Castanea sativa Mill. bark extract cardiovascular effects in a rat model of high-fat diet

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Abstract: Ellagitannins may have a beneficial impact in cardiovascular diseases. The aim of the study was to evaluate the effect of high-fat diet (HFD) and the efficacy of Castanea sativa Mill. bark extract (ENC) on cardiac and vascular parameters. Rats were fed with regular diet, (RD, n = 15), HFD (n = 15), RD + ENC (20 mg/kg/day by gavage, n = 15), and HFD + ENC (same dose, n = 15) and the effects on body weight, biochemical serum parameters, and inflammatory cytokines determined. Cardiac functional parameters and aorta contractility were also assessed on isolated atria and aorta. Results showed that ENC reduced weight gain and serum lipids induced by HFD. In in vitro assays, HFD decreased the contraction force of left atrium, increased right atrium chronotropy, and decreased aorta K+-induced contraction; ENC induced transient positive inotropic and negative chronotropic effects on isolated atria from RD and HFD rats and a spasmolytic effect on aorta. In ex vivo experiments, ENC reverted inotropic and chronotropic changes induced by HFD and enhanced Nifedipine effect more on aorta than on heart. In conclusion, ENC restores metabolic dysfunction and cardiac cholinergic muscarinic receptor function, and exerts spasmolytic effect on aorta in HFD rats, highlighting its potential as nutraceutical tool in obesity.

Keywords: cholinergic receptor, chronotropy, hydrolysable tannins, inotropy, oxydative stress, vascular relaxation

1- Introduction

Hydrolysable tannins are a heterogeneous group of water-soluble high-molecular-weight (500–3,000 Da) polyphenolic compounds, accounting for more than 500 natural products that, as recently reported, are found in large amounts of dietary sources, such as red fruits, berries, and nuts (Smeriglio, Barreca, Bellocco, & Trombetta, 2017).

In common diet of western countries, ellagitannin intake is generally about 5 mg/day (Garcia-Munoz & Vaillant, 2014), while higher consumption is reported in northern Europe, mainly due to seasonal berries, and in tropical areas, for local blackberries (Crozier, Del Rio, & Clifford, 2010). Ellagitannins contribute to prevent oxidative stress, inflammation, atherogenesis and other cardiovascular events

(Larrosa, Garcia-Conesa, Espin, & Tomas-Barberan, 2010), limit apoptosis and transduction of pathological pathways to cancer (Neergheen, Bahorun, Taylor, Jen, & Aruoma, 2010): however, in the intestine, they are poorly absorbed (Espin, Larrosa, Garcia-Conesa, & Tomas- Barberan, 2013) and partially transformed into ellagic acid, metabo- lized by colonic bacteria into urolithins, that are adsorbed and are mostly responsible for the systemic effects (Garcia-Munoz & Vaillant, 2014). Tannins from Castanea sativa Mill. (ENC) have long been studied in our laboratory: we have shown that they exert antioxidant and cytoprotective effects in cardiomyocites (Chiarini et al., 2013), produce positive inotropic and negative chronotropic effects in guinea-pig atria (Chiarini et al., 2013), contract gallbladder and relax sphincter of Oddi in lithogenic diet fed rats, reducing the risk of gallstones formation (Micucci et al., 2014). We have shown the protective effects of ENC on liver and intestinal parameters in high- fat diet (HFD) obese rats showing that it decreases histological steatosis, increases phase I and II and antioxidant enzyme, as well as reduces oxidative stress status in ileum and restores altered intestinal contractility (Budriesi et al., 2018). In the present paper, we address the cardiovascular effects of ENC.

The imbalance in the autonomic system is commonly reported in obesity, aging, and cardiovascular diseases with consequent worsen- ing of cardiological outcomes (Floras & Ponikowski, 2015). Feeding rats with HFD results in a reduced parasympathetic activity, mainly through an imbalance in cholinergic receptors (Pellizzer, Straznicky, Lim, Kamen, & Krum, 1999; Schwartz, Young, & Landsberg, 1983) and ENC, restoring the muscarinic function, can contribute to parasympathetic activity recovery (Budriesi et al., 2018).

The aim of the present research is to investigate:

a. In vitro: ENC effects on cardiac functional parameters and aorta contractility on isolated tissues from rats fed with regular diet (RD) and HFD.

b. Ex vivo: rats cardiac functional parameters, aorta contractility and body weight, biochemical, inflammatory, and oxidative state in RD and HFD rats after ENC x os (21 days) administered.

2- Materials and Methods

2.1 Plants Material

Wood extract of ENC was provided by SilvaTeam S.p.a. (San Michele Mondovì, Italy). ENC has been chemically described in previous stud- ies (Budriesi et al., 2010) and characterized by HPLC-DAD-MS analysis (Chiarini et al., 2013). The hydrolizable tannins fraction was composed by phenolic compounds such as castalin (1.47 ± 0.06), vescalin (5.01 ± 0.11), castalgin (4.96 ± 0.08), vescalgin (5.01 ± 0.11), ellagic acid (3.64 ± 0.10), and gallic acid (3.68 ± 0.12) expressed as gallic acid equivalent g (gGAE)/100 g of extract. In particular, ellagic plus hexahydroxydiphenic and gallic acids were 28.2 and 5.9% respectively. Detailed information is available at SILVATEAM website (http://it.silvateam.com/).

