IN VITRO AND ANIMAL STUDIES

Sweeteners modulate bioactivity of endothelial progenitor cells but not induce detrimental effects both on inflammation and behavioural changes

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ABSTRACT
This study sought to determine the possible detrimental effects of several low- or non-caloric sweeteners on endothelial progenitor cells (EPCs), inflammation and behavioural changes in mice. C57BL/6 male mice received low and high dose of natural and artificial sweeteners for 4 weeks. EPCs, physical and biochemical variables, inflammation and behavioural changes were evaluated. A significant reduction of about 25% of EPCs was found when mice received a moderate amount of all sweeteners (p < .05). This reduction was more strongly significant when a double dose of glucose, aspartame, rebaudioside A and cyclamate (p < .005) in comparison to fructose and sucrose (p < .05) was administered. During inflammation carrageenan-induced, all sweeteners produced a significant increase of EPCs compared to the control group (p < .05). Consumption of glucose and sugar substitutes affect mouse EPC number according to the absence or presence of an inflammatory status, but does not induce detrimental effects on inflammation and behavioural changes.

Introduction

The growing interest on sugar intake and its impact on the prevalence of overweight, obesity, diabetes and other related diseases, induced many people to consume fructose and sugar substitutes, like artificial or natural sweeteners, in order to reduce the risk of metabolic disorders (Gardner et al. 2012; Swithers 2013, 2016; Hess et al. 2018). To lower calorie intake, a commonly adopted strategy includes replacement of dietary simple sugars with low- or non-caloric natural or artificial substitutes (Feehley and Nagler 2014). However, possible adverse effects due to increase sweetener consumption on obesity, diabetes, and other related disorders are still debated (Swithers 2016). Indeed, some studies suggest that especially artificial sweeteners are not always effective for body-weight control and may also lead to undesirable consequences (Gardner et al. 2012; Swithers 2013, 2016). For instance, recent studies in mice and humans (Suez et al. 2014) indicate that non-caloric artificial sweeteners may promote obesity-associated metabolic changes (Suez et al. 2014). Recently, the possible risk of cancer associated with the use of artificial sweeteners has been largely discussed due to the different results obtained in animal models and human studies (Soffritti et al. 2010; Mishra et al. 2015). As far as natural sweeteners, some glycosides extracted from leaves of Stevia Rebaudiana (steviol, stevioside and rebaudioside A) have not only been established as non-caloric sweeteners and reported to exhibit some other pharmacological activities (Feehley and Nagler 2014). Indeed, they may have anti-diabetic, anti-hypertensive, anti-inflammatory, anti-diarrheal, diuretic and immunomodulatory actions, even though these effects are small and the available controlled
clinical trials do not permit any conclusion on both their safety and effectiveness (Brahmachari et al. 2011; Feehley and Nagler 2014).

Endothelial progenitor cells (EPCs) are immature cells that can differentiate into mature endothelial cells and be recruited from bone marrow to the vascular injury/tissue ischaemia site to promote endothelial regeneration and neovascularisation (Napoli et al. 2011; Fadini et al. 2012b; Hristov and Weber 2004).

To date, several studies have described the detrimental effects of high glucose concentrations on endothelial cells as well as on their progenitors (Chen et al. 2007; Balestrieri et al. 2008). Indeed, EPCs may promote the integrity and function of the endothelium, thus playing a role in the prevention of cardiovascular diseases. EPC dysfunction is known to be a critical event during diabetes mellitus (DM), because leads to the initiation and progression of atherosclerotic vascular disease and impaired neovascularisation after ischaemia induced by hyperglycaemia (Sheetz and King 2002; Hristov and Weber 2004). In patients with type 1 and 2 DM, circulating EPCs are reduced and their functions are impaired in terms of decreased proliferation, adhesion, and vasculogenesis (Fadini et al. 2005; Ii et al. 2006; Balestrieri et al. 2008; Liguori et al. 2008; Napoli et al. 2011; De Pascale et al. 2016). In several studies from our group and others, it was demonstrated that number and migratory capability of this specific sub-population of progenitor cells, which circulate in the blood stream and targets injured endothelium sites (Takahashi et al. 1999; Hristov et al. 2003; Casamassimi et al. 2007), inversely correlate with risk factors for coronary heart disease (Hill et al. 2003; Werner et al. 2005; Liguori et al. 2008). EPCs from type 1 DM patients, showed a potential role for glucotoxicity in impairing EPC function (Fadini et al. 2005). However, the molecular mechanisms underlying the reduced numbers of EPCs and their function in diabetes are poorly understood still. Other evidences, also demonstrated that diabetes impair the mobilisation of EPCs in response to tissue ischaemia or cytokines, such as granulocyte colony-stimulating factor (Fadini et al. 2012a, 2013; Fadini 2014). Moreover, endothelial nitric oxide synthase (eNOS) dysfunction and altered cytokine gradients, such as stromal-derived factor 1α (SDF-1α), vascular endothelial cell growth factor (VEGF) and erythropoietin, may play major roles in the impairment of EPC mobilisation.

