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PAPER

## Effect of a cocoa polyphenol extract in spontaneously hypertensive rats

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In this study, we evaluated the short-term effect of a cocoa polyphenol extract (CPE), in spontaneously hypertensive rats (SHR). Male 17–22-week-old SHR were administered by intragastric gavage water, 50 mg kg<sup>-1</sup> Captopril or CPE at different doses (13, 26, 80 and 160 mg kg<sup>-1</sup>). The systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded by the tail cuff method before the administration and also 2, 4, 6, 8, 24, 48 and 72 h post-administration. Highly significant decreases in the SBP and in the DBP were observed when captopril or CPE was administered to SHR. The cocoa extract produced a dose dependent effect in the SBP of the SHR up to the dose of 80 mg kg<sup>-1</sup>. Nevertheless this dose of CPE did not decrease the arterial blood pressure in the normotensive Wistar Kyoto rats. The decrease in the SBP caused by 80 mg kg<sup>-1</sup> of CPE in the SHR ( $-39.1 \pm 3.7$  mm Hg) was maximum 6 h post-administration, and the initial values of SBP were recovered 72 h post-administration of this extract. Paradoxically, 160 mg kg<sup>-1</sup> of the cocoa extract caused a decreased antihypertensive effect than lower doses of CPE. In addition, the decrease in DBP was always more accentuated when the dose of CPE administered was lower. Our results suggest that CPE may be used as a functional food ingredient with beneficial effects for controlling arterial blood pressure.

### Introduction

Cardiovascular disease is the most common cause of death in industrial societies.<sup>1–3</sup> The intake of polyphenols has been inversely related with the reduced risk of this disease.<sup>4–6</sup> In fact, many epidemiological studies associate an increased consumption of foods and beverages rich in flavonoids, with a reduced risk of cardiovascular death.<sup>7–9</sup> Moreover, the use of products with a natural origin that may cause scarce side-effects, is an attractive possibility to be considered in treating several pathologies.<sup>10,11</sup>

Cocoa is one of the foods that possess a major content of flavanols,<sup>12,13</sup> and the cardiovascular benefits of cocoa and cocoa derivatives has been extensively studied.<sup>14–16</sup> It was reported that cocoa consumption reduced cardiovascular mortality in the indigenous populations of Kuna islands,<sup>17</sup> and more recently cocoa consumption has also been associated with lower cardiovascular mortality.<sup>18–20</sup> Cocoa is rich in flavan-3-ols and procyanidins, (–)-epicatechin being the main component.<sup>21</sup> Other examples of sources rich in these kind of flavonoids are wine and tea. However, cocoa has been shown to have the highest content of flavanols.<sup>22,23</sup>

The presence of these polyphenols is important for the healthy effects associated with cocoa consumption. Moreover, it has recently been published that these kind of flavonoids are able to induce a progressive, and sustained reduction in blood pressure when administered chronically to humans or to different rat models of hypertension, including spontaneously hypertensive rats (SHR) and L-NAME-treated rats.<sup>24–26</sup> Nevertheless, it should be kept in mind that the cocoa flavanol ingestion will depend on the initial flavanol content of the cocoa beans and the manufacturing process.<sup>27</sup>

In previous works we have demonstrated the antihypertensive properties of a polyphenol rich cocoa powder, after short<sup>28</sup> and long-term treatment,<sup>29</sup> and we have also studied the possible mechanisms implicated in their antihypertensive effect.<sup>30</sup> However, extracts are easier to handle in the food industry than other traditional functional ingredients, due to some of their physicochemical properties, such as their high solubility. Moreover, the organoleptic profile of the final application is usually not modified when extracts are used as food ingredients, and they could be added to many applications. Therefore, the aim of this study is to evaluate the possible short-term antihypertensive effect of a cocoa polyphenol extract (CPE) derived from a polyphenol rich cocoa powder, after single oral administration, in SHR.