2.2 Animals and experimental design

Sixty male Sprague–Dawley rats (purchased from Envigo RMS S.R.L. San Pietro al Natisone [Udine, Italy]), age 9 weeks, 270–300 g weight at the beginning of the study were used. After 7 days housing (conditions: 12 hr-light/12 hr-dark cycle, ambient temperature 22°C, 60% humidity, water and food ad libitum), they were randomly divided into two groups fed with regular diet (RD, n = 30) or HFD (HFD, n = 30) for 10 weeks. RD or HFD were supplied by Mucedola (Mucedola, s.r.l. Via Galileo Galilei, 6, Settimo Milanese MI, Italy). RD was composed by 18.7% crude protein, 5.6% crude fat,

4.5% crude fiber, while HFD was composed by 23% crude protein, 34% crude fat, 5% crude fiber. For more detailed information, see www. mucedola.it/. Afterward, rats were then further randomly split into further four groups: RD rats orally treated for 21 days with vehicle (tap water) (n = 15) or with ENC 20 mg/kg/day (n = 15) by gavage; HDF rats orally treated for 21 days with vehicle (tap water) (n = 15) or with ENC 20 mg/kg/day (n = 15) by gavage (Figure 1). The oral route (gavage) was used to mimic a possible administration of an integrated medicine of natural chestnut extract in man. The frequency (once a day) has been established according to the literature with the final aim to forecast the maximum compliance by the patient and, at the same time, to reduce as much as possible the stress caused by the manipulation and administration itself in the treated animals. The dose of ENC was extrapolated by using the translational dose calculation (Reagan-Shaw, Nihal, & Ahmad, 2008), which expresses the human equivalent dose (HED, mg/kg). HED {HED = Animal dose • [Animal Km • (Human Km)−1]} was calculated either by multiplying or dividing the animal dose with the Km ratio values, where Km was 6 for rat and 37 for human, respectively (Nair & Jacob, 2016). We have used therefore the body surface area (BSA) normalization method and the doses are relevant for human translation. As Abidov, Ramazanov, Seifulla, and Grachev (2010) used 300 mg/day in man, a value in agreement with the human dose of 225 mg/day, the resulting final dose for rat was thus calculated to be 20 mg/kg. After treatment, the animals were sacrificed by decapitation. Body weight and food consumption were evaluated from day 0 to the sacrifice. Skin, fur, eyes, mucous membranes or salivation, and growth rate were checked in animals upon ENC treatment, in order to evaluate ENC safe profile.

The protocol followed the guidelines of the Institutional Ethics Committee of the University of Bologna (Protocol 21/79/14) about the protection of animals used for scientific purposes and conformed the European Union Guidelines for the Care and the Use of Laboratory Animals (European Union Directive 2010/63/EU).

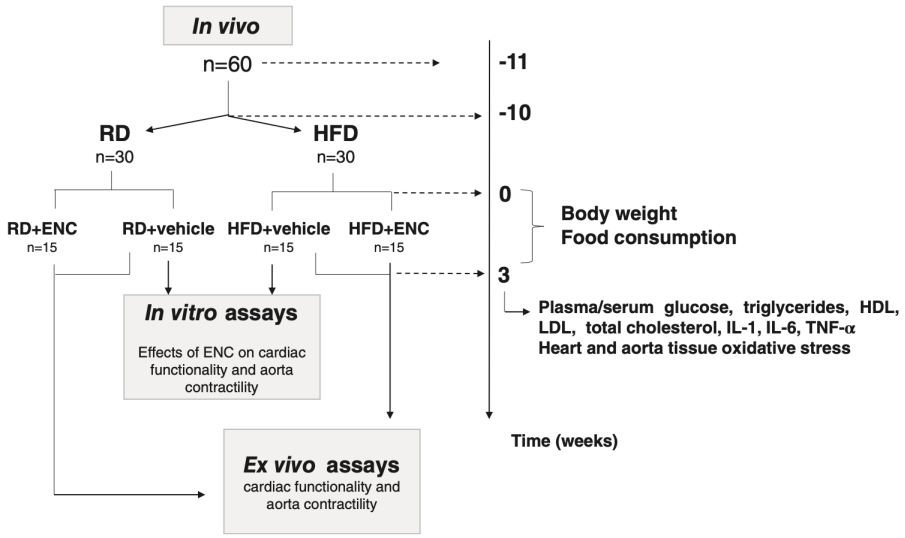

Figure 1, Schematic experimental design

2.3 Functional studies on cardiovascular parameters

Based on previous results (Budriesi et al., 2010), we monitored the cardiovascular activity:

- by evaluating the in vitro effect of ENC on inotropy and chronotropy activity and on K+-depolarized vascular smooth muscle in tissues from RD and HFD fed rats;

- by studying the effect of ENC treatment on the cardiovascular system by monitoring the Nifedipine profile and by studying the functionality of parasympathetic system on heart using agonist carbachol and antagonist atropine on tissue taken from treated rat.

2.3.1 Heart

The hearts were rapidly removed and set up as previously described (Micucci et al., 2016). Briefly, the contractile activity was recorded iso- metrically by means of force transducer (FT 0.3, Grass Instruments Corporation, Quincy, MA) using Power Lab® software (AD- Instruments Pty Ltd, Castle Hill, Australia). The change on chronotropy was detected on spontaneously right atria.

Atrial muscle preparations were used:

• To examine the inotropic and chronotropic activity of the ENC (0.01–10 mg/ml), dissolved in PSS.

• To study effect of Nifedipine, carbachol and atropine on tissue from ENC fed rats.

Nifedipine was first dissolved in dimethylsulfoxide (DMSO) and then diluted with PSS. According to this procedure, the concentration of DMSO in the bath solution never exceeded 0.3%. Appropriate con- trols with 0.3% DMSO were run in parallel. At this concentration, DMSO did not produce appreciable inotropic and chronotropic effects (data not shown).

During the generation of cumulative concentration response curves to ENC, Nifedipine and CCh, the increasing extract or drug concentrations was added, after a steady state was reached. To assess that ENC was not able to affect the functionality of the hearts, the effects of Nifedipine and CCh on tissues were checked after washing out the highest concentration of ENC. This was always found to be within the physiological range (data not shown).

