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Assessment of DNA damage using comet assay in middle-aged overweight/obese subjects after following a hypocaloric diet supplemented with cocoa extract

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Abstract

Nutrient excess and unbalanced diets can result in overproduction of reactive oxygen species (ROS), which are associated with oxidative stress. Cocoa extract contains antioxidants that inhibit the harmful effects of ROS. This trial analysed the effect of cocoa extract consumption integrated as a bioactive compound into ready-to-eat meals, on oxidative stress at the level of DNA in overweight/obese subjects. Fifty volunteers [57.26 (5.24) years, 30.59 (2.33) kg/m2] participated in a 4-week double-blind, randomised, placebo-controlled parallel nutritional intervention. Half of the volunteers received meals supplemented with 1.4g/day cocoa extract, while the other half received control meals, both within a 15% energy restriction diet. Lymphocytes were isolated and endogenous strand breaks, oxidised bases and resistance to H2O2-induced damage were measured by the comet assay. The intake of ready-to-eat meals supplemented with cocoa extract did not show relevant changes in the oxidative status of DNA. However, in the cocoa group, oxidised bases negatively correlated with methyl epicatechin-O-sulphate (r = −0.76; P = −0.007) and epicatechin sulphate (r = −0.61; P = −0.046). When volunteers of both groups were analysed together, a marginal decrease (P = 0.072) in oxidised bases was observed, which attributed to weight loss. Subjects who started the intervention with higher levels of damage showed a greater reduction in oxidised bases after 4 weeks (P = 0.040) compared to those who had lower baseline levels. In conclusion, even if 1.4g of cocoa supplementation for 4 weeks did not show notable changes in terms of antioxidant status of DNA, the energy restriction showed a slightly decrease in oxidised bases and this was seen to a greater extent in subjects who started the intervention with higher levels of damage. On the other hand, the inverse associations found between oxidised bases and some cocoa-derived metabolites suggest that a protective effect might be seen in a longer period of time or in subjects with higher baseline DNA damage. Trial registration: www.clinicaltrials.gov (NCT01596309).

Introduction

Impaired oxidative stress is a state where homeostasis between antioxidants and oxidants is altered and free radical production increases above the defence of the body (1). This situation can be produced by psychological, physiological and environmental stressors, which favour reactive oxygen species (ROS) production (2).
Molecules, cells, tissues and systems of the organism suffer damage from oxidative stress, which alter their function and increase the risk of disease and mortality (2). In this sense, oxidative stress has been directly implicated in disease onset and also in the ageing process (4,5). On the other hand, ROS are also involved in immune protection and in cell signalling pathways, therefore suppressing or enhancing them too much might be harmful for the organism (6).

Obesity is a disease characterised by excess accumulation of adipose tissue, almost always produced by an imbalance between energy intake and expenditure (7). Nutrient excess, particularly from high-fat and high-carbohydrate meals, acts as a stress factor, promoting adipocyte proliferation and causing excessive ROS production (8–10), with an impact on the development of obesity-related diseases (11).

However, the organism has an antioxidant system that copes with and manages the damage caused by excessive ROS production (1). This system consists of endogenous (enzymatic and non-enzymatic) defences and exogenous antioxidants (vitamins, polyphenols and minerals), which act as ROS scavengers (3). Exogenous antioxidants are principally found in plant-derived foods and are currently receiving great attention as they may provide protection against oxidative stress and related diseases including cancer, cardiovascular disease, inflammatory disorders and the ageing process (12,13). In this sense, cocoa extract is a rich source of antioxidants due to its high flavanol content (14). Indeed, different studies have observed a negative association between cocoa consumption and oxidative stress (15–17). In agreement with our previous results, cocoa extract decreases plasma oxidised low-density lipoprotein (oxLDL) cholesterol in middle-aged subjects (18), suggesting that it might also act as an antioxidant at the DNA level.

Moreover, ROS can cause the formation of strand breaks (SBs) and oxidised bases in the DNA leading to genome destabilisation (19). ROS-induced promutagenic lesions (e.g. 8-oxoguanine) can play a significant role in the development of cancer and other diseases (19,20). Dietary antioxidants can be in part responsible for disease prevention by acting directly on ROS, stimulating the cellular endogenous defences or even improving the DNA repair capacity (21,22). Many nutritional intervention trials have successfully used the comet assay to monitor endogenous SBs and oxidised bases, antioxidant resistance and DNA repair capacity (22,23).

