

Preventive Antioxidant Effects of Cocoa Polyphenolic Extract on Free Radical Production and Cognitive Performances after Heat Exposure in Wistar Rats

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ABSTRACT: The preventive effects of ACTICOA powder (AP), a cocoa polyphenolic extract, on free radicals produced by leucocytes in rats after heat exposure (HE) and its protective effects on subsequent cognitive impairments were assessed. AP or vitamin E, the antioxidant reference, was orally administered to rats for 14 d before HE at 40 °C temperature during 2 h. The day after HE, free radical production by leucocytes in rats treated with AP or vitamin E was significantly reduced as compared to control. Unlike controls, AP- and vitamin E-treated rats discriminated between active lever and inactive levers in a light extinction paradigm. In the Morris water maze, escape latencies before reaching the hidden platform by AP- and vitamin E-treated rats decreased throughout testing. The daily oral administration of AP or vitamin E protected rats from cognitive impairments after HE by counteracting the overproduction of free radicals.

Keywords: cocoa polyphenolic extract, cognitive performances, free radicals, heat exposure, rat

Introduction

Dietary flavonoids derived from fruits, vegetables, red wine, and green tea decrease the risk of death from stroke (Sirving and others 1996). Cocoa-derived products have been identified to be rich in flavonoids, particularly flavan-3-ol (-)-epicatechin (epicatechin) and its polymers (Adamson and others 1999; Hammerstone and others 1999) and they may act as potent antioxidants (Kondo and others 1996; Arteel and Sies 1999). The antioxidant activity of cocoa powder is well known but its physics and chemistry are complex, change during the lifetime of the bean, and depend on its processing (Wang and others 2000; Zhu and others 2002).

An imbalance between formation of reactive oxidant species (ROS) and antioxidant defense is the source of oxidative stress (Sies 1985). ROS include superoxide anion, hydrogen peroxide, and hydroxyl radical normally produced in low quantities (Biesalski 2000). Under normal conditions, superoxide dismutase and catalase metabolize free radicals (Jiménez-Escrig 2006). Polyunsaturated fatty acids, phospholipids, free cholesterol, proteins, DNA (Lodovici and others 2001), and small molecules are targets for free radicals, which impair cell functions (Halliwell and others 1995; Madhavi and others 1996). An increase in free radicals with enhanced superoxide production and lipid peroxidation occurs in heat-exposed anesthetized rats (Yang and Lin 2002; Chang and others 2004; Wang and others 2005). This increase in ROS is accompanied by a rise in reactive nitric species (Canini and others 1997). Furthermore, extensive lesions in cortex, thalamus, hypothalamus, and striatum have been described in cases of heatstroke (Lin and others 1995). Free radi-

cals are involved in heat-induced brain lesions, as indicated by their attenuation following α -tocopherol administration (Niu and others 2003). Moreover, high amounts of ROS may result in memory impairments (Gonenç and others 2005). In particular, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP)-treated rats with high amounts of free radicals were deficient in light extinction and water maze tests (Messaoudi and others 1996). ROS generated by leucocytes can be measured by several methods, including the highly sensitive real-time chemiluminescence probe Pholasine (Swindle and others 2002). Learning and memory processes of rats can be determined by the light extinction procedure, which assesses the ability of rats to discriminate between active and inactive levers in a brightly lit cage (Messaoudi and others 1999; Benton and others 2003), and the Morris water maze test is used to assess spatial learning (Morris 1984).

The aim of this study was to evaluate the antioxidant activity of ACTICOA powder (AP), a cocoa polyphenolic extract obtained using the ACTICOA™ process from cocoa beans, on the overproduction of free radicals by leucocytes and on cognitive performances after HE.

Material and Methods

Sixty-four male Wistar/Han IGS rats (Charles River Laboratories, 69-St-Germain sur l'Arbresle, France), weighing 300 to 320 g, were used. Rats were housed in polycarbonate cages measuring 48 × 27 × 20 cm (U.A.R., 91—Epinay-Sur-Orge, France) in a regulated environment (humidity 50% ± 5%; temperature 22 ± 1 °C) with an inverted light cycle (lights from 20:00 to 08:00). The rats were allowed free access to standard food (M20 pellets, Dietex, 95—Saint Gratien, France) and tap water freely throughout the experiment.