2.3.2 Aorta preparations and assays

The thoracic aorta was removed and placed in Tyrode solution as pre- viously described (Micucci et al., 2016) After the equilibration period, guinea-pig aortic strips were contracted by washing in PSS containing 80 mM KCl (equimolar substitution of K+ for Na+). When the contrac- tion reached a plateau (about 45 min or 15 min respectively), the preparations were used to examine the spasmolytic effect of ENC (0.01–10 mg/ml) or Nifedipine. Both samples were added cumula- tively to the bath allowing for any relaxation to obtain an equilibrated level of force. The effects of Nifedipine on aorta strips were checked at the end of each experimental session after washing out the highest concentration of ENC. The functionality of the tissue was always found to be within the physiological range (data not shown).

2.4 Biochemical serum parameters and inflammatory plasma markers

Glucose, triglyceride, HDL-cholesterol, and total cholesterol concen- trations were measured using Dimension RxL Max system Kit (Siemens Healthcare Diagnostics, Newark, DE), following manufac- turer's instructions. Serum transaminases were evaluated by Sigma- Aldrich colorimetric assay. For each sample, a volume of 200 µl was used.

Inflammatory plasma pro-atherogenic cytokines: TNF-α, interleu- kin (IL)-1 and IL-6, and antiatherogenic cytokine IL10 (Tousoulis, Oikonomou, Economou, Crea, & Kaski, 2016) were evaluated by the Luminex MAGPIX® system (Millipore Corp., Billerica, MA).

2.5 Oxidative stress evaluation in heart and aorta

The total antioxidant power of tissue homogenates was deter- mined by FRAP assay (Benzie & Strain, 1996), following the absorbance at 595nm of FeII-tripyridyltriazine. As previously described, all solutions were prepared freshly. The sample was added to the FRAP reagent and the absorbance at 595 nm was measured (Time 0); then the reaction mixture was incubated at 37□C for 3 min in continuous stirring and the final absorbance was measured (Time 30). The absorbance at Time 30 minus Time 0 was calculated and the final values were expressed as ΔAbs on mg wet tissue weight. All measurements were performed in triplicate ± SD.

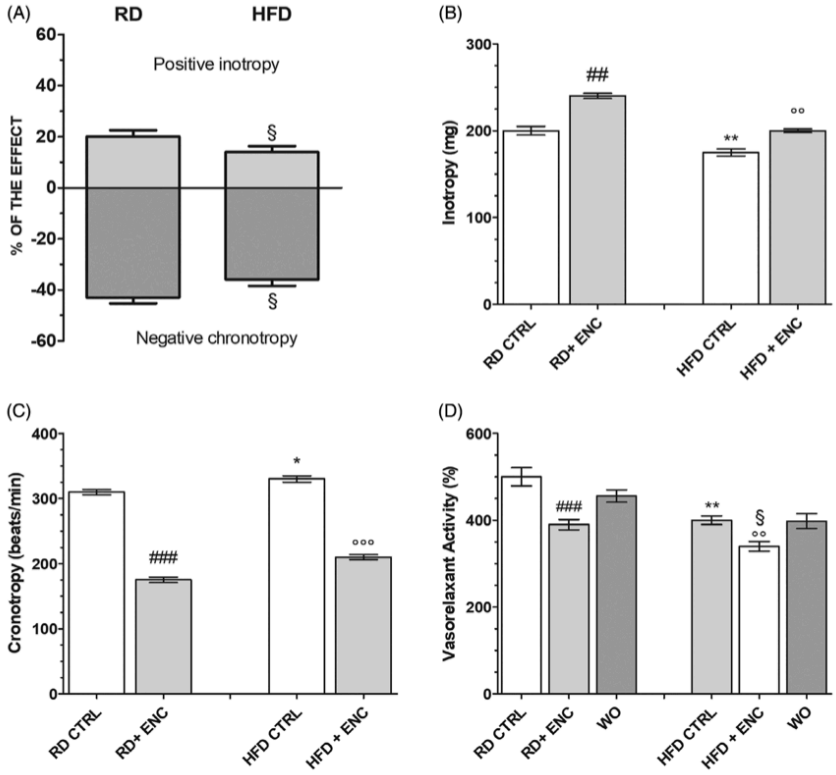

Figure 2, In vitro effects of ENC (1 mg/ml, 30 min incubation) on spasmolytic effect on aorta K+ (80 mM)-induced contraction. (a) Percent effects elicited by ENC of positive inotropy and negative chronotropy activities. (b) Positive inotropic and (c) negative chronotropic effects expressed as mg contraction and beats/min, respectively. (d) Spasmolytic effect of ENC on aortic strips precontracted with potassium (80 mM) expressed as mg of contraction. “Data are expressed as mean ± SD (n = 3–4). RD CTRL or HFD CTRL: basal activity. *p < .05, **p < .01 vs. RD CTRL; ##p < .01, ###p < .001 vs. RD CTRL; ##p < .01, ###p < .001 vs. HFD CTRL; §p < .05 vs. RD (Panel c) and §p < .05 vs. RD + ENC (Panel d); WO, washout. Panel a, Student t-test. Panel b, c, d, ANOVA followed by Bonferroni post hoc test”

2.6 Data presentation and statistical analysis

The experimental design was subjected to “a priori power analysis” to ensure that the size of treated and control groups was adequate (G*Power v. 3.1.9.2, effect size 0.45, α 0.05, power [1 − ß] 0.8.).

Plasma cytokine content was reported as percent changes of the baseline value [100 (%)] of RD rats.

The potency of ENC and drugs (EC50) was calculated from con- centration response curves (ENC) or log concentration response cur- ves (drugs) (Probit analysis using Litchfield and Wilcoxon

[Tallarida & Murray, 1987]) or from concentration response curves by GraphPad Prism® (Motulsky, 2007) software.

Functional-induced contractility of agonist carbachol (CCh) was expressed as pEC50 values. The antagonism activity of atropine against CCh was expressed as pA2 values determined from Schild plots (Arunlakshana & Schild, 1959) constrained to slope −1.0, as required by theory (Tallarida & Murray, 1987).

Data are reported as mean ± SD. Comparison has been per- formed by using the two-tailed unpaired Student's t test or by ANOVA followed by Bonferroni's post hoc tests as appropriate (GraphPad Prism® software). In all comparisons, p value lower than .05 was considered significant.