### Material and methods

#### Cocoa polyphenol extract

The extract CPE used for this study was obtained from a polyphenol-rich cocoa powder produced from unfermented,

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blanch-treated, non-roasted cocoa beans which preserves polyphenol degradation.<sup>31</sup> The theobromine content of the extract, determined by High-Performance Liquid Chromatography (HPLC), was 90.6 mg g<sup>-1</sup>. Total polyphenols, measured by Folin-Ciocalteu's method and flavan-3-ols content (monomers and procyanidins B1 and B2), measured by HPLC-DAD according to Cienfuegos-Jovellanos *et al.*,<sup>28</sup> of CPE were 509.8 and 232.1 mg g<sup>-1</sup> respectively. Table 1 shows these data and also the antioxidant capacity of this extract, expressed as  $\mu\text{mol}$  of Trolox equivalents (TE) per gram of CPE, that was determined by the hydrophilic ORAC (H-ORAC) assay according to Ramos *et al.*<sup>32</sup> All the analyses have been performed in triplicate and the results are reported on wet basis.

### Experimental procedure in rats

In this study we have used twenty 17–20-week-old male SHR, weighing 314  $\pm$  3 g, and ten 17–20-week-old male normotensive Wistar-Kyoto (WKY) rats, weighing 337  $\pm$  6 g. All these animals were obtained from Charles River Laboratories Spain. The animals were maintained at a temperature of 23 °C with 12 h light/dark cycles, and consumed tap water and a standard diet (A04 Panlab, Barcelona, Spain) *ad libitum* during the experiments. CPE was dissolved in water and orally administered by gastric intubation, between 9 and 10 am. Distilled water was used as negative control, and Captopril (Sigma, USA) (50 mg kg<sup>-1</sup>), a known antihypertensive drug, was given as positive control. Different doses of CPE (13, 26, 80 and 160 mg kg<sup>-1</sup>) were administered. The volume orally administered to the rats was always 1 mL/rat either of water, or of the appropriate solution of CPE or Captopril. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded in the rats by the tail cuff method,<sup>33</sup> before administration and 2, 4, 6, 8, 24, 48 and 72 h post-administration. Before the measurement, the rats were kept at 38 °C for 10 min in order to detect the pulsations of the tail artery. To establish the value of SBP and DBP, five measurements were taken, and the average of all of them was obtained. To minimize stress-induced variations in blood pressure all measurements were taken by the same person in the same peaceful environment. Moreover, to guarantee the reliability of the measurements we established a training period of two weeks before the actual trial time, and during this period the rats were accustomed to the procedure.

**Table 1** Theobromine, total polyphenol content, flavan-3-ols and antioxidant capacity of the cocoa polyphenol extract studied

Compounds	mg g <sup>-1</sup> wet matter <sup>a</sup>
Theobromine	90.6 $\pm$ 0.2
Total polyphenol content <sup>b</sup>	509.8 $\pm$ 4.0
Epicatechin	133.5 $\pm$ 1.1
Catechin	10.8 $\pm$ 1.0
Procyanidin B1	9.1 $\pm$ 0.2
Procyanidin B2	78.7 $\pm$ 1.2
Antioxidant capacity <sup>c</sup>	12134 $\pm$ 379

<sup>a</sup> The values are expressed as the mean  $\pm$  SD ( $n = 3$ ). <sup>b</sup> Measured by Folin-Ciocalteu's method. <sup>c</sup> Hydrophilic ORAC (H-ORAC) assay, expressed as  $\mu\text{mol}$  of Trolox equivalents (TE) per gram of CPE.

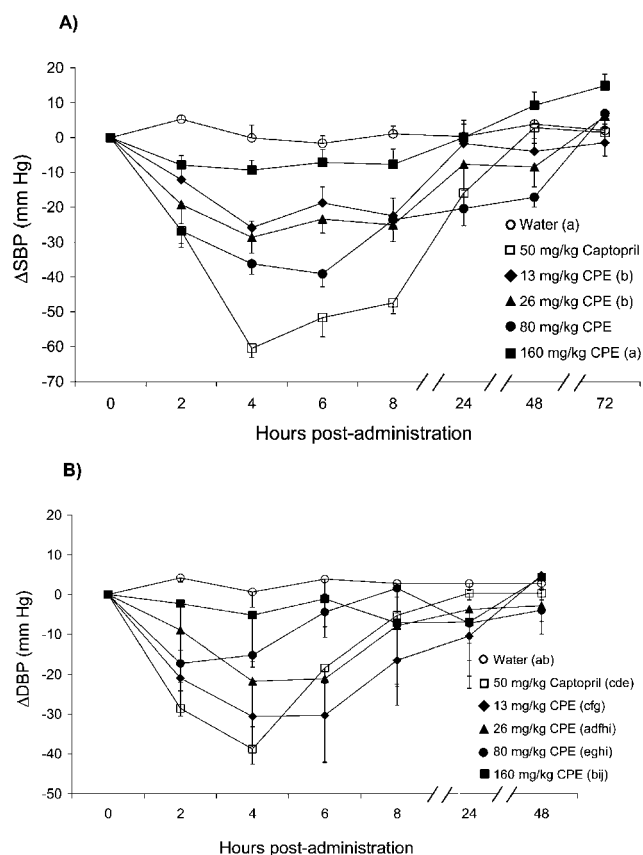
All the above-mentioned experiments were performed as authorized for scientific research (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food).