In vitro studies demonstrated that high glucose resulted in cellular injury in EPCs by inducing oxidative stress (Sorrentino et al. 2007), apoptosis (Rosso et al. 2006) and the down-regulation of eNOS (Ignarro and Napoli 2005; Huang et al. 2011).

In addition, EPC function was impaired in mouse cerebral ischaemia after long-term treatment with fructose and some artificial and natural sweeteners such as rebaudioside A (Dong et al. 2015). On this basis, the effect of low- or non-caloric sweeteners on EPCs is a relevant issue to understand whether these compounds may be, at the same time, helpful to reduce the risk of overweight, obesity, inflammation and not harmful to the cardiovascular system.

The goal of this study was to determine the effects of low- or non-caloric sweeteners (both natural and artificial) on EPC number, inflammation and behavioural tests in an experimental mouse model.

**Methods**

**Experimental design**

Twelve week old C57BL/6 male mice (28–30 g) (Harlan, Italy), were housed one per cage under controlled illumination (12:12 h light:dark cycle) and environmental conditions (room temperature 22 ± 1°C, humidity 60 ± 10%) for at least 1 week before experimental treatments. Mice were randomly assigned (without blinding) to treatment and control groups and were given commercial sweeteners in drinking water for 8 weeks: sucrose (Suc) (0.75 g/kg die-bw p.o., n = 6), d- (+)-glucose (Glu) (0.80 g/kg die-bw p.o., n = 6), d-fructose (Fru) (0.80 g/kg die-bw p.o., n = 6), aspartame (Asp) (3.75 mg/kg die-bw p.o., n = 6), sodium cyclamate (Cyc) (7.00 mg/kg die-bw p.o., n = 6), rebaudioside A (RebA) (2.80 mg/kg die-bw p.o., n = 6). The dosages were calculated in accordance with acceptable daily intake (ADI) established by the European Food Safety Authority and with the reported cytotoxicity of all sweeteners (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:295:0205:0211:EN:PDF). Indeed, for experiments conducted with sucrose a 0.1 mg ml⁻¹ solution was used in order to meet with FDA defined ADI for saccharin in humans (5 mg/kg (body weight), according to the following calculation:

\[
\text{ADI} = \frac{5 \text{ mg kg}^{-1} \text{ day}^{-1} \times \text{average mouse weight 0.03 kg}}{\text{Average daily liquid intake 2 ml}}
\]

\[
= 0.075 \text{ mg ml}^{-1} \rightarrow 0.1 \text{ mg ml}^{-1}
\]

The different non-caloric sweetener concentrations were chosen based on the sweetening power, as described elsewhere [DuBois and Prakash 2012; Food and Drug
Administration, [http://www.fda.gov/](http://www.fda.gov/); European Food Safety Authority, [http://www.efsa.europa.eu/](http://www.efsa.europa.eu/) (Figure S1).

A separate set of animals received a drinking solution containing higher sweetener concentrations (double concentration). Mouse chow and tap water were available ad libitum. Control group was always administered pure drinking water and the same batch of diet as the treated groups. After 8-week treatment, animals underwent behavioural evaluations and sacrificed for analyses. Intra-abdominal fat, liver, kidney, lung and brain were excised weighed and stored at $-80 \, ^\circ \text{C}$ for the molecular analysis. The experimental procedures were approved by the Animal Ethics Committee of the University “L. Vanvitelli” in Naples. (PR. 896/2016) Animal care was in compliance with Italian Legislative Decree (D.L. 116/92) and European Commission Directive (O.J. of E.C. L358/1, 18/12/86) regulations on the protection of laboratory animals. All efforts were made to minimise animal suffering and the number of sacrificed animals.