3- Results

3.1 *In vitro* experiments

ENC induced positive inotropic and negative chronotropic effects in atria and spasmolytic effect in K+-depolarized (80 mM) aorta from RD and HFD rats. On the left atrium, ENC (1 mg/ml, 30 min incubation) induced a positive inotropic effect (+20% in RD and +14% in HFD), while it induced on the right atrium a negative chronotropic effect (−43% in RD rats and −36% in HFD rats) (Figure 2a). HFD significantly reduced left atrium inotropy (−25%, $p < .01$ vs. RD) (Figure 2b) and increased right atrium chronotropy (+6%, $p < .05$ vs. RD) (Figure 2c). Moreover, the effects on inotropic and chronotropic heart activities elicited by ENC were significantly lower in tissues from rat fed with HDF. All the reported effects were reversible after 30 min washing out (data not shown).

Table 1, Nifedipine, carbachol, and atropine activities on cardiovascular preparation from RD and HFD rats treated or not with ENC

		RD	RD + ENC[a]	HFD	HFD + ENC[a]
Ca^{++} channels					
Left atrium[b]	Activity[c]	86 ± 1.2[d]	93 ± 1.2	94 ± 2.6	91 ± 1.9
Negative inotropy	EC_{50}[e]	0.12	0.0035##	0.036**	0.0097°
	95% conf lim	0.09–0.16	0.0019–0.0060	0.024–0.054	0.0048–0.019
Right atrium[f]	Activity[c]	83 ± 5.8	81 ± 0.7	88 ± 3.7	91 ± 1.9
Negative chronotropy	$EC_{5\mu0}$[e]	0.55	0.78	0.42	0.50
	95% conf lim	0.34–0.66	0.64–1.21	0.30–0.48	0.39–0.62
Aorta[g]	Activity[c]	88 ± 1.4	80 ± 2.1	77 ± 1.9	87 ± 2.3
Vasorelaxant effect	EC_{50}[e]	0.0054	0.0048	0.0072*	0.0040
	95% conf lim	0.0037–0.0060	0.0041–0.0057	0.0066–0.0079	0.0039–0.0057
CCh receptor					
Left atrium	CCh[h]	7.39 ± 0.02	7.35 ± 0.04	7.10 ± 0.02**	7.25 ± 0.03*°
Inotropic effect	Atropine[i]	8.58 ± 0.05	8.45 ± 0.03	8.01 ± 0.04**	8.55 ± 0.01*°°
Right atrium	CCh[e]	6.05 ± 0.03	6.00 ± 0.01#	5.96 ± 0.03*	6.08 ± 0.02**°°
Chronotropic effect	Atropine[h]	8.22 ± 0.02	8.25 ± 0.03	8.13 ± 0.05*	8.20 ± 0.02

[a]RD and HFD rats have been given ENC (20 mg/kg/day).
[b]Decrease in developed tension on isolated left atrium driven at 1 Hz at 5 μM concentration, expressed as percent changes from the control (n = 3–4). The 5 μM concentration gave the maximum effect in most groups.
[c]Data are reported as mean ± SD.
[d]At 10 μM concentration.
[e]EC_{50} (μM) was calculated from log concentration response curves (Probit analysis by Litchfield and Wilcoxon with n = 3–4) (Arunlakshana & Schild, 1959; Tallarida & Murray, 1987). When the maximum effect was <50%, the EC_{50} or IC_{50} values were not calculated.
[f]Decrease in atrial rate on spontaneously beating right atrium at 10^{-5} M concentration, expressed as percent changes from the control (n = 3–4). The 5 μM concentration gave the maximum effect in all groups.

[g]Percent inhibition of calcium-induced contraction on K^+-depolarized (80 mM) vascular smooth muscle (aortic strips) at 0.1 μM concentration. This concentration gave the maximum effect in all groups.
[h]Data are expressed as pEC_{50}. $pEC_{50} = -\log EC_{50}$. EC_{50} values are the means ± SD of independent experiments (n = 3–4) and were calculated by a non- linear regression curve-fitting computer program (Arunlakshana & Schild, 1959).
[i]Data are expressed as pA_2. pA_2 values ± SD (n = 3–4) were calculated from Schild plots (Tallarida & Murray, 1987), constrained to slope − 1.0 (Motulsky, 2007) (pA_2 is the positive value of the intercept of the line derived by plotting log (DR − 1) vs. log [antagonist]. The log (DR − 1) was calculated from three different antagonist concentrations, and each concentration was tested from four to six times. Dose-ratio (DR) values represent the ratio of the potency of the agonist carbachol (EC_{50}) in the presence of the antagonist and in its absence. Parallelism of concentration response curves was checked by linear regression, and slopes were tested for significance ($p < .05$).
*$p < .05$, **$p < .01$ vs. RD (same time); [▽]$p < .05$, [▽▽]$p < .01$ vs. HFD; #$p < .05$, ##$p < .01$ vs. RD (ANOVA followed by Bonferroni post test).

We have previously shown that ENC (1 mg/ml) induced vasorelaxant activity on aorta strips from guinea-pig (Chiarini et al., 2013). In the present work, HFD reduced K+-induced contraction (80 mM) (Figure 2d) ENC (1 mg/ml, 30 min incubation) significantly decreased K+-induced contraction by about 20% in RD and 15% in HFD rats (Figure 2e). All the reported effects were reversible after 30 min washing out.

ENC enhanced Nifedipine-mediated effects more on heart than on aorta. Nifedipine intrinsic activity and potency on atria inotropic and chronotropic activity have been studied and aorta K+-depolarized (80 mM) from RD and HFD rats treated with vehicle or with ENC (Table 1).