### Statistical analysis

The results are expressed as mean values  $\pm$  standard error of the mean (SEM) for a minimum of 8 rats, and were analyzed by a two-way analysis of variance (ANOVA), using the GraphPad Prism software. In addition, in order to compare the different treatments and to assess the effect of time within each treatment, some data were also analyzed by a one-way ANOVA, and differences between the groups were assessed by the Bonferroni test. Differences between the means was considered to be significant when  $P < 0.05$ .

### Results

Fig. 1 shows the changes in SBP and DBP obtained in SHR after the administration of the different assayed compounds. Before administration of the different products, the SHR showed SBP



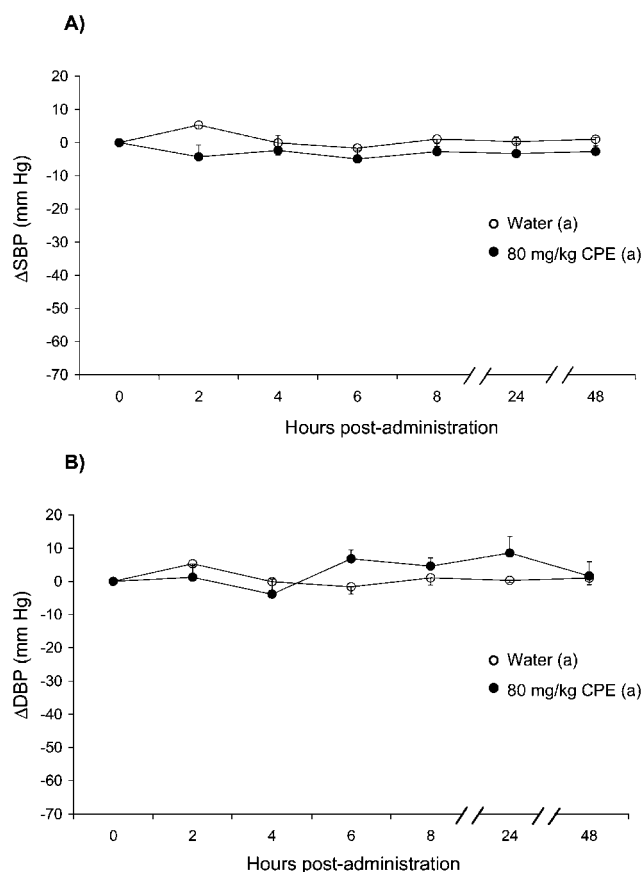
**Fig. 1** Decrease in systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) caused in spontaneously hypertensive rats after the administration of different products. Water (○), Captopril (50 mg kg<sup>-1</sup>) (□) or different doses of CPE: 13 mg kg<sup>-1</sup> (◆), 26 mg kg<sup>-1</sup> (▲), 80 mg kg<sup>-1</sup> (●) and 160 mg kg<sup>-1</sup> (■). Data are expressed as mean  $\pm$  SEM. The experimental groups always have a minimum of 8 animals. Same letters indicate no statistical differences ( $P > 0.05$ ).  $P$  estimated by two-way ANOVA.