Suc, Glu, Fru, Asp and Reb.A were purchased from Sigma-Aldrich (St Louis, MO), and Cyc was purchased from SUPELCO (Bellefonte, PA).

**Evaluation of EPC number**

Blood was withdrawn by tail puncture and collected in heparinised tubes before animal was sacrificed. Tail blood sample (50 μl) was incubated for 30 min in the dark with Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against mouse (Ly-6A/E) Phosphatidylinositol-anchored protein (Sca-1) (BD Pharmingen, San Diego, CA) and Phycoerythrin (PE)-conjugated antibody against mouse Fetal liver kinase 1 (Flk-1) (BD Pharmingen). Isotype-identical IgG antibodies were used as controls (BD Pharmingen). Erythrocytes were lysed using FACS lysing solution (Becton-Dickinson, Franklin Lakes, NJ), and the remaining cells were washed with PBS and analysed on a FACSCanto II (Becton-Dickinson) using Diva software (BD Pharmingen). Each analysis included approximately 10,000 events (total number of cells).

**Carrageenan-induced mouse paw oedema**

Peripheral inflammatory reaction was induced in the left hind paw of each sweeter-treated and control animal by a single 20 μl intraplantar injection of a 2% (w/v) λ-carrageenan saline solution accordingly to previous studies (Corea et al. 2005; Palazzo et al. 2011; Luongo et al. 2013). Volumes of animal’s paws were measured immediately before subplantar injection, and 1, 2, 3, 4 and 6 h thereafter by using a hydro-plethysmometer (IITC Inc./Life Science, Woodland Hills, CA). The degree of the induced oedema was assessed by calculating the differences between the volumes of injected and non-injected paws at the indicated time points. Animals were sacrificed after 6 h post carrageenan injection and biochemical evaluations were carried out.

**Von Frey filament test**

As described before (Zhu et al. 2016), mechanical allodynia was measured by assessing paw withdrawal thresholds (PWTs) to mechanical stimuli using von Frey filaments (Stoelting Co., Wood Dale, IL). Ten calibrated von Frey filaments with approximately equal logarithmic incremental bending forces (i.e. 0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4 and 2.0 g bending force) were used. Mice were acclimated on an elevated wire mesh floor covered with a clear Plexiglas chamber for at least 20 min, and then fibres with sequentially increasing stiffness were applied to for 5 s the plantar surface of the hind paw, adjacent to the incision point, with enough force to slightly bend the fibre. Each filament was tested three times per paw, and the PWT was defined as the minimal force that caused at least two withdrawals out of three consecutive trials. If mice did not produce withdrawal response to the filament with maximal force 2.0 g, the PWT was recorded as 2.0 g.

**Biochemical measurements**

Blood was collected from the caudal vena of all mice after 6 h fasting. Blood samples were fractioned by centrifugation at 3000 rpm for 10 min. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), total cholesterol (T-Chol), high-density lipoprotein cholesterol (HDL), triglycerides (TG), total bilirubin (T-bil) and alkaline phosphatase (ALP) were measured by enzymatic colourimetric assays (according to the manufacturer’s protocol; Menarini dry chemistry), with an automated analyser (Spotchem EZ SP-4430, Wokingham, UK). Blood glucose levels were determined collecting blood from tail vein and using a glucometer (ACCU-CHECK Compact Plus, Roche Diagnostics, Tokyo, Japan).

**Behavioural testing**

The behavioural tests were scheduled in order to avoid carry-over effects from prior testing experience, based on previous experimental protocols (Guida et al. 2015, 2017). All behavioural tests were
performed only for administration of higher sweetener doses after 8 week-treatment. At the end of treatments, mice were sacrificed for further evaluations.

**Depressive-like behaviours**

**Tail suspension test (TST)**

Mice were individually suspended by the tail on a horizontal bar (50 cm from floor) using adhesive tape placed approximately 1 cm from the tip of the tail. The duration of immobility, recorded in seconds, was monitored during the last 4 min of the 6-min test by a time recorder. Immobility time was defined as the absence of escape-oriented behaviour. Mice were considered to be immobile when they did not show any body movement, hung passively and completely motionless.