After a 7-d acclimatization period from their arrival, rats were weighed and randomly divided into 4 groups ($n = 16$) depending on treatment and exposure or not to experimental HE: Control (vehicle rats without HE), Veh/HE (vehicle rats with HE), Vit-E/HE (200

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mg·kg⁻¹/d of α -tocopherol); AP/HE (22.9 mg·kg⁻¹/d of AP). The dose of AP was chosen according to the daily calculated consumption of antioxidant from chocolate in humans and reported to rat body weight. The vitamin E dose was chosen according to the literature.

Rats used in this study were treated according to rules provided by the ASAB Ethical Committee (2006) and the Canadian Council on Animal Care (1993). All standard operating procedures were in compliance with the European Community 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.

The cocoa polyphenol extract was provided by Barry Callebaut France (Louviers, France). Solvent-free AP was isolated with the patented ACTICOA™ process from nonroasted cocoa beans harvested in the Ivory Coast. The crude composition of AP was 31.1% proteins, 13.9% fat, 13.6% carbohydrates, 4.0% fiber, and 36.9% total polyphenols. The total polyphenol composition of AP was 88.5% procyanidins, 10.0% epicatechin, 1.0% epicatechin gallate, and 0.5% catechin. Rats were treated with AP dissolved in water. Alpha-tocopherol (Xinchang Pharmaceuticals Factory, China) was dissolved in olive oil and used as the antioxidant reference (Niu and others 2003).

Vitamin E, AP, and vehicle were administered *per os* (PO) in each group for 14 consecutive days (D1 to D14), with treatments ending the day before HE. During the treatment period, rats were allowed free access to standard food pellets and water. Rats were weighed daily and their food intake was calculated weekly from Monday to Friday.

On D15, after overnight fasting, rats belonging to HE groups were exposed to a temperature-controlled chamber (55 × 48 × 40 cm) at 40 °C with 50% relative humidity for 2 h. Immediately after HE, rectal temperature was measured and rats with a body temperature between 41.5 and 42.0 °C were assumed to have undergone severe heat stress and were included in the study. Rats were then allowed to recover in their home cage. The group without HE was put in the same chamber at 20.0 °C during 2 h.

For the free radical production by leucocytes after HE, on D16, a blood sample of 500 μ L was taken by the tail vein in heparinized tubes on each rat. Based on the chemiluminescence of Pholasin, a photoprotein of the marine rock-boring mollusc *Pholas dactylus*, a kit (Cell Activation Test Kits for Whole Blood with Adjuvant-K, Knight Scientific, Plymouth, UK) was used to measure the free radical production by leucocytes by the activation of the NADPH oxidase complex from diluted blood, according to the method developed by Swindle and others (2002). Pholasin can react with a variety of ROS to form oxypholasin and light as a by-product. The protein reacts only once with ROS and then the luminescent product is degraded. The luminescence (mV) was recorded.

All cognitive tests were performed with experimenters blinded to treatment variables.

The light extinction test was performed on D18, 3 d after HE, according to the procedure previously described by Messaoudi and others (1996).

The Morris water maze test was performed on D20 and D21 according to the modified procedure of Morris (1984). Briefly, rats were trained to find a hidden platform (1 cm beneath water surface at 25 °C) in a tank (150 cm diameter) filled with water from the same starting position for 5 trials per day with a maximal duration of 180 s. On the 1st trial, the platform was placed against the wall of the tank. The platform was placed 5 cm away from the wall in a constant quadrant position during the 2nd trial and 10 cm away for the final 3 trials. On each trial, rats were let on the platform for 30 s and then were immediately placed again in the water for the next trial. When unable to find the platform, rats were placed on it for 30 s.

On D21, to test the long-term memory of rats over a single trial (trial 6), the platform was placed 10 cm away from the wall of the tank. Trial 3 of D20 served as reference trial. The dependent variable recorded was the latency before reaching the platform.

For the statistical analyses, analysis of variance (ANOVA) was used to compare cognitive performances and free radical production by leucocytes in the 4 tested groups. When significant, Dunnett's *t*-test was used to compare treated groups to the control one. For repeated measures, paired *t*-tests (2-tailed) were used in each group to compare the discrimination in the light extinction test and the short-term and the long-term memory in the Morris water maze test. All statistical analyses were carried out with StatView® 5 software (SAS Inc., USA). The results were expressed as mean \pm standard error of the mean (SEM). For all comparisons, differences were considered to be significant at *P* < 0.05.

Results and Discussion

No differences in the body weight and food intake were observed for the 4 groups of rats during the 14-day treatment period (data not shown), indicating lack of any serious discomfort.