values of  $216.9 \pm 3.3$  mm Hg ( $n = 20$ ) and DBP values of  $158.8 \pm 3.8$  mm Hg ( $n = 20$ ). The values of SBP and DBP obtained after oral administration of bidistilled water were very similar to those obtained before its administration. Captopril caused a clear decrease in SBP and DBP in SHR. The maximum decrease in SBP and DBP caused by  $50 \text{ mg Kg}^{-1}$  of this drug were observed 4 h post-administration. These variables returned to baseline 48 h after the administration of Captopril. The oral administration of CPE also resulted in a significant decrease of the SBP and the DBP in the SHR. The decrease in SBP caused by the extract was dose-dependent only up to the dose of  $80 \text{ mg kg}^{-1}$ . The change in the SBP caused by this dose of CPE was  $-39.1 \pm 3.7$  mm Hg. This decrease in SBP was the maximum decrease in this variable obtained with this extract, and it was observed 6 h post-administration. Nevertheless, 72 h post-administration of  $80 \text{ mg kg}^{-1}$  CPE, the values of SBP were very similar to those observed before the administration of this extract. The maximum decrease of SBP caused by  $13 \text{ mg kg}^{-1}$  CPE ( $-25.9 \pm 1.9$  mm Hg) and the maximum decrease of SBP caused by  $26 \text{ mg kg}^{-1}$  of CPE ( $-28.6 \pm 4.5$  mm Hg) were reached 4 h post-administration. Paradoxically, the dose of  $160 \text{ mg kg}^{-1}$  of CPE had the lowest antihypertensive effect ( $-9.3 \pm 2.7$  mm Hg). In addition, the decrease in DBP was always more accentuated when the dose of CPE administered was lower. Therefore,  $13 \text{ mg kg}^{-1}$  CPE caused the maximum decrease in the DBP in the rats ( $-30.6 \pm 7.7$  mm Hg), and this decrease in this variable was observed 4–6 h post-administration of this dose of CPE.

The administration of CPE did not modify the arterial blood pressure in the normotensive WKY rats (Fig. 2). This variable was similar in the WKY rats treated with this product and in the WKY rats administered water.

## Discussion

The health benefits of cocoa reported in recent studies have increased the interest to obtain products with high cocoa polyphenol content.<sup>34,35</sup> A human study demonstrated that only  $30 \text{ mg day}^{-1}$  of flavan-3-ols (up to 5-mers) reduced blood pressure in humans.<sup>24</sup> According to previous results of our research group an important content of polyphenols was founded in CPE, in particular of low molecular weight procyanidins.<sup>31</sup> In fact, the concentration of total polyphenols and flavan-3-ols of CPE was very high when compared to other cocoa derivatives such as different cocoa powders or chocolates.<sup>36–38</sup> Moreover, the total flavan-3-ols content (monomers and procyanidins B1 and B2) and the (–)-epicatechin content of CPE were 2 and 7 times higher than the respective values previously reported for the original polyphenol rich cocoa powder.<sup>28</sup> As (–)-epicatechin is the most abundant flavanol in cocoa, the potential of this compound to be responsible for the cocoa blood pressure lowering effect is of singular relevance, as explained by Fraga *et al.*<sup>20</sup> In this context, the high content of flavan-3-ols, and in particular the high content in (–)-epicatechin, measured in CPE, point out that it should be possible to use a small amount of this extract to decrease arterial blood pressure. It should be also noted that the doses of CPE selected for this study had the same (–)-epicatechin content as the doses of a polyphenol rich cocoa powder that had exhibited an antihypertensive effect.<sup>28</sup> In addition, low molecular weight procyanidins are probably an important component of



**Fig. 2** Decrease in systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) caused in Wistar-Kyoto rats after the administration of water (○), or  $80 \text{ mg kg}^{-1}$  CPE (●). Data are expressed as mean  $\pm$  SEM. The experimental groups always have a minimum of 8 animals. No statistical differences were observed.

CPE, because Cooper *et al.*, in 2008, postulated that the healthy properties attributed to cocoa would be related to the high amount of monomeric and dimeric compounds.<sup>39</sup> In fact, the bioavailability of cocoa polyphenols is strongly related with their molecular size, and, in general, the smaller polyphenols (monomers and dimers) are found in higher concentration in the blood than other polyphenols. Polyphenol monomers and dimers have therefore more possibilities to reach their target organs in the body. A high amount of these compounds of low molecular weight in cocoa derivatives, especially for the monomer (–)-epicatechin, could also be accompanied by a dose-dependent increment in plasma antioxidant capacity<sup>40,41</sup> and by a dose-dependent decrease in plasma lipid oxidation.<sup>40</sup> This monomer is also considered responsible, at least in part, of many of the vascular beneficial effects associated with cocoa consumption,<sup>18,20,29,42–44</sup> and their antihypertensive effects have also been demonstrated in rats subjected to  $N\omega$ -nitro-L-arginine methyl ester pre-treatment<sup>20</sup> and in SHR rats (unpublished data).