**Forced swimming test (FST)**

Mice were placed in a cylinder (30 cm × 45 cm) filled with water at a temperature of 25–27 °C, for a 6-min period. The duration of immobility in seconds was monitored during the last 4 min of the 6-min test. Immobility period was defined as the time spent by the animal floating in the water without struggling and making only movements necessary to keep its head above the water. Immediately afterwards, the trial mice were placed under a heating lamp to dry.

**Muscle strength by wire hang**

Mice will be placed by the forelimbs on a stainless steel bar (50 cm length, 2 mm in diameter, and elevated 30 cm from the surface) at a point midway between the supports and observed for 60 s. Three consecutive trials will be performed, with a 60 s interval between trials. The amount of time spent hanging will be recorded and scored from 1 to 5 according to the following scheme: 1. hung onto the bar with two forepaws; 2. in addition to 1, attempted to climb onto the bar; 3. hung onto the bar with two forepaws and one or both hind paws; 4. hung onto the bar with all four paws with tail wrapped around the bar; 5. escaped to one of the supports located on the edges of the apparatus.

**Obsessive-compulsive/anxiety behaviour**

**Marble-burying test**

Mice were individually placed in a cage (21 × 38 × 14 cm length × width × height) containing 5 cm layer of sawdust bedding and 15 glass marbles (1.5 cm in diameter) arranged in three rows. Mice were left undisturbed for 15 min under dim light. An observer blind to the treatment counted the time spent in digging behaviour and the number of marbles buried (at least two or third buried in the sawdust) and the latency at to first dig. At the end of the test the animal was removed to its own cage.

**Working memory by “Y maze”**. The apparatus consisted of three enclosed black arms 30 × 5 × 15 cm (length × width × height) converging on an equilateral triangular black centre (5 × 5 × 5 cm). At the beginning of each experimental session, each mouse was placed in the centre platform and the number of spontaneous alternations (defined as number of successive triplet entry into each of the three arms without any repeated entries) was monitored in a 5 min test session. The percentage of alternation was calculated as the percentage of the ratio of the number of alternations/(total number of arm entries-2). The alternation percentage was calculated as a parameter for the working memory-related behaviour.

**Body weight**

Body weight (BW) was measured (±0.1 g) before the sweetener treatments (day 0), after 21 d of treatments and at the end of the treatment (day 56) (Thermo Fisher Scientific, Waltham, MA).

**Blood pressure measurements**

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured in restrained mice by means of tail-cuff method (Blood Pressure Analysis System, BP-2000, Visitech System, Physiological Research Instruments, Apex, NC). Mice were accustomed to the blood pressure measurement device for 5–6 d. The measurements were undertaken before and after sweetener treatments. Each one of 10 measurements was preceded by 10 preliminary measurement, which was discarded.

**Statistical analysis**

Data are expressed as mean ± standard error of mean (SEM) in both plots and tables. One-way ANOVA followed by Dunnett’s post hoc test was applied to evaluate statistical significance between the control and sweetener-treated groups. For multiple comparisons two-way ANOVA followed by Bonferroni post hoc test was performed for carrageenan-induced paw oedema and mechanical allodynia. Whereas the statistical analysis for the rest of behavioural test was assessed by one-way ANOVA, post-hoc Newman–Keuls Multiple
Comparison Test. Differences between means were considered significant at $p \leq .05$.

**Results**

**Sweetener effects on EPC number**

To determine whether moderate or high intake of sweeteners induced variations of circulating EPC number, we carried out flow cytometry analysis on peripheral blood samples from experimental animals. EPC count was performed by measuring the number of cell positive for an endothelial marker protein (Flk-1) and a stem cell antigen (Sca-1).

As shown in Figure 1(a,b), relative percent values of EPCs in mice receiving moderate or larger amounts of all sweeteners were lower than control mice by about 30–50%. Moreover, acute inflammation, induced by intraplantar injection of carrageenan, prompted a larger reduction of EPC number in control animals of about 80% (Figure 1(c)). Interestingly, dietary supplementation with moderate doses of sweeteners strongly reduced this effect of carrageenan on EPCs that was statistically significant for all sweeteners except for Asp (Figure 1(c)).

**Sweetener effects on physical and biochemical variables**

**Body weight**

Body weight was significantly increased after 56 d of treatment with low dose of sweeteners for all mice groups ($F_{6,33} = 37.54, p = .042$) (Figure 2(a)). Similarly, mice treated with double dose of sweeteners revealed a significant gain of body weight already after 28 d of treatment and an even larger gain after 56 d ($F_{6,33} = 51.24, p = .001$) (Figure 2(b)).