Left atria of HFD fed rats seemed to be more sensible to Nifedipine-mediated inotropic effects as the potency of the drug differs by about one order of magnitude (0.12 μM of RD vs. 0.036 μM in HFD, $p < .01$). ENC treatment further lowered Nifedipine EC50 values (0.036 vs. 0.0097 μM, $p < .05$) in HFD, as well as in RD rats ($p < .01$). On the contrary, in right atria, Nifedipine negative chronotropic potency was similar in RD and HFD rats, with no differences also upon ENC administration (Table 1). The effects of the calcium antagonist were also studied at vascular level. In HFD fed rats, Nifedipine potency, lower than in RD fed rats ($p < .05$), was increased by ENC treatment, which reported the EC50 to values close to those observed in RD rats. As previously outlined, however, despite statistically different, this difference may be not biologically relevant (Table 1).

3.2 *Ex vivo* experiments

3.2.1 ENC effects on cardiovascular parameters in HFD

ENC treatment restored inotropy in HFD and decreased aorta K+- induced contraction. As shown in Figure 2, HFD decreased inotropy on left atrium, driven at 1 Hz, increased chronotropy on spontaneously beating right atrium and reduced aorta contraction induced by high K+ concentration (80 mM). ENC treatment significantly increased inotropy both in RD and HFD rats. Interestingly, in the latter group, ENC reestablished the activity to values close to those observed in RD rats (Figure 3a). In right atria, however, ENC did not affect chronotropy in both RD and HFD fed rats (Figure 3b).

ENC assumption restored cardiac parasympathetic cholinergic receptor function. To evaluate the effects of ENC treatment on para-sympathetic system in atria of both RD and HFD fed rats, we focused on muscarinic cholinergic receptors. In left atrium, HFD seemed to reduce the sensitivity of the tissue to CCh- and atropine-mediated effects as the potency and the pA2 values of the drugs were significantly affected (Table 1). ENC, however, restored the potency of the agonist CCh and pA2 of the antagonist atropine to RD values.

In the right atrium, ENC treatment increased carbachol and atropine potency in HFD rats ($p < .01$ for carbachol and atropine in left atrium and for carbachol potency in right atrium), bringing the values

closer to those of RD fed rats (Table 1). As above observed, these values are statistically different, but this significance may be not “bio- logically” relevant.

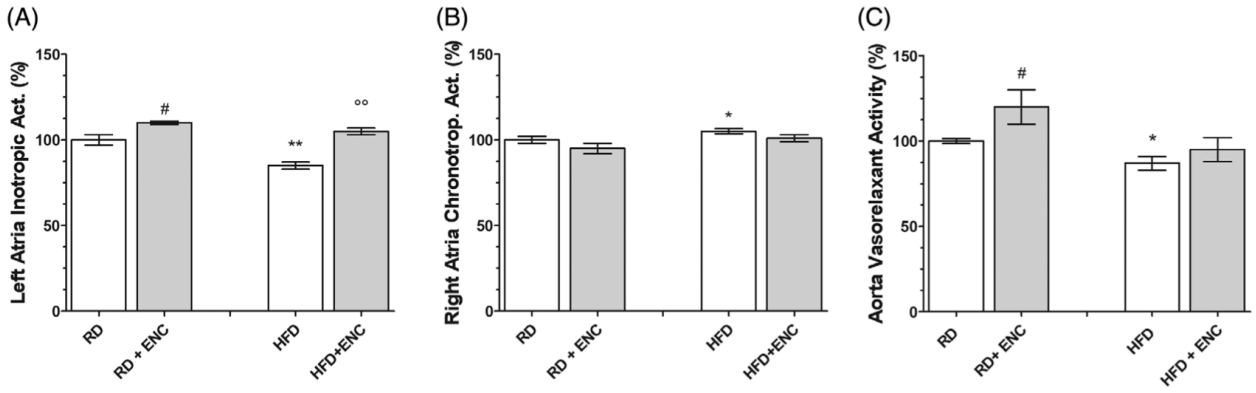

Figure 3, Ex vivo cardiovascular parameters on RD and HFD rats with vehicle and with ENC. The inotropy was tested on left atria driven at 1 Hz (a), while the chronotropy on spontaneously beating right atria (b). The effect of high potassium-induced contraction response was tested on aorta (c). The activity of tissues from RD rats was taken as 100% of the effect. Data are expressed as mean ± SD (n = 3–4). *p < .05, **p < .01 vs. RD; °°p < .01 vs. HFD; #p < .05 vs. RD (ANOVA followed by Bonferroni post test)

3.2.2 ENC reduces weight gain in HFD

HFD rats presented an average medium weight higher than RD rats (p < .001) both at the beginning of the experiment (week 0), and par- ticularly after 3 weeks (week 3). Interestingly, HFD rats body weight was significantly lowered by ENC (p < .05), which induced also a sig- nificant decrease in food intake (p < .05) and a lower increase in weight gain (p < .01) (Table 2). ENC did not affect the same parame- ters in RD rats. Finally, 21 days of ENC treatment did not cause any death in either RD or HFD rats. Animals showed no signs of changes in the skin, fur, eyes mucous membrane, behavior patterns, tremors, salivation, showing a safe profile of ENC treatment.

Table 2, Effects of ENC on body weight, food intake, and weight gain

	RD		RD + ENC[a]	HFD		HFD + ENC[a]
Week	0	3	3	0	3	3
Weight (g)	420 ± 5	440 ± 20	430 ± 15	580 ± 5***	630 ± 15°°***	590 ± 15°***
Food intake (g/day)	15 ± 1	15 ± 2	15 ± 2	17 ± 1	19 ± 2	15 ± 1.3°
Weight gain (g)	–	24 ± 2	20 ± 2	–	40 ± 5***	30 ± 4**°°

Notes: Data are reported as mean ± SD (n = 4–5). ***p < .001; **p < .01 HFD vs. RD same time; °p < .05, °°p < .01 HFD + ENC vs. HFD21 (ANOVA followed by Bonferroni post test). ***p < .001; **p < .01 vs. RD same time; °p < .05, °°p < .01 vs. HFD21 (ANOVA followed by Bonferroni post test). [a]RD and HFD rats have been given 3 weeks ENC (20 mg/kg/day by gavage).