To our knowledge, there is only one study that has investigated the effect of cocoa on DNA resistance to oxidative stress (24). This study evaluated cocoa as a food (dark chocolate vs. white chocolate) and analysed the acute and chronic (2 weeks) effect in normal weight young healthy subjects. They found that dark chocolate significantly improved antioxidant resistance to H$_2$O$_2$-induced damage, but only after 2 h of ingestion and this was correlated with the amount of epicatechines in plasma.

The present trial is apparently the first study analysing the effect of cocoa extract consumption within ready-to-eat meals and a hypocaloric diet, over oxidative stress at the level of DNA in overweight/obese middle-aged subjects. The comet assay was applied to measure the endogenous level of SBs and 8-oxoguanine as well as the antioxidant resistance in lymphocytes from overweight/obese middle-aged subjects before and after the 4-week intervention.

**Materials and methods**

**Subjects**

As described elsewhere (18), 50 subjects (23 men and 27 women) were recruited during the spring 2012 to participate in the study. The inclusion criteria were as follows: age between 50 and 80 years, body mass index between 27.0 and 35.5 kg/m$^2$ and <5% of weight variation during the 3 months prior to the intervention. The exclusion criteria encompassed history of metabolic disorders: diseases such as gastrointestinal diseases, diabetes, cancer or inflammatory diseases, food allergies, cognitive alterations, current slimming, hormone replacement, anti-inflammatory or blood pressure lowering treatments, medication that could influence appetite or nutrient absorption, inability to perform the follow-up and being a smoker. The trial was carried out in the Metabolic Unit of the University of Navarra (Spain) and all of the participants gave prior written informed consent. The study was approved by the Research Ethics Committee of the University of Navarra (ref. no. 006/2012) and followed the Helsinki Declaration guidelines. The trial was registered at www.clinicaltrials.gov (NCT01596309).

**Study design**

The design of the study was a 4-week double-blind, randomised, placebo-controlled parallel nutritional intervention. Of the 50 volunteers, half received meals supplemented with 1.4 g/day cocoa extract and the other half received control meals, which were supplied weekly by Tutti Pasta S.A. (Navarra, Spain). One group received ready-to-eat meals supplemented with cocoa extract (cocoa group) and the other one received the same meals but without cocoa extract supplementation (control group) within the same hypocaloric diet. Test meals consisted of a variety of ready-to-eat meals: dishes (such as pasta, rice and creams) and desserts (custards with different flavours), which were designed considering likes and dislikes of this collective and to facilitate functions such as chewing and swallowing. Each dish and each dessert was supplemented with 0.7 g of cocoa extract in the case of cocoa group. From those meals, volunteers had to consume one dish and one dessert per day, consuming 1.4 g of extract daily. The meals had the same appearance and differed only on the code label, ensuring the double-blind model of the study. In the same time, and considering that volunteers were overweight/obese, the same hypocaloric diet (~15% E restriction) was prescribed to improve the health status of the participants and to incentive subjects to follow the study. Diet, which was designed including the study products, provided 45% of total energy value from carbohydrates, <30% of energy from lipids and 22–25% of energy from proteins. The Harris–Benedict equation and the corresponding individualised physical activity factor, which was estimated by a 24-h physical activity questionnaire, were used to calculate the resting metabolic rate and to adjust energy requirements (25). One week prior to the beginning of the study, the volunteers had to exclude cocoa and cocoa containing products from their habitual diet and 3 days before the start of the trial, they were asked to consume a low-polyphenol diet without energy restriction. The low-polyphenol diet was prescribed by a trained nutritionist. The diet consisted of five meals where most polyphenol-rich foods were excluded. Moreover, volunteers were provided with a list of forbidden polyphenol-rich foods such as cocoa and derived products, coffee, tea, spices, wine, antioxidant supplements, fruits and vegetables rich in flavanols, etc., which were excluded for 3 days prior to the intervention and during the intervention. In addition, volunteers were asked not to change their physical activity pattern. Adherence to the intervention was assessed by a notebook where volunteers self-reported the name of the test dish and dessert they consumed daily. Nutrient intake was analysed by a 3-day validated food-recall questionnaire, which was completed at the beginning and the end of the study. These data were analysed using the DIAL software (Alce Ingenieria SL, Madrid, Spain) according to other studies (26).
Total polyphenol content and characterisation of the cocoa extract

The cocoa extract added to meals contained 645.30 (32.27) mg of total polyphenols and the analytical characterisation was provided by Nutrasur S.A. (Murcia, Spain) (Table I). Folin–Ciocalteu method was used to analyse the total polyphenol content and high-performance liquid chromatography to quantify flavanoids and theobromine. More details have been described elsewhere (18).