**Blood pressure**

SBP and DBP were monitored over the entire duration of the treatments. Blood pressure monitoring revealed that sweeteners did not affect neither the SBP nor the DBP values as compared to the control group as reported in Table 1 ($F_{6,33} = 11.2, p = 0.82$).

**Serum biochemistry**

We measured several parameters in the blood of the experimental animals to evaluate glucose and lipid metabolism, as well as liver and kidney functions (Table 2). As far as glucose metabolism, except for the low dose of fructose, which lowered glycaemia, low doses of RebA and Cyc, as well as high doses of Suc and RebA increased glycaemia. Interestingly, no treatment increased plasma TG, but low and high doses of sucrose and fructose, as well as high doses of Cyc reduced plasma TG. With respect to cholesterol metabolism, treatments were either ineffective, or lowered T-Chol and HDL-cholesterol, except for high doses of Suc, which increased T-Chol. LDL-cholesterol did not change following most treatments, but increased after administration of high doses of Suc, Asp or RebA. Liver function was preserved after most treatments, except for low and high doses of Suc, and high doses of Asp, which increased serum levels of AST. Kidney function, according to BUN levels, was not altered following all treatments under investigation.

**Sweetener effects on the carrageenan-induced paw oedema**

In order to verify the impact of the sweeteners under investigation on inflammatory reaction, we injected paws of control and sweetener-treated mice with carrageenan, an inducer of acute inflammation. As expected, intra-plantar injection of carrageenan-induced substantial oedema in mice characterised by a significant increase in paw volume that reached the peak at 4 h post-carrageenan injection, compared with the uninjected contro-lateral paw ($p < .001$). However, the administration of the sweeteners did not affect the of carrageenan-induced paw oedema, as compared to the control group ($H_{2O}$) ($F_{6,33} = 2.19, p = .061$) (Figure 3(a)). Finally, the paw volume did not differ among the different sweetener-treated mice 4 h after carrageenan injection as reported in Table 3.

**Sweetener and paw withdrawal threshold 6 h after carrageenan injection**

PWT before carrageenan injection was $1.8 \pm 0.1$ g and it significantly decreased on the ipsi-lateral side 6 h after the inflammation induction. Conversely, no effect was observed in the contro-lateral paw. In Table 4 was reported all measurements showing that the administration of our sweeteners did not change the withdrawal threshold in carrageenan-injected mice as compared to the control group ($F_{6,33} = 1.76, p = 0.17$; Figure 3(b)). On the other hand, the PWT of ipsi-lateral paw to carrageenan-injection resulted significantly reduced 6 h after inflammation induction as compared to the contralateral paw (dashed line) ($F_{6,33} = 576.3, p < .001$).
Effects of excess sweeteners exposure on behaviour

To test whether the sweetener exposure affected mouse behaviour, we carried out different behavioural tasks.

Depressive-like behaviours

Eight weeks of sweetener treatment did not significantly change the time of immobility in the tail suspension test, as well as, in the forced swimming test compared with the group-receiving vehicle (Figure S2(a,b)). The wire hang test showed that the muscle strength was not compromised by treatment ($F_{(6,35)} = 5.02$, $p = .85$; $F_{(6,14)} = 1.43$, $p = .27$) (Figure S2(a,b)).

Spatial memory

Sweetener-treated mice did not show any difference in percentage of alternation in arm entries compared with controls in the Y-maze (Figure S3).

Obsessive-compulsive behaviour

Finally, we observed that the number of buried marbles, the number of digging events and the latency to digging were not changed following treatments, as
compared with controls $F(6,14) = 0.68, p = .67$; $F(6,14) = 1.43, p = .27$; $F(6,14) = 0.48, p = .81$) (Figure S4).

**Discussion**

The results of this study reveal, for the first time, that administration of both natural and artificial sweeteners affects EPC number according to the presence or absence of inflammation but does not induce adverse effects on behavioural changes.