Table 3, Laboratory findings and anti-atherogenic and pro-atherogenic cytokines

	RD	RD + ENC[a]	HFD	HFD + ENC[a]
Glucose (mg/dl)	120 ± 20	115 ± 25	170 ± 30	160 ± 25
Total cholesterol (mmol/L)	3.24 ± 0.20	3.16 ± 0.08	3.68 ± 0.20*	3.20 ± 0.19°
LDL cholesterol (mmol/L)	0.88 ± 0.03	0.89 ± 0.02	1.10 ± 0.04*	0.90 ± 0.10°
HDL cholesterol (mmol/L)	2.40 ± 0.07	2.48 ± 0.03	2.10 ± 0.06**	2.29 ± 0.03°
Triglyceride (mmol/L)	1.27 ± 0.04	1.27 ± 0.16	2.07 ± 0.10**	1.44 ± 0.05°°
IL-1α[b]	100 ± 10	100 ± 10	400 ± 20***	270 ± 15§§§ °°°
IL-6[b]	100 ± 10	105 ± 10	650 ± 15***	420 ± 20§§§°°°
TNFα[b]	100 ± 20	100 ± 15	490 ± 20***	400 ± 10§§§°°
IL-10[b]	100 ± 20	100 ± 22	50 ± 15**	86 ± 15§°

[a]ENC (20 mg/kg/day by gavage). Data are reported as mean ± SD (n = 3–4).
[b]Expressed as percent changes of the baseline value [100 (%)] of RD-fed rats. *p < .05, **p < .01,
***p < .001 vs. RD; §§§p < .01, §p < .05 vs. RD + ENC; °p < .05, °°p < .01, °°°p < .001 vs. HFD (ANOVA followed by Bonferroni post test).

3.2.3 ENC reduces HFD increased metabolic and inflammatory markers

ENC contrasted the increase in metabolic and inflammatory markers in HFD. In rats fed with RD, ENC treatment did not affect glucose, triglycerides, total, LDL, and HDL-cholesterol. HFD increased triglycerides ($p < .01$), total and LDL cholesterol ($p < .05$) and decreased HDL cholesterol ($p < .01$); this effect, however, was reverted by ENC. Glucose values were not affected neither by HFD or HFD plus ENC treatment. In the HFD group, ENC decreased total cholesterol serum levels ($p < .05$) and LDL levels ($p < .05$) (Table 3). HDL cholesterol was increased ($p < .05$) by ENC in HFD group. Triglycerides were increased by about 63% in HFD with respect to RD rats ($p < .01$); interestingly, this increase was significantly reverted by ENC ($p < .01$) (Table 3). The antiatherogenic cytokines IL-6, IL1α and TNF-α were much higher in HFD rats than in RD rats. In the former group, however, 3 weeks of treatment with ENC significantly reverted this increase. Interestingly, the proatherogenic IL-10 was significantly lower in overweight animals ($p < .001$ vs. RD) and ENC restored IL-10 plasma content to values closer to RD rats. Finally, ENC did not affect cytokines plasma levels in RD-fed rats (Table 3).

3.2.4 ENC reduces oxidative stress in HFD heart and aorta

ENC decreased HFD-induced oxidative stress in heart and aorta. In heart tissue, which seemed less sensitive to oxidative stress induced by HDF, ENC restored the total antioxidant capacity after HFD (Figure 4). Also, the aorta tissue from HDF fed rats showed a lower ferric reducing capacity, confirming the presence of an oxidative stress status, which however was strongly recovered by ENC (Figure 4). Interestingly, ENC was able to improve the ferric reducing capacity of both aorta and heart tissue of RD fed rats.

4- Discussion

A high-fat diet leads to the so-called metabolic syndrome, an unhealthy confluence of conditions that may include insulin resistance and high blood sugar, high levels of cholesterol and triglycerides in the blood, hypertension, and cardiovascular disease. High-fat diets have been used for many decades in rodents to resemble the human metabolic syndrome and its complications, cardiac hypertrophy, cardiac dysfunction and fibrosis, myocardial necrosis, and hepatic steatosis (Aguila & Mandarim-de-Lacerda, 2003; Buettner, Parhofer, & Woenckhaus, 2006; Kobayasi et al., 2010; Woods, Seeley, Rushing, D'Alessio, & Tso, 2003). Patients suffering from metabolic syndrome have a clinical picture determined by vascular phenomena, inflamma- tion, and oxidative stress affecting several organs. Natural substances can play a key role in disease prevention; they are helpful if associated with conventional therapy in outgoing disease. In fact, they affect a broad-spectrum of molecular networks in case of risk factor such as overweight or pre-obesity, and act on known molecular targets with the same natural pleiotropic substances in case of a more severe disorder such as obesity (Farzaei et al.,

2019). This fits with the proto- col used in the present study, in which rats were fed with HFD for 10 weeks to induce the so-called metabolic syndrome and then orally treated with ENC for 3 weeks to verify whether the natural extract could revert some of the effects caused by the high-fat diet.