Isolation of lymphocytes

Venous blood samples were collected in EDTA tubes (BD Vacutainer®) at fasting state (10 h) between 8:00 and 9:30 a.m. For the determination of endogenous DNA damage and the resistance to H₂O₂-induced damage, lymphocytes were isolated from blood samples using the Lymphoprep sedimentation method. Briefly, blood was mixed with the same volume of phosphate buffered saline (PBS), carefully added on top of Lymphoprep™ (Axis-Shield) and centrifuged. The layer of lymphocytes was collected and washed with PBS by centrifugation. Lymphocytes were counted, suspended in freezing medium (Roswell Park Memorial Institute medium supplemented with 10% inactivated foetal bovine serum, antibiotics—100 U/ml penicillin and 100 µg/ml streptomycin—and 10% dimethyl sulfoxide) and aliquots frozen at −80°C using Mr. Frosty™ freezing containers (Thermo Fisher Scientific Inc.). Samples were kept at −80°C until their analysis. After lymphocyte collection, samples were kept on ice and all processes were carried out at 4°C to avoid DNA repair.

Comet assay—endogenous SBs and oxidised bases

The medium-throughput comet assay format of 12 minigels per slide (two rows of six minigels on a glass microscope slide) was used as previously described (27). The 12-Gel Comet Assay Unit™ (Severn Biotech Ltd) was used to perform the enzyme incubation of each minigel separately.

On the day of analysis, samples were thawed, washed with cold PBS by centrifugation and suspended in cold PBS at 2.5 x 10⁵ cells/ml. Thirty microlitres of the cell suspension were placed on agarose-precoated lysis solution on a Coplin jar at 4°C overnight. Slides were then transferred with the rest of the slides to the electrophoresis solution for 40 min in the same solution in a 4°C cold room. Slides were neutralised by washing them in PBS for 10 min and rinsed in distilled water for a further 10 min. DNA was fixed by immersing the slides in 70% ethanol for 15 min and in absolute ethanol for a further 15 min before letting them dry overnight.

Comets were stained with 1 µg/ml 4′,6-diamidino-2-phenylindole by adding a drop on top of each minigel and a single large glass coverslip (2.4 x 60 mm) was used to cover all the minigels on one slide. Comets were visualised under a fluorescence microscope (Nikon Eclipse 50 i) and the semi-automated image analysis system Comet Assay IV (Perceptive Instruments) was used to evaluate them. Fifty comets were evaluated per gel, so 100 comets were evaluated per sample. Percentage of DNA in tail was used to describe each of the comets and the median was calculated to describe each sample. Net FPG-sensitive sites were calculated by subtracting the median of the percentage of tail DNA after buffer incubation from the median of the percentage of tail DNA after FPG incubation.

| Table I. Polyphenol composition of the cocoa extract (1.4 g) used in the study. |
|-------------------------------------|-------------------------------|
| Component                          | Value                         |
| Theobromine (mg)                   | 140.42 (7.02)                 |
| Total polyphenols as catechin (mg) | 645.30 (32.27)                |
| Total flavanols as catechin (mg)   | 414.26 (20.71)                |
| Epicatechin (mg)                   | 153.44 (7.67)                 |
| Catechin (mg)                      | 14.56 (0.73)                  |
| Dimer B2 (mg)                      | 99.40 (4.97)                  |
| Dimer B1 (mg)                      | 13.44 (0.67)                  |
| Oligomeric procyanidins (mg)       | 133.53 (6.68)                 |

From Ibero-Baraibar et al. (18) with permission from Elsevier.

a Determined by high-performance liquid chromatography assay (chromatographic technique).

b Determined by the Folin–Ciocalteu reagent (colorimetric assay).

detects mainly 8-oxoguanine and also other purine oxidation products. Two sets of slides were run in each experiment: each set contained five samples and a positive or a negative control (i.e. in each experiment, 10 samples, a positive and a negative control were analysed). Negative controls were untreated lymphocytes from a healthy young female non-smoker. Positive controls were lymphocytes from the same donor treated with the photosensitiser Ro 19-022 plus light to induce 8-oxoguanine, which were isolated as explained before.