On D16, a difference in the free radical production by leucocytes was observed for the 4 groups of rats (*P* < 0.001). As shown in Table 1, Veh/HE-treated rats displayed a higher production of free radicals than in the other groups.

The antioxidant effect of AP was evaluated on the basis of biological and clinical criteria. First, we assessed on D16 *in vitro* free radical production of stimulated leucocytes in HE rats. The increase of free radical production by leucocytes from Veh/HE-treated rats indicated a high oxidative reactivity after HE. The preventive treatment with antioxidants reduced leucocyte reactivity, since the free radical contents of AP/HE- and Vit-E/HE-treated rats were significantly lower than those of Veh/HE-treated rats (Table 1). The protection against induced overproduction of free radicals after sub-chronic administration of AP and of Vit-E was similar. Such an activity is unlikely to be caused by the vehicle solution, as Niu and others (2003) demonstrated that water or corn oil injections had no effect on heatstroke responses. Instead, leucocyte reactivity in free radical production is likely to be related to the inflammatory reaction occurring after HE (Lin and others 1995; Niu and others 2003).

Cocoa is one of the richest flavonoid-containing foods available (Serafini 2004), presenting high epicatechin concentrations and a

Table 1 – Free radical production by leucocytes on D16 (luminescence, mV) (mean \pm SEM)

Groups	Veh/HE (<i>n</i> = 13) ^a	Control (<i>n</i> = 14) ^a	AP/HE (<i>n</i> = 14) ^a	Vit-E/HE (<i>n</i> = 13) ^a
Free radical content	1257.5 \pm 163.8	310.1 \pm 19.6	300.8 \pm 11.3	311.2 \pm 20.6
		ANOVA: <i>F</i> _(3,50) = 445.25; <i>P</i> < 0.001		
Dunnett's <i>t</i> -test (vs. Veh/HE)	–	<i>t</i> = 21.51	<i>t</i> = 21.83	<i>t</i> = 20.67
Significance		<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> < 0.0001

^a Some samples were hemolyzed and were not tested.

source of other dietary polyphenols such as catechin and quercetin that may also contribute to its antioxidant activity (Lamuela-Raventos and others 2001). A cocoa polyphenol solution inhibited production of ROS by activating granulocytes and lymphocytes (Sanbongi and others 1997). Thus, polyphenols contained in AP may protect cells during HE by reducing inflammatory responses. Consistent with this hypothesis is the finding of the protective activity of vitamin E. In severe heatstroke induced in rats, pretreatment with vitamin E significantly attenuated the heat-induced increase of free-radical formation and lipid peroxidation in the brain and also results in the increase of the levels of cytokines in the plasma. This results in short-term diminution of heatstroke-induced arterial hypotension, cerebral ischaemia, and neuronal damage, together with prolonged survival times (Niu and others 2003). However, long-term effects cannot be ruled out, because vitamin E given prior to HE reduced heat-induced cytokine production and thus the inflammatory reaction. It would have been of interest to measure levels of plasma cytokines before and after the HE in our study.

The diminished free radical production of stimulated leucocytes observed here may reflect this antioxidant-reduced inflammatory response. However, we observed in our model only a relative efficiency of vitamin E. This could be explained by the following conjectures: (i) animals are not deficient in vitamin E, (ii) heat stress was less severe than that provoked by Niu and others (2003). On the contrary, administration of AP was more efficient than vitamin E, indicating possible different mechanisms of action. Polyphenols may have directly protected cells from the heat-induced damage. Milbury and others (2003) demonstrated that polyphenols from almond skins enhance LDL resistance to oxidation by radical quenching. Although the mechanisms of action of polyphenols are not well known, AP and vitamin E might be combined to obtain synergistic actions.

Table 2—Total number of lever pressings (ALP + ILP)^a (mean ± SEM)

Groups	Veh/HE (n = 16)	Control (n = 16)	AP/HE (n = 16)	Vit-E/HE (n = 16)
Total lever pressings	42.8 ± 5.8	32.9 ± 6.3	26.8 ± 4.5	42.4 ± 5.8
ANOVA: $F_{(3,60)} = 1.88$; NS				

^aALP, active lever pressing; ILP, inactive lever pressing.