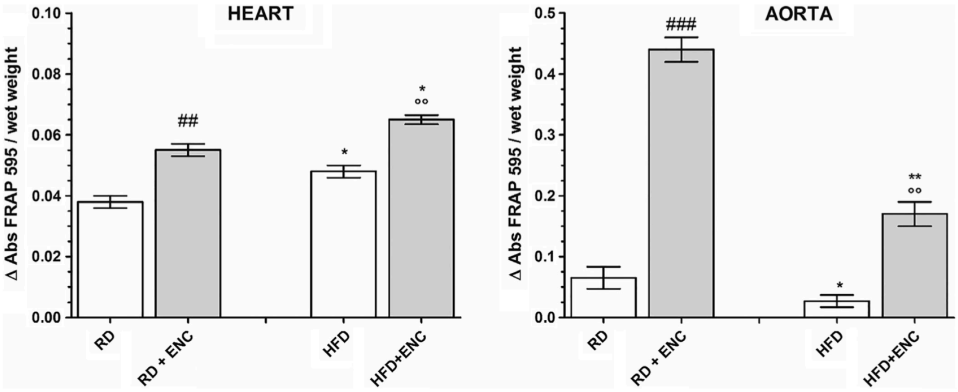

Figure 4, Effects of ENC on heart and aorta tissue oxidative stress of RD- or HFD-fed rats, by FRAP assay in rats with RD and HFD without or with ENC. Data are reported as mean ± SD (n = 3–4). *p < .05 vs. RD; ##p < .01, ###p < .001 vs. RD; §p < .05, §§ p < .01 vs. RD + ENC (ANOVA followed by Bonferroni post test)

The results showed that ENC reduces rats' weight by 25% and this completely match with the purpose of weight reduction for the prevention of cardiovascular diseases, being overweight and espe- cially obesity important risk factors (International guidelines of the for the prevention of atherosclerosis). Interestingly, it was demonstrated also that ENC causes a 30% reduction in triglycerides, an effect com- parable to that elicited by berberine, that reduce them by 29 and 35% respectively, following oral administration in hypercholesterolemic subjects (Kong et al., 2004). High levels of triglycerides (>150 mg/dL) are considered an independent coronary risk factor, which becomes particularly critical if associated with reduced HDL cholesterol (<40 mg/dL) or as part of the so-called metabolic syndrome.

Our results are consistent with the following reports: triglycerides are an independent risk factor for cardiovascular disease also by genetic mutations and Mendelian randomization; there is evidence from randomized clinical trials that lowering triglycerides reduces clinical events (Holmes, Ala-Korpela, & Smith, 2017) and finally, there is evidence of the metabolic relationships between the triglyceride- and cholesterol-rich ApoB lipoproteins (Sniderman et al., 2018).

The objectives of EURODIET [Nutrition and lifestyle modifications in the prevention of cardiovascular disease are strongly highlighted in the recent recommendations of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS)] (Mach et al., 2020) as well as WHO guidelines for the assessment and management of cardiovascular risk (WHO, 2007) stress how the reduction of these parameters is crucial for reducing cardiovascular risk. A healthy diet based on a correct use of foods, in fact, might lead to a decrease in cholesterol and triglycerides up to 10%, which in turn is compatible with a 20% reduction in the cardiovascular risk. In the light of these considerations, we can speculate that the ability of ENC to lower body weight and triglycerides might constitute interesting and clinically relevant properties.

In the present study, we have shown that ENC can counteract the cardiovascular and metabolic alterations induced by HFD in rat.

Similar cardiovascular effects are observed both in in vitro, due to C. sativa Mill-tannin extract and in ex vivo studies, possibly due to intestinally absorbed ENC derivatives.

In fact, tannins and ellagitannins, once ingested, provide pharmacological effects both locally and systemically (Serrano, Puupponen-Pimia, Dauer, Aura, & Saura-Calixto, 2009). In the small intestines, ellagitannins are hydrolyzed and partly absorbed (Espin et al., 2013) and reach the colon where they are metabolized by the microbiota (Marin, Miguelez, Villar, & Lombo, 2015) to urolithins (Larrosa et al., 2010). Urolithins enter the enterohepatic circulation system and may be recovered in blood, urine, and tissues (Cerda, Espin, Parra, Martinez, & Tomas-Barberan, 2004). The heart presents high capacity to accrue urolithins (Gasperotti et al., 2015), that reduce the inflammatory state of cardiac tissue and prevent the occurrence of the early signs of cardiac dysfunction, in a rat model of streptozotocin induced diabetes (Savi et al., 2017). Moreover, Urolithin A reduces myocardial injury in in vitro and in vivo models of ischemia/reperfusion (Tang et al., 2017).

In mouse, HFD induces obesity, increases systemic inflammation (Xu et al., 2003), and worsens cardiac contractile dysfunction through alteration of Ca2+ homeostasis (Xu et al., 2003), increases cardiac oxy- gen needing, with consequent reduced cardiac efficiency (Cole et al., 2011). We confirmed that HFD, compared with RD, increased serum parameters and clinical signs of metabolic disease in rats and that ENC contrasted this metabolic state. Control of food intake results by a balance between the central nervous system and the enteric nervous system (Cuomo & Sarnelli, 2004) and proanthocyanidins delay the gastric emptying and reduce intestinal motility (Serrano et al., 2016). In a previous study (Budriesi et al., 2018), we have shown that ENC decreases the gastric tone in HFD rats, thus decreasing gastric emptying. Since gastric motility and emptying regulate intestinal exposure of nutrients, it controls satiety and plays a role in the regulation of body weight (Janssen et al., 2011). Moreover, ENC reduces spontaneous and induced contractility in iso- lated guinea-pig ileum and proximal colon (Micucci et al., 2017), suggesting that ENC can decrease food intake through the control of digestive contractility: consistently, ENC reduced food intake and weight gain in HFD rats in the present study.

Cardiovascular disease is closely linked to the development and progression of systemic inflammation (Tousoulis et al., 2016). ENC reduces plasma cytokines levels with pro-atherogenic role, such as TNF-α, IL-1, and IL-6 and increased cytokines with antiatherogenic activities, such as IL-10: ENC therefore can decrease the progress of atherosclerosis by serum lipid regulation and alteration of markers of inflammation.

We have shown that ENC antagonized intestinal M3-cholinergic receptors in a noncompetitive reversible manner in guinea-pig ileum and proximal colon smooth muscle (Budriesi et al., 2010) and that ENC improved cardiac function, inducing transient negative chronotropic and positive inotropic effects when studied in the guinea-pig atria ex vivo (Chiarini et al., 2013). Therefore, we speculated that ENC may act differently in intestinal M3, the most expressed subtypes mus- carinic receptors on the luminal side of epithelial cells, than in cardiac M2 cholinergic receptors, the most expressed on cardiomyocytes. Therefore, in the experiments here presented, we considered M2- receptor as a target to test the cardiac response.