Lymphocytes were lysed by immersing the slides in a lysis solution (2.5 M NaCl, 0.1 M Na₂EDTA, 0.1 M Tris base, pH 10 and 1% Triton X-100) in a Coplin jar at 4°C overnight. Slides for incubation with enzyme reaction buffer or FPG were washed three times, 5 min each, with the enzyme reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0) at 4°C. The slides were then transferred to the 12-Gel Comet Assay Unit™ and placed on a cold metal plate. Thirty microlitres of reaction enzyme buffer or FPG was added to the corresponding wells and chambers were transferred to the incubator at 37°C for 30 min. Then, slides were removed from the units and incubated with the electrophoresis solution (0.3 M NaOH, 1 mM Na₂EDTA, pH > 13) for 40 min at 4°C in a Coplin jar. Slides prepared for measuring SBs were in lysis during the washing and the incubation of slides with reaction enzyme buffer or FPG, but they were transferred with the rest of the slides to the electrophoresis buffer to carry out the 40-min alkaline incubation. Slides were then transferred to an electrophoresis tank and electrophoresis was then carried out in the electrophoresis solution at 1.2 V/cm for 20 min in a 4°C cold room. Slides were then neutralised by washing them in PBS for 10 min and rinsed in distilled water for a further 10 min. DNA was fixed by immersing the slides in 70% ethanol for 15 min and in absolute ethanol for a further 15 min before letting them dry overnight.

Comets were stained with 1 µg/ml 4′,6-diamidino-2-phenylindole by adding a drop on top of each minigel and a single large glass coverslip (2.4 x 60 mm) was used to cover all the minigels on one slide. Comets were visualised under a fluorescence microscope (Nikon Eclipse 50 i) and the semi-automated image analysis system Comet Assay IV (Perceptive Instruments) was used to evaluate them. Fifty comets were evaluated per gel, so 100 comets were evaluated per sample. Percentage of DNA in tail was used to describe each of the comets and the median was calculated to describe each sample. Net FPG-sensitive sites were calculated by subtracting the median of the percentage of tail DNA after buffer incubation from the median of the percentage of tail DNA after FPG incubation.

Comet assay—antioxidant resistance

Cells were thawed, washed, centrifuged, suspended in cold PBS, embedded in agarose and set in minigels on a glass microscope slide as described elsewhere (27). Different slides containing different samples were analysed in one experiment. Each slide in each experiment contained six samples, two minigels per sample, except one of the slides that contained five samples and one negative control (see previous section: Comet assay—endogenous SBs and oxidised bases).

After cells were embedded in gels and set on slides, cells were exposed to 100 µM H₂O₂ for 5 min at 4°C by immersing the slides on a Coplin jar. Slides were then thoroughly washed with cold PBS, by performing different washes in different Coplin jars, and immersed in lysis solution on a Coplin jar at 4°C overnight. Slides were then transferred to the electrophoresis tank and incubated with the electrophoresis solution for 40 min in a 4°C cold room before performing the electrophoresis at 1.2 V/cm for 20 min in the same solution in a 4°C cold room. Slides were neutralised and rinsed, DNA was fixed and stained and comets were appropriately analysed.
Statistical analysis
The sample size was primarily calculated for the main study, considering oxidised LDL marker as the main variable (18). Thus, the sample size was estimated taking into account a reduction in oxidised LDL levels of 14.1 U/l and an interquartile range of 16.3 U/l, according to the study carried out by Khan et al. (16). With a bilateral confidence index of 95% (α = 0.05) and a statistical power of 80% (β = 0.80), the sample size was estimated to be 44 subjects. A possible drop-out rate of 15% was considered, establishing the final sample size to be 50 subjects. Participants were randomly assigned to each group using the “random between 1 and 2” function in the Microsoft Office Excel (Microsoft Iberica, Spain). In the control group, one subject dropped out because of poor adherence to the diet, while in the cocoa group, two subjects were excluded, one for the poor adherence to the diet and the other for personal reasons.

Net FPG-sensitive sites of all the volunteers were obtained in a separate experiment and so data were normalised by reference to the values obtained for the positive control, lymphocytes in the respective experiment, as described elsewhere (A. R. Collins, N. El Yamani, Y. Lorenzo, S. Shaposhnikov, G. Brunborg and A. Azqueta, submitted for publication). The same approach was used to normalise the data of the antioxidant status experiment. In this case, values obtained for the negative controls (untreated lymphocytes from a healthy donor treated with 100 μM H$_2$O$_2$), included in each of the experiments, were used as reference values.