Hyperglycaemia plays a crucial role in endothelial dysfunction through induction of endothelial cell apoptosis, necrosis and senescence (Chen et al. 2007; Balestrieri et al. 2008; Tamura et al. 2009; Napoli et al. 2011; Yiu and Tse 2014; Maeda et al. 2015). Over the past 50 years, consumption of sugar has tripled worldwide. A growing body of epidemiological evidence disputes that excessive sugar consumption affects human health beyond simply adding calories (WHO/FAO, 2015). Indeed, other pathologies can be triggered: hypertension (fructose increases uric acid, which raises blood pressure); high TG and insulin resistance through synthesis of fat in the liver; diabetes from increased liver glucose production combined with insulin resistance; the ageing process, caused by damage to lipids, proteins and DNA through non-enzymatic binding of fructose to these molecules (Livesey 2011; Bursa/C19/C et al. 2018).

Therefore, the use of natural and artificial sweeteners gains a growing interest in the worldwide. We show that both moderate and high intake of sweeteners induced variations of circulating EPCs. In our experimental conditions, relative percentage values of EPCs in animals that receive moderate amounts of all sweeteners were lower than control mice (of about 30%). Coherently, also double doses of these same sweeteners caused a reduction of EPC number compared to the moderate doses (of 30–50%). It is generally accepted that added sugars promote weight gain and increase cardiometabolic risk (Popkin and

**Figure 2.** Variation in body weight of treated mice with different doses of sweeteners. Body weight curves of C57BL/6J mice treated for 6 weeks with water ($H_2O$) or different sugar solutions [Glucose (GLU), Fructose (FRU), Sucrose (SUC), Aspartame (ASP), Rebaudioside-A (REB-A) and Cyclamate (CYCL)], at day 0, 21 and 56. The data represented are related to moderate doses (a) and double doses (b) of administered sweeteners. Data are expressed as mean±SEM assessed by one-way ANOVA followed by Bonferroni post-hoc test. *$p<.05$; **$p<.01$ and ***$p<.001$ vs control ($H_2O$) group. $^*p<.05$ and $^{**}p<.001$ vs day 0.
Table 1. Systolic and diastolic blood pressure values in the different study groups.

| Treatment | Water | Glucose | Fructose | Aspartame | Cyclamate |
|-----------|-------|---------|----------|-----------|-----------|
| Systolic blood pressure (mmHg) | 102.0 ± 3.8 | 105.2 ± 5.5 | 101.8 ± 3.9 | 107.8 ± 10.5 | 113.7 ± 3.9 |
| Diastolic blood pressure (mmHg) | 75.7 ± 2.2 | 75.0 ± 2.7 | 74.3 ± 1.3 | 71.9 ± 1.8 | 70.2 ± 2.4 |

The values were obtained by the average of ten measurements in all groups of animals treated with low- and high-doses of sweeteners. Data were expressed as mean and standard error of mean (SEM) assessed by one-way ANOVA followed by Bonferroni post-hoc test.

Table 2. Sweetener effects on biochemical parameters into analysed animal groups.

| Treatment | Glucose (mg/dL) | Fructose (mg/dL) | Aspartame (mg/dL) | Cyclamate (mg/dL) |
|-----------|----------------|------------------|-------------------|------------------|
| Low-dose  | 152.0 ± 6.1    | 134.2 ± 7.3      | 130.4 ± 10.6      | 135.3 ± 3.3      |
| High-dose | 220.0 ± 8.4    | 153.6 ± 8.5      | 147.6 ± 10.6      | 157.9 ± 5.4      |
| T-Chol (mg/dL) | 170.1 ± 14.6  | 130.4 ± 10.6      | 160.4 ± 12.6      | 175.4 ± 14.6     |
| HDL (mg/dL) | 130.4 ± 10.6    | 163.4 ± 17.1      | 159.4 ± 13.1      | 149.2 ± 13.7     |
| LDL (mg/dL) | 222.0 ± 8.5    | 135.3 ± 6.3      | 159.4 ± 13.1      | 149.2 ± 13.7     |
| AST (U/L) | 153.6 ± 8.5    | 147.6 ± 10.6      | 175.4 ± 14.6      | 178.3 ± 0.6      |
| ALT (U/L) | 130.4 ± 10.6   | 163.4 ± 17.1      | 159.4 ± 13.1      | 149.2 ± 13.7     |
| BUN (mg/dL) | 222.0 ± 8.5   | 135.3 ± 6.3      | 159.4 ± 13.1      | 149.2 ± 13.7     |
| T-bil (mg/dL) | 12.2 ± 3.2   | 18.0 ± 12.4      | 21.2 ± 3.2        | 21.0 ± 10.2     |

Values are given as mean ± SEM. Statistically significant changes are highlighted in bold.