It is well known that theobromine was commonly used to treat hypertension because of its ability to relax smooth muscle tissue and dilate blood vessels. In fact, this methylxanthine could be responsible for the decrease in blood pressure reported after the short-administration of dark chocolate.<sup>45</sup> In the present study, an antihypertensive effect of CPE was demonstrated, but

theobromine is present in this extract only in a low concentration (90.6 mg g<sup>-1</sup>) and could therefore hardly justify its antihypertensive properties. A recent study shows that a theobromine-enriched flavanol-rich cocoa with 979 mg of theobromine could decrease central systolic blood pressure in healthy individuals. Nevertheless, the flavanol-rich cocoa with a natural dose of theobromine consisting in 106 mg of this methylxanthine did not significantly change it.<sup>46</sup> As in the study carried out with the original polyphenol rich cocoa powder,<sup>28</sup> in the present study, a dose dependent antihypertensive effect of CPE could not be demonstrated. On the contrary, we could observe that the highest dose of this polyphenolic compound (160 mg kg<sup>-1</sup>) was not the most effective one to decrease arterial blood pressure. In this context, different studies have demonstrated that a high quantity of polyphenols could exhibit pro-oxidant properties instead of antioxidant properties.<sup>47–49</sup>

The lowering blood pressure effect exhibited by CPE would be mainly due to the presence of flavan-3-ols. As mentioned before, the doses of both products, the original polyphenol rich cocoa powder and the extract used in the present work, are equivalent in (–)-epicatechin content, but the potential contribution for the antihypertensive effect of other polyphenolic compounds, and the synergy between them, cannot be ruled out. It has been also published that polyphenols are able of inducing a progressive, and sustained reduction in blood pressure when administered chronically to humans or to different rat models of hypertension, including SHR and L-NAME-treated rats.<sup>24–26</sup> It is important to note that hypertension is a chronic pathology that requires chronic treatment, and the use of strategies with long lasting antihypertensive effects is always desirable. In this context, it is important to highlight that the decrease in arterial blood pressure caused by CPE lasted for a longer period of time than the antihypertensive effect previously reported for the original polyphenol rich cocoa powder. In accordance with this idea, the antihypertensive properties of CPE may be more favourable than the original cocoa powder for controlling high blood pressure levels.

The administration of 80 mg kg<sup>-1</sup> of CPE to normotensive WKY rats did not change the arterial blood pressure of these animals. This indicates that the effect of CPE is specific for the hypertensive condition.

In this study we have also demonstrated that CPE presents an extraordinary *in vitro* antioxidant capacity (12134 μmol TE/g), which can be considered as a very high one when compared with the antioxidant capacity of other cocoa derivatives such as milk chocolate, dark chocolate or unsweetened chocolate (74, 219 and 490 μmol TE/g respectively), or when compared with the antioxidant capacity of a natural cocoa powder (820 μmol TE/g), all of them analyzed by the H-ORAC method.<sup>37</sup> Moreover, the antioxidant capacity of CPE was much higher than those reported for other foods different from cocoa, such as cereals, legumes, vegetables, or fruits.<sup>50</sup> This suggests that an antioxidant mechanism could be implicated in the antihypertensive effect observed when CPE is administered. However, we cannot discard that other mechanisms of action are involved in this effect, and more studies are necessary in order to elucidate the blood pressure lowering mechanisms of CPE.

In this paper, the *in vitro* antioxidant capacity and antihypertensive effect of CPE in SHR has been demonstrated. Our

results suggest that this extract could be used as a functional food ingredient with potential therapeutic benefit in the prevention and treatment of hypertension. In particular, CPE could be useful for prehypertensive patients who do not need the prescription of blood pressure lowering medications so far, and we want also to point out that our results clearly support that it can be consumed by normotensive subjects without promoting any change in arterial blood pressure. Undoubtedly clear advantages are applicable to CPE, and this extract could be used preferentially to the original polyphenol rich cocoa powder as a food antihypertensive ingredient. However, further studies after long-term treatment are necessary in animals and humans before to use CPE as antihypertensive functional ingredient. In addition, studies to elucidate the mechanisms implicated in the antihypertensive effect of CPE should also be carried out. In fact, we are currently conducting some studies using young SHR, in order to investigate if CPE is useful to prevent the development of hypertension and to clarify the mechanisms implicated in their antihypertensive effect.

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