Normality of the variables was assessed using the Shapiro–Wilk test. Data are expressed as mean (SD). According to whether variables were normally distributed or not, comparisons between baseline and the end point were analysed by paired Student’s t-test or Wilcoxon signed-rank test, and comparisons between both groups were performed with and independent sample t-test or Mann–Whitney U test. Data related to the comet assay variables were reported for each group separately (intervention and controls) as well as for both groups together from an observational point of view. SBs, oxidised bases and the resistance to H$_2$O$_2$-induced damage were also compared before and at 4 weeks of intervention after dividing the whole group by the median of each variable at baseline (SBs: 0.28% of DNA damage in tail; oxidised bases: 16.32% of DNA damage in tail and resistance to H$_2$O$_2$-induced damage: 18.01% of DNA damage in tail). P values are reported unadjusted and adjusted for age, sex, intervention group (control/cocoa) and weight loss.

Correlation analyses were performed between oxidised bases or resistance to H$_2$O$_2$-induced damage and cocoa-derived metabolites (epicatechin glucoronide, catechin sulphate, epicatechin sulphate and methyl epicatechin-O-sulphate) at the end of intervention. The analysis of metabolites forms part of another publication (I. Ibero-Baraibar, in preparation). Metabolites were measured in the cocoa (n = 11) and control (n = 12) groups, but those metabolites were detected only in the cocoa group. Thus, the associations are shown only for cocoa group. These correlations were assessed by Pearson correlation test (normally distributed variables) or Spearman correlation test (non-normally distributed variables). SPSS 15.0 for Windows (SPSS Inc., Chicago, USA) software was used to analyse the data considering P < 0.05 as significant.

Results
General characteristics of participants
The general characteristics of the participants are summarised in Table II. No differences were found between groups. As shown in Figure 1, out of the 50 volunteers (25 subjects in each group) who started the intervention, 47 completed the study, 24 subjects in the control group and 23 in the cocoa group (18). The adherence to meal consumption was 98.4 (SD 2.2)% in control and 98.5 (SD 3.3)% in cocoa group. No differences were observed between groups in energy [control: 1513 (305) kcal vs. cocoa: 1535 (238) kcal], carbohydrate [control: 160 (35) g vs. cocoa: 167 (28) g], protein [control: 83 (18) g vs. cocoa: 84 (13) g] and lipid [control: 58 (14) g vs. cocoa: 58 (13) g] intake during the intervention. None of the volunteers presented any side effects, taste dislike or changes in the physical activity pattern during the study as was reported elsewhere (18).

Comet assay: endogenous SBs, oxidised bases and antioxidant resistance
There were no significant differences in DNA SBs, base oxidation or antioxidant resistance to H$_2$O$_2$-induced damage in either control or cocoa group after 4 weeks of intervention (Table III). A tendency to significance (P = 0.062) was only observed in the decrease of oxidised bases in control group and when both groups were analysed together as a collective sample (P = 0.072) (Figure 2). Moreover, when comparisons were performed dividing the collective sample by the median of each variable at baseline, significant difference was observed between baseline and the end of intervention in oxidised bases in subjects who were above the median (P = 0.039), as shown in Table IV. When this comparison was adjusted for age, sex, group and weight loss, the result remained significant (P = 0.040). Thus, subjects who had higher oxidised bases at baseline presented a significant reduction of damage after 4 weeks of intervention (Table IV). Concerning the SBs and antioxidant resistance to H$_2$O$_2$-induced damage, no differences were observed (Table IV).

Correlations of cocoa-derived metabolites in plasma with oxidised DNA bases and antioxidant resistance
As shown in Table V, significant correlations were found in the cocoa group between oxidised bases (FPG-sensitive sites) and methyl epicatechin-O-sulphate (r = −0.76; P = 0.007) and epicatechin sulphate (r = −0.61; P = 0.046) at 4 weeks of intervention. However, no significant associations were found between resistance to H$_2$O$_2$-induced damage and cocoa-derived metabolites.