Hawkes 2016). Differently, lack of consensus exists about the health effect of low-calorie sweeteners (Popkin and Hawkes 2016). Interestingly, the results of the present study highlight a common ground for both caloric and low-calorie sweeteners that could contribute to the vascular damaging effects of excessive intake of such food additives.

Moreover, our data showed that acute inflammation, induced by intraplantar injection of carrageenan, prompted a larger reduction of EPC number in
control animals of about 80%. Interestingly, dietary supplementation with moderate doses of sweeteners strongly reduced this effect of carrageenan on EPCs that was statistically significant for all sweeteners except for Aspartame. Inflammation and connected oxidative stress are relevant features of cardiovascular diseases that participate to vascular injury and atherosclerosis (Napoli 2003). However, the interplay between inflammation and EPCs involves a delicate balance of EPC mobilisation and homing. Indeed, EPC number can either increase or decrease following inflammatory stimuli, depending on the type, intensity, and duration of the stimulus (Lin et al. 2013; Balestrieri et al. 2013). The higher number of EPCs that we observed in mice with an inflammatory condition, and that were ingesting most types of sweeteners, is highly suggestive of a potential beneficial role of the tested sweeteners on vascular biology in the presence of inflammatory disorders.

Body weight was significantly increased after 56 d of treatment with low dose of sweeteners for all mice

**Table 3.** Effect of sweeteners drinking solutions on carrageenan-induced paw oedema in mice.

| Treatment       | Dose     | Time 0 min | Time 4 h  |
|-----------------|----------|------------|-----------|
| Water           | None     | 13.3 ± 12.0 µl | 110.0 ± 19.0 µl |
| Glucose         | 0.80 g/kg, o.s. | 2.5 ± 11.5 µl | 125.4 ± 45.8 µl* |
| Sucrose         | 0.75 g/kg, o.s. | 1.9 ± 5.8 µl | 155.7 ± 41.0 µl** |
| Fructose        | 0.80 g/kg, o.s. | 6.7 ± 12.0 µl | 70.1 ± 23.3 µl*** |
| Aspartame       | 3.75 mg/kg, o.s. | 16.7 ± 12.0 µl | 170.3 ± 37.1 µl*** |
| Rebaudioside A  | 3.80 mg/kg, o.s. | 2.3 ± 5.8 µl | 90.3 ± 11.5 µl*** |
| Cyclamate       | 7.00 mg /kg, o.s. | 1.1 ± 5.0 µl | 140.0 ± 40.0 µl*** |

Data represent mean ± error of mean (SEM) (n = 4–6 for each group) of paw volume before carrageenan paw injection (time 0 min) and at the pick of oedema (time 4 h).

*, ** or *** indicate significant (p < .05, p < .01 or p < .001, respectively) differences as compared to the basal value of paw volume before injection of carrageenan.

**Table 4.** Paw withdrawal threshold (PWT) in water or sweeteners drinking solutions-treated mice, after paw carrageenan injection in mice.

| Treatment      | Dose     | PWT (ipsi) | PWT (contra) |
|----------------|----------|------------|--------------|
| Water          | None     | 0.07 ± 0.02 g*** | 1.5 ± 0.08 g |
| Glucose        | 0.80 g/kg, o.s. | 0.08 ± 0.04 g*** | 1.0 ± 0.05 g |
| Sucrose        | 0.75 g/kg, o.s. | 0.02 ± 0.01 g*** | 1.4 ± 0.02 g |
| Fructose       | 0.80 g/kg, o.s. | 0.03 ± 0.02 g*** | 1.0 ± 0.04 g |
| Aspartame      | 3.75 mg/kg, o.s. | 0.09 ± 0.04 g*** | 1.0 ± 0.02 g |
| Rebaudioside A | 3.80 mg/kg, o.s. | 0.05 ± 0.02 g*** | 1.4 ± 0.07 g |
| Cyclamate      | 7.00 mg /kg, o.s. | 0.01 ± 0.00 g*** | 1.0 ± 0.04 g |

Data represent mean ± error of mean (SEM) (n = 4–6 for each group) of paw withdrawal threshold (PWT) after carrageenan paw injection.