The activities of autonomic nervous systems are often altered in various cardiovascular disease and obesity (Rodriguez-Colon, Bixler, Li, Vgontzas, & Liao, 2011). HFD has been found to reduce parasym- pathetic activity (Hartnett, Gao, Schnack, & Li, 2015) and to induce a similar elevation in heart rate in mice, suggesting a potential impair- ment in the autonomic control of the cardiovascular function (Bruder- Nascimento, Ekeledo, Anderson, Le, & Belin de Chantemèle, 2017).

These data are consistent with the human literature reporting increased heart rate in obese patients (Nault et al., 2007). Further- more, cholinergic receptor function is reduced since carbachol and atropine had a lower impact. ENC treatment restores parasympathetic cholinergic receptor function as well as vasorelaxation of aorta, as demonstrated by ex vivo experiments. The possibility that a down- regulation of M2 receptors occurs in the heart of HFD fed rats should be taken into consideration. Data from the literature suggest that in rat atrial cardiomyocytes, insulin induces a concentration- and time-dependent decrease in the expression of M2 mRNA, suggesting that hyperinsulinemia could directly contribute to cardiovascular morbidity in obese patients (Pathak et al., 2005). As HFD rats are characterized by five-fold increased insulin levels (Beaudry, D'souza, Teich, Tsushima, & Riddell, 2013), it is reasonable to hypothesize that insulin (but also many other factors as well) might drive a downregulation of muscarinic receptor expression at the heart or other tissues. As mentioned before, HFD has been found to reduce parasympathetic activity, which was accompanied by a trend toward a reduction in atrial M2 receptors in mice (Hartnett et al., 2015). Lin, Wu, Chang, Cheng, and Tong (2018) showed that a decrease in bladder muscarinic receptor expressions was accompanied by reduced contractile responses to acetylcholine and bethanechol in HFD rats. Authors hypothesized that HFD feeding probability affected first the bladder muscarinic receptors and then the smooth muscle. Despite differences in the strain (mice vs. rat), age, duration of the treatment and composition of the HFD and analyzed tissues might hamper a correct comparison of the data, the hypothesis that ENC could restore muscarinic function by remodulating M2 receptors expression cannot be ruled out.

HFD decreases left atrium inotropy, increases right atrium chronotropy, and reduces the contraction of aorta in response to high K+ concentration.

In both groups of animals, however, a decrease in chronotropic activity was observed upon ENC treatment, although it did not reach statistical significance. This was probably due to urolitins rather than ENC itself. As the heart presents high capacity to accumulate urolithin, and in consideration of its cardiac-preventing functional impairment activity (see above), it is possible that a longer time of ENC treatment is needed to achieve significant cardiac urolithins con- tent and thus significant results in term of chronotropic activity.

Furthermore, cholinergic receptor function is reduced since carbachol and atropine have a lower impact. ENC treatment restores parasympathetic cholinergic receptor function as well as vasorelaxation of aorta, as demonstrated by ex vivo experiments.

Important to note that some components of ENC, in particular GA block the AT1 receptor and this, in turn may improve cardiac function in obesity (Jin et al., 2017; Kang et al., 2015). Moreover, GA can decrease blood pressure in SHR owing to both an endothelium- dependent vasorelaxant effect and to ACE-inhibiting properties (Wang, Jiang, Wang, & Yao, 2007). The modulation exerted by GA on AT1 or ACE activity, however, occurs at concentration two–three orders of magnitude higher than that used in the present study. Despite it is unlikely that GA might reach blood or brain level sufficiently high to interact with RAAS system after oral administration of ENC. Therfore, the possibility of the involvement of RAAS system in the ENC induced effects cannot be ruled out.

Our findings support evidence that ENC enforces negative inotropy effects of Nifedipine in atria. Moreover, in terms of spasmolytic effect in K+ precontracted aorta, Nifedipine is more effective than in atria obtained by ENC fed rats, consistent with the observed vasorelaxant potency of urolithins (Van Rymenant et al., 2017).

In conclusion, the present results showed that upon ENC treatment of HFD rats, metabolic dysfunction is partially reverted in terms of body weight and triglycerides reduction, suggesting that

it might be comparable to many substances already marketed to reduce cholesterol and triglyceride levels (Kong et al., 2004). Moreover, ex vivo and in vitro results demonstrated that ENC restores cardiac cholinergic muscarinic receptor function and exert spasmolytic effect on aorta, suggesting an interesting potential protective effect also at vascular level. Finally, in HFD, ENC decreases spontaneous food assumption, upregulates hepatic antioxidant enzymes and increases the liver oxidative defenses, restoring liver phase I and II enzymes activity, reduces intestinal oxidative stress, and restores the intestinal motor function (Budriesi et al., 2018), as depicted in Figure 5.

Taken together, the metabolic and gastrointestinal findings, associated to the observed cardiovascular results in the present study, highlight that ENC can be a promising nutraceutical tool in obesity.

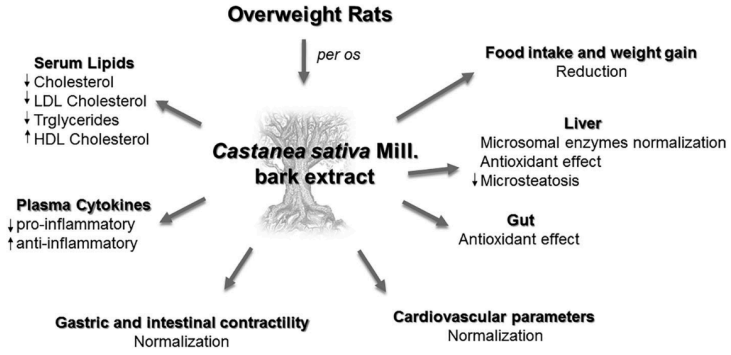

Figure 5, Overview of ENC activity in fat induced obese rats

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