Discussion
A number of nutritional interventions have used the comet assay technique to assess the effect of various antioxidants on oxidative damage and antioxidant resistance (22,23,28). Cocoa extract consumption is negatively associated with biochemical markers of oxidative stress in blood (15–17). However, to our knowledge, only the study of Spadafranca et al. (24) has assessed the effect of chocolate at the level of DNA damage. Thus, ours is apparently the first study assessing the influence of cocoa extract consumption as a bioactive compound, integrated into ready-to-eat meals and together within a hypocaloric diet, on the antioxidant status at the DNA level of overweight/obese subjects.

We previously have observed that the consumption of ready-to-eat meals supplemented with cocoa extract within a hypocaloric diet (~15% energy restriction) causes a significant decrease in oxLDL levels in the group supplemented with cocoa extract (18). However, we show here that the cocoa supplementation did not result in a significant effect on the measures reflecting the antioxidant status at DNA level (SBs, oxidised bases and antioxidant resistance). Nevertheless, there were negative associations between
Table II. General characteristics of participants at baseline (n = 47) and at 4 weeks in each dietary intervention group: control (n = 24) and cocoa (n = 23) groups.

| Characteristics         | Control group (n = 24) | Cocoa group (n = 23) | ΔP
|-------------------------|------------------------|----------------------|--------
|                         | Baseline (n = 24)      | 4 weeks (n = 24)     | Baseline (n = 23) | 4 weeks (n = 23) |
| Age (years)             | 56.96 (5.00)           | 56.96 (5)***         | 58.13 (5.58)      | 58.13 (5.58)***  | 0.452
| BMI (kg/m²)             | 30.34 (1.97)           | 29.40 (2.09)**       | 30.73 (2.46)      | 29.74 (2.34)**   | 0.744
| Waist/height (cm/m²)    | 0.63 (0.04)            | 0.60 (0.04)**        | 0.64 (0.04)       | 0.61 (0.04)**    | 0.432
| Glucose (mg/dl)         | 98.35 (9.99)           | 95.67 (9.48)         | 98.85 (10.03)     | 96.73 (10.96)*** | 0.868
| Total-c (mg/dl)         | 233.54 (33.50)         | 192.04 (30.44)***    | 247.87 (53.38)    | 206.43 (41.42)*** | 0.509
| LDL-c (mg/dl)           | 162.09 (32.27)         | 130.40 (25.45)***    | 179.18 (50.98)    | 147.40 (35.31)*** | 0.992
| TG (mg/dl)              | 114.75 (38.60)         | 87 (33.18)***        | 109.13 (49.14)    | 79.87 (35.12)*** | 0.887

Data are expressed as mean (SD). According to the normality of the variables, comparisons between groups were performed with an independent sample t-test or Mann–Whitney U test (ΔP). BMI, body mass index; LDL-c, low-density lipoprotein cholesterol; TG, triglycerides; total-c, total cholesterol. P < 0.05 was considered as significant.

aP value comparing baseline and 4-week within each intervention group (control and cocoa). **P < 0.001; n.s., not significant.
bComparison of differences (4-week value − baseline value) between control and cocoa groups.
cNon-normally distributed variables.

Table III. SBs, oxidised bases and antioxidant resistance (resistance to H₂O₂-induced damage) of DNA before and after 4 weeks of intervention in control (n = 24) and cocoa (n = 23) groups.

| Variables (%) of DNA in tail | Control group (n = 24) | Cocoa group (n = 23) | ΔP
|------------------------------|------------------------|----------------------|--------
|                              | Baseline | 4 weeks | P | Baseline | 4 weeks | P |
| SBs*                         | 2.03 (5.66) | 1.91 (6.25) | 0.747 | 0.72 (1.02) | 0.88 (1.11) | 0.273 | 0.712 | 0.404 | 0.267
| Oxidised bases               | 17.49 (8.57) | 15.23 (5.90) | 0.062 | 16.35 (5.48) | 15.62 (6.37) | 0.542 | 0.592 | 0.718 | 0.355
| Antioxidant capacity        | 18.20 (11.87) | 20.71 (15.61) | 0.390 | 21.21 (12.12) | 20.83 (12.19) | 0.445 | 0.369 | 0.696 | 0.158

Data are expressed as mean (SD) of % of DNA in tail. According to the normality of the variables, comparisons between baseline and end point of the study were analysed by paired Student’s t-test or Wilcoxon signed-rank test (P) and comparisons between both groups were performed by independent sample t-test or Mann–Whitney U test (P, P, ΔP).

