gerbils(49). In the mouse hippocampal model system for oxidative stress, many flavonoids protected HT-22 cells from glutamate-induced toxicity as well as other oxidative insults(50).

Urinary free dopamine

Dopamine is an essential neurotransmitter enabling smooth, controlled movements as well as efficient memory, attention
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and problem-solving function. Three months after the start of the treatment period, urinary free dopamine levels were paradoxically much higher in control than AP24-treated rats. At 21 months of age (6 months of treatment), no significant difference was observed between the dopamine levels of the two groups. At 24 and 27 months of age, urinary dopamine concentration declined in control rats, but remained relatively high and stable in AP24-treated rats. On the basis of the present results, the variability of urinary dopamine is probably not due to the salt content in the extract. Urinary dopamine levels exhibited by control rats are concordant with the age-related decrease found in Wistar rats. Some studies directly associate the level of free dopamine in urine with the severity of the Parkinsonian syndrome. Hoehn et al. concluded that, although many peripheral sources contribute to urinary free dopamine, a small decrease in the level may actually reflect the severity of the disturbance of central dopamine metabolism and the known deficiency of dopamine in the neurons of the Parkinsonian brain. Green tea polyphenols inhibited the uptake of [3H]dopamine and 1-methyl-4-phenylpyridinium by dopamine transporters and partially protected embryonic rat mesencephalic dopaminergic neurons from 1-methyl-4-phenylpyridinium-induced injury. Similarly, potent neuroprotective properties of green tea polyphenols were demonstrated with respect to striatal dopamine depletion and substantia nigra dopaminergic neuronal loss caused by 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine in mice. Reactive oxygen species are involved in the decline of functions associated with ageing and flavonoid administration attenuated apoptotic injury of mesencephalic dopamine neurons caused by oxidative stress. Joseph et al. examined the brain tissues of blueberry-supplemented rats and found that dopamine levels were significantly higher than those of control rats.

Despite distinct chemical structures, cocoa antioxidant effects are similar to those of tea or blueberry, providing antioxidant defences against reactive oxygen species. Cocoa polyphenolic extracts may therefore protect vulnerable structures such as the nigro-striatal system against dopamine depletion in psychopathological and neurological disorders. The lifespan of AP24-treated rats was prolonged relative to placebo by approximately 11% over the 27-month test period. The mechanisms underlying prolongation of the lifespan of the Acticoa powder remain to be fully established. It was demonstrated that cocoa powder enhances the level of antioxidative activity in rat plasma and among human subjects, a flavonoid-rich chocolate increased plasma antioxidant capacity and reduced the amounts of plasma 2-thiobarbituric acid-reactive substances. Lee et al. showed that cocoa powder and cocoa extracts exhibit greater antioxidant capacity than many other flavanol-rich foods and food extracts, such as green and black tea, and red wine.

However, the beneficial effects of the cocoa extract are attributable not only to antioxidant properties of its polyphenol constituents (procyanidins and methylxanthines), but also via inhibition of nitric oxide-stimulated protein kinase C activity, since flavanols improved nitric oxide-dependent vasodilatation even in the presence of pre-existing endothelial dysfunction. Cocoa polyphenols may improve vasodilation, dependent on epicatechin. The cocoa polyphenolic extract may also act on the cardiovascular system through a variety of mechanisms including reduced LDL oxidation, improved endothelium-dependent relaxation, modulation of cytokines and eicosanoids involved in the inflammatory response, and inhibition of platelet activation.

Conclusion

Since AP24 administration did not show any influence on weight and on food and water consumption of treated rats throughout the experimental period, the benefits observed with the AP24 cocoa extract are not due to dietary restriction. The present results suggest that Acticoa powder may be beneficial in retarding age-related brain impairments, including cognitive deficits in normal ageing and perhaps neurodegenerative diseases. On the basis of the present results, it is of interest to carry out further in-depth preclinical and clinical studies on the neurobehavioural actions of Acticoa powder in order to determine whether age-related dysfunctions may be prevented and to elucidate the mechanisms of cocoa polyphenols in neuroprotection.

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