Table 3—Lever discrimination (mean ± SEM)

Groups	Veh/HE (n = 16)	Control (n = 16)	AP/HE (n = 16)	Vit-E/HE (n = 15) ^a
ALP ^b	23.2 ± 3.4	20.4 ± 4.0	15.6 ± 2.8	27.3 ± 3.5
ILP ^c	19.6 ± 2.9	12.5 ± 3.1	11.19 ± 1.9	17.60 ± 2.5
Paired <i>t</i> -test	<i>t</i> = 1.55	<i>t</i> = 2.40	<i>t</i> = 2.85	<i>t</i> = 3.81
(ALP vs. ILP)	NS	<i>P</i> < 0.05	<i>P</i> < 0.05	<i>P</i> < 0.005

^aOne rat was discarded from the statistical analysis as it had not pressed the inactive lever during the test.

^bALP, active lever pressing.

^cILP, inactive lever pressing.

Table 4—Water maze test: latency to reach the platform (s) (mean ± SEM)

Groups	Veh/HE (n = 16)	Control (n = 16)	AP/HE (n = 16)	Vit-E/HE (n = 16)	
Day 20	Trial 3	21.0 ± 4.6	27.4 ± 4.5	22.8 ± 4.5	26.4 ± 5.2
	Trial 4	13.9 ± 1.3 ^{NS}	18.5 ± 2.9*	17.5 ± 3.8 ^{NS}	17.8 ± 2.4 ^{NS}
	Trial 5	14.2 ± 2.1 ^{NS}	14.4 ± 3.0*	11.9 ± 1.7*	13.6 ± 1.4*
Day 21	Trial 6	12.5 ± 1.7 ^{NS}	12.1 ± 2.3**	10.2 ± 1.3*	14.7 ± 2.2 ^T

Paired *t*-test (2-tail.); significance vs. trial 3.

NS = not significant, ^T Trend; *P* < 0.10; **P* < 0.05; ***P* < 0.01.

In the light extinction test, as shown in Table 2, no significant difference was observed in the total number of lever pressings in the 4 groups of rats.

As indicated in Table 3, Control-, Vit-E/HE-, and AP/HE-treated groups displayed a significant lever discrimination by pressing more often on the active lever than on the inactive one (*P* < 0.05, *P* < 0.05, and *P* < 0.005, respectively). On the contrary, Veh/HE-treated rats showed no lever discrimination.

Thus, HE caused cognitive impairment in Veh/HE-treated rats without significantly changing total pressing activity. This deleterious effect was prevented by AP and vitamin E. This vitamin is already known to limit cognitive impairment in elderly subjects (Ortega and others 2002; Grodstein and others 2003).

In the Morris water maze test, as shown in Table 4, escape latencies significantly decreased over trials in Control, AP/HE, and Vit-E/HE groups (*P* = 0.002, *P* = 0.02, and *P* = 0.02, respectively), but not in the Veh/HE group. Control rats improved their latency from trial 3 to trials 4, 5, and 6 (Table 4) contrary to the Veh/HE group. The AP/HE group improved their performances from trial 3 to trials 5 and 6, but not from trial 3 to 4. The Vit-E/HE group only improved from trial 3 to 5. However, ANOVAs did not show significant group differences on any trial.

Rats receiving vitamin E or AP obtained similar benefits as in the control group and better ones than the Veh/HE group, which did not show any significant improvement throughout testing. The beneficial action was limited to repeated measures and not to intergroup comparisons in any specific trial. Suzuki and others (2004) demonstrated the protective effect of tea catechins on damage of cerebral tissue depending on plasma epigallocatechin-gallate concentrations in rats. Our results suggest preservation of short- and long-term memory processes following antioxidants. The improved cognitive outcome may be the consequence of (i) preservation of brain function as a result of reduced HE or inflammatory aggression, or (ii) enhanced cerebral plasticity.

AP given to rats before HE may be effective in the prevention of the clinical outcome. Further investigations on aged animals are needed to address the question of mechanisms of action as well as optimal dosages and duration of the beneficial treatment period. It would also be of interest to carry out much more analyses such as oxidative stress biomarkers and plasma antioxidants, before and after the HE, to assess how AP is affected by the induced stress and how AP antioxidant levels are correlated with the parameters of oxidative stress and cognitive impairment. The present study suggests that polyphenols from AP provide a clinical interest in the prevention of heatstroke, usually occurring in previously healthy active subjects. Vinson and others (1999) observed a wide variation in total phenol content depending on type of chocolate and cocoa. Further preclinical and clinical experiments are necessary to support understanding of the effects of cocoa-derived polyphenols on health measures.

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