***indicates significant (p < .001) differences as compared to contralateral paw PWT following carrageenan injection. Ipsi and contra indicate the same or the opposite site to carrageenan injection, respectively.
groups. Similarly, mice treated with double dose of sweeteners revealed a significant increase of body weight already after 28 d of treatment, and an even larger increase after 56 d of treatment. Interestingly, intra-abdominal fat weight was significantly higher in Glu, Fru and Cyc treated groups compared to control (p < .01). These results are particularly striking because low-calorie sweeteners are supposed to prevent or, at least, reduce body weight gain. Nevertheless, similar findings are present in the literature in both animal models and humans and low-caloric sweeteners have been associated with weight gain, diabetes and other metabolic dysfunctions (Mattes and Popkin 2009; Pearlman et al. 2017). Moreover, one of the main limitations making the outcomes difficult to interpret in human studies is that sweeteners consumption is commonly based on dietary recall. Many products like soft drinks, mouthwash, toothpaste, sauces and frozen dinners contain both artificial and natural sweeteners and consumers may not be aware of their consumption.

Furthermore, blood pressure monitoring revealed that sweeteners did not affect the SBP or the DBP values as compared to the control group.

Overall, all analysed treatments did not have a negative impact on glucose and lipid metabolism, as well as liver and kidney function. However, increased glycaemia after ReBA and Cyc, and the HDL-cholesterol-lowering effect of low doses of all treatments are worrying in terms of cardiovascular risk and deserve further investigation. As far as the lipid changes that were observed in this study, the peculiar HDL-cholesterol-rich profile, which is typical of mice, must be taken into account when trying to extrapolate these findings to humans.

Finally, to test whether the sweetener exposure affected mouse behaviour, we carried out different behavioural tasks. Fifty-six days of sweetener treatment did not significantly change the time of immobility in the tail suspension test, as well as, in the forced swimming test compared with the group-receiving vehicle. Also, sweetener-treated mice did not show any difference in percentage of alternation in arm entries compared to controls in the Y-maze, and the number of digging events and the latency to digging were not changed following treatment as compared with control group during obsessive-compulsive behaviour. We decided to perform such studies because the information about how sugars and sugar substitutes affect behaviour and cognition, both in humans and in experimental animals is quite limited. Although it is accepted that sugar does not have negative effect on behaviour in humans (Benton 2008), rodents may develop cognitive dysfunction but not long-term effects on anxiety or mood following sugar ingestion (Kendig et al. 2014). The lack of significant changes in behavioural phenotype observed in this study, might be due to the healthy mice condition. Indeed, there is evidence for a common soil between metabolic changes and neurodegenerative as well as chronic inflammatory diseases (Guida et al. 2017, 2018; Boccella et al. 2018). Therefore, further investigations are needed to better understand the possible impact of the sugars in mice with mild pathologies (i.e. mild cognitive impairment, mild traumatic brain injury) in which we already have a slight, but significant, change in the psychiatric behaviour (Guida et al. 2017).

In this work, we have expanded the study beyond sugars and demonstrated that neither caloric nor low-calorie sweeteners generate behavioural effects in mice.

Conclusions

Although sweetened beverages and food have an adverse effect on health, particularly by enhancing the risk both of cardiovascular and metabolic diseases, they are increasingly consumed around the world. Because excessive calorie intake is regarded as the major factor of the health negative influence of dietary added sugars, low- or non-caloric sweeteners have attracted the attention of the scientific arena as well as food industry and consumers. It is worrying that there is no consensus on the health effects of these so-called non-nutritive sweeteners and, at the same time, diet beverages and light formulations of food containing such compounds are widely advertised and increasingly consumed around the world. Aiming at studying the effects of sugars and sugar substitutes on health, we have evaluated the response of several variables in mice that were administered such compounds in the drinking water. Under our experimental conditions, this study highlights the negative effect of lower and higher intake of sweeteners on EPC number, which is an important factor of vascular repair and regeneration. Interestingly, the same compounds showed a potentially protective effect on the EPC compartment, by reducing the inflammation-induced fall of EPC number. It is therefore clear that apparently opposite effects can be elicited by these sweeteners according to the health status and/or inflammatory condition of the recipient and that such subtle issue must be evaluated also in both clinical epigenetic (Sommese et al. 2017, 2018) and controlled studies in order to
promote a safe and effective use of natural and artificial sweeteners in humans.

**Disclosure statement**

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