*Control group vs. cocoa group at baseline.
*Control group vs. cocoa group at 4 weeks.
*P value of Δcontrol group vs. Δcocoa group; Δ = 4-week value − baseline value.
*Non-normally distributed variables.
cocoa-derived plasma metabolites (methyl epicatechin-O-sulphate and epicatechin sulphate) and oxidised bases after 4 weeks of intervention, suggesting that the increase of antioxidants in blood could cause a decrease in DNA oxidation, even though we did not observe any differences between the cocoa and control groups. In this context, the investigation of methyl epicatechin-O-sulphate and epicatechin sulphate in an isolated manner would be interesting to clarify their role in this aspect, since epicatechin has been related to DNA oxidative protection (24). Spadafranca et al. performed an intervention study where 20 healthy subjects (10 males and 10 females) consumed a standardised diet for 2 weeks followed by a period of two more weeks taking 45 g of either dark or white chocolate daily.

Table IV. Comparison of SBs, oxidised bases and antioxidant resistance (resistance to H\textsubscript{2}O\textsubscript{2}-induced damage) before and after 4 weeks of intervention in the whole sample divided by the median of each variable at baseline.

<table>
<thead>
<tr>
<th>Variables</th>
<th>&lt;Median(^{a}) (n = 23)</th>
<th>&gt;Median(^{a}) (n = 24)</th>
<th>(P)</th>
<th>Baseline</th>
<th>4 weeks</th>
<th>(P)</th>
<th>(\Delta )P (\Delta )P</th>
<th>(\Delta )P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBs</td>
<td>0.13 (0.80)</td>
<td>0.36 (0.45)</td>
<td>0.687</td>
<td>2.59 (5.55)</td>
<td>2.41 (6.20)</td>
<td>0.197</td>
<td>0.072</td>
<td>0.402</td>
</tr>
<tr>
<td>Oxidised bases</td>
<td>11.64 (3.10)</td>
<td>11.85 (5.68)</td>
<td>0.401</td>
<td>22.00 (6.22)</td>
<td>18.84 (4.24)</td>
<td>0.103</td>
<td>0.039</td>
<td>0.040</td>
</tr>
<tr>
<td>Antioxidant resistance(^{a})</td>
<td>9.31 (4.42)</td>
<td>12.25 (9.98)</td>
<td>0.315</td>
<td>29.61 (7.54)</td>
<td>28.94 (20.87)</td>
<td>0.439</td>
<td>0.124</td>
<td>0.448</td>
</tr>
</tbody>
</table>

\(^{a}\)Median of SBs at baseline: 0.28%; median of oxidised base damage at baseline: 16.32%; median of antioxidant resistance at baseline: 18.01%.

\(^{b}\)P value of comparing baseline and 4-week values within subjects who are below the median and who are above the median.

\(^{c}\)Unadjusted \(P\) value of comparing difference values (4-week value − baseline value) of who are below the median and who are above the median.

\(^{d}\)Adjusted \(P\) value for age, sex, intervention group (control/cocoa) and \(\Delta \)weight of comparing difference values (4-week value − baseline value) of who are below the median and who are above the median.

\(^{e}\)Non-normally distributed variables.

Table V. Correlation analyses between oxidised bases and antioxidant resistance (resistance to H\textsubscript{2}O\textsubscript{2}-induced damage) (% of DNA in tail) with cocoa-derived metabolites at fourth week of intervention in cocoa group (n = 11).

<table>
<thead>
<tr>
<th>Cocoa-derived metabolites (nmol/l)</th>
<th>Oxidised bases (fourth week)</th>
<th>Antioxidant resistance(^{a}) (fourth week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl epicatechin-O-sulphate</td>
<td>(r = -0.76; P = 0.007)</td>
<td>Rho = −0.38; (P = 0.247)</td>
</tr>
<tr>
<td>Epicatechin sulphate</td>
<td>(r = -0.61; P = 0.046)</td>
<td>Rho = −0.11; (P = 0.750)</td>
</tr>
<tr>
<td>Epicatechin glucoronide</td>
<td>(r = -0.52; P = 0.099)</td>
<td>Rho = −0.35; (P = 0.298)</td>
</tr>
<tr>
<td>Catechin sulphate(^{a})</td>
<td>Rho = 0.36; (P = 0.274)</td>
<td>Rho = 0.22; (P = 0.519)</td>
</tr>
</tbody>
</table>

Correlation analyses were performed by Pearson or Spearman correlation test depending on the normality of the variables. \(P < 0.05\) was considered as significant.

\(^{a}\)Non-normally distributed variables.

Figure 2. SBs, oxidised bases and antioxidant resistance (resistance to H\textsubscript{2}O\textsubscript{2}-induced damage) before and after 4 weeks of intervention in whole sample. Data are presented as mean (SD).
dark chocolate does not affect antioxidant resistance, at least in that period of time and with that dose of polyphenols. In this context, our results are in accordance with the results obtained by Spadafranca et al. since we did not observe differences in the antioxidant resistance after 4 weeks of daily cocoa extract consumption.

On the other hand, some interesting results were observed when control and cocoa groups were pooled and analysed as a collective sample. In this sense, a tendency to significance was observed in the oxidised bases, which decreased after 4 weeks of intervention. Moreover, when subjects were stratified by the median of each variable at baseline, subjects with higher values of oxidised bases showed greater reduction in the damage after 4 weeks of intervention. The weight loss associated with the hypocaloric diet followed by the volunteers could be responsible for this result. A similar phenomenon was observed in the study by de la Iglesia et al. (29), where after categorising subjects by the weight loss median, those subjects who had lost more body weight showed a greater decrease in some more metabolic parameters.

Concerning the possible limitations of our study, a double-blinded randomised controlled cross-over trial would have been an appropriate design as each patient would serve as his or her own control, reducing the effect of confounding variables and improving the statistical power. However, the expected weight loss due to the prescribed hypocaloric diet in our study meant that a double-blinded randomised controlled parallel trial was more suitable. Nevertheless, limitations of our study design do not seem to be sufficient to account for a lack of observable differences between cocoa-supplemented and placebo groups. Thus, the intervention period of 4 weeks could be a short period of time, but it is similar to that in various studies that have demonstrated antioxidant protection using other extracts or plant-derived products (28,30). Moreover, the number of subjects in our study is comparable with those in other studies that have shown significant effects (28,30,31). However, as a limitation, our subjects were apparently healthy overweight/obese and they did not present high values of DNA damage at baseline (32). It is likely that the antioxidant effect of cocoa extract could be more relevant in subjects with higher baseline rates of oxidative DNA damage. In this sense, a recent meta-analysis found that the effect of plant foods and beverages on non-enzymatic antioxidant resistance was three times higher in subjects with oxidative stress when compared with healthy subjects (33). However, studies using the comet assay on either healthy (24,31,34) or unhealthy subjects (35,36) show mixed results. In retrospect, it might have been informative to look for short-term effects of supplementation—as was done by Spadafranca et al. (24) with cocoa or Del Bo et al. (31) with blueberry.

On the other hand, the role of dietary antioxidants in human health remains controversial (37,38). Fruit and vegetable sources in the diet are associated with lower rates of chronic diseases, and this is often attributed to their content of antioxidants and a resulting protection against oxidative stress (39,40). However, large-scale human trials with antioxidant supplements have shown, if anything, an increase in mortality (37,41).

Endogenous SBs may be a poor indicator of the effects of antioxidants as in some circumstances, antioxidant resistance of lymphocyte DNA to H$_2$O$_2$-induced damage or measuring endogenous base oxidation is more likely to reflect antioxidant intake (23). Changes in the antioxidant status at DNA level were not noted, but we have demonstrated a decrease in plasma oxLDL levels (18). This outcome could be explained by the fact that DNA is equipped with different mechanisms for the continuous repair of oxidative DNA damage (42). However, in the blood, the oxidation of LDL cholesterol depends mainly on the diet, lifestyle patterns, etc.

**Conclusion**

The consumption of ready-to-eat meals supplemented with 1.4 g of cocoa extract within a hypocaloric diet for 4 weeks did not have direct benefits on antioxidant status at the DNA level in middle-aged apparently healthy but overweight/obese subjects. However, in the cocoa-supplemented group, a negative association between cocoa-derived metabolites and oxidised bases was found, which suggests a possible beneficial effect of cocoa extract in a longer period of time or in subjects with higher baseline oxidised bases. Interestingly, the energy restriction could be the cause of the slightly decrease on oxidised bases, being more relevant in subjects who started the intervention with higher levels of DNA damage. Thus, further studies are justified to assess the antioxidant effect of cocoa extract on oxidative damage to DNA.

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