Grape-seed procyanidins prevent the cafeteria diet-induced decrease of glucagon-like peptide-1 production

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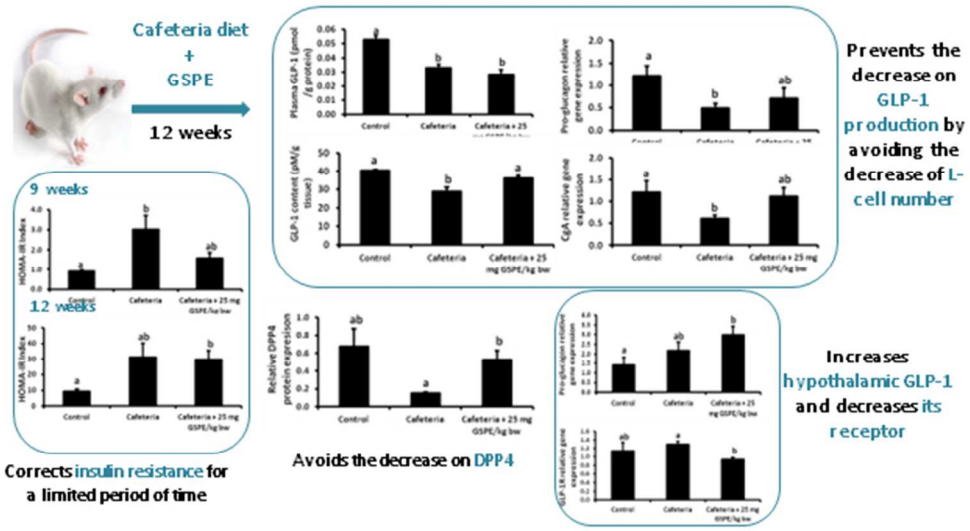

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Running title: GSPE modulates GLP-1 production

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Abstract

Grape-seed procyanidins (GSPE) have been reported to improve insulin resistance in cafeteria rats. Since glucagon-like peptide-1 (GLP-1) is involved in glucose homeostasis, the preventive effects of GSPE on GLP-1 production, secretion, and elimination were evaluated in a model of diet-induced insulin resistance. Rats were fed a cafeteria diet for 12 weeks and 25 mg of GSPE/kg of body weight was administered concomitantly. Vehicle-treated cafeteria-fed rats and chow fed rats were used as controls. The cafeteria diet decreased active GLP-1 plasma levels, which is attributed to a decreased intestinal GLP-1 production, linked to reduced colonic enteroendocrine cell populations. Such effects were prevented by GSPE. In the same context, GSPE avoided the decrease on intestinal dipeptidyl-peptidase 4 (DPP4) activity, and modulated the gene expression of GLP-1 and its receptor in hypothalamus. In conclusion, the preventive treatment with GSPE abrogates the effects of the cafeteria diet on intestinal GLP-1 production and DPP4 activity.

Keywords: GSPE/ GLP-1/ DPP-4/ insulinemia

Introduction

Procyanidins, a class of phenolic compounds, have been shown to improve glucose homeostasis in several models of glucose-homeostasis disruption [1]. Chronic corrective treatment using grape-seed procyanidins ameliorates insulin resistance in a cafeteria diet model [2]. These effects might be partially explained by their insulin-like effect on insulin-sensitive cell lines [3,4] as well as through their effects on pancreatic β-cell function, and by decreasing insulin secretion and production and lipid accumulation in the pancreas that is induced by the cafeteria diet [5]. Moreover, we have previously reported that the chronic treatment of healthy rats with GSPE increases the insulin/glucose ratio after oral glucose administration compared to intraperitoneal glucose infusion, suggesting an incretin-like effect [6]. However, the effect of procyanidins on incretins in an insulin resistance context has not been described yet.

The main incretin hormones are glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), which are secreted by the intestinal L- (ileum and colon) and K-cells (duodenum and jejunum), respectively [7]. Plasma levels of GLP-1 have been reported to be reduced in type 2 diabetic patients [8,9] and in mice with high-fat diet (HFD)-induced insulin resistance [10], and exogenous infusions of GLP-1 were shown to improve glycemia [11,12] and insulin resistance [13] in these contexts. Along this line, therapies, including the use of bioactive food components, which would enhance the action of GLP-1, are of great interest to diminish the development of these pathologies. An *Ilex paraguariensis* leaf extract, which is rich in the phenolic compound 3,5-*O*-dicaffeoyl-*D*-quinic acid, was previouslyshown to improve glycemia and insulinemia in HF-fed mice, a result that was correlated with increased plasma levels of active GLP-1 [14,15]. Moreover, Dao et al. reported that chronic treatment with

resveratrol, a polyphenolic compound found in red grapes, improves portal and intestinal levels of active GLP-1 and pro-glucagon gene expression in HF-fed mice [10].

The aim of this study was to assess whether preventive treatment with GSPE can counteract the effects of a cafeteria diet on glucose homeostasis, focusing on the GLP-1 system.

Materials and Methods

Reagents

The grape seed procyanidin extract (GSPE) was obtained from *Les Dérivés Résiniques et Terpéniques* (Dax, France) and was previously fully characterized by our research group [16].

Glucose plasma concentrations were assayed using an enzymatic colorimetric kit (GOD-PAP method from QCA, Tarragona, Spain). Insulin plasma concentrations were determined using Rat Insulin ELISA/Ultrasensitive Rat Insulin ELISA (Mercodia, Uppsala, Sweden). Total GIP and active GLP-1 plasma levels were measured using Rat/Mouse GIP (total) ELISA and GLP-1 (active) ELISA kits, respectively (Millipore, Madrid, Spain).

DPP4 activity was determined using the colorimetric substrate H-glycylprolyl-pnitroanilide p-tosyalte (Bachem, Bubendorf, Switzerland).

Animal experimental procedures

Female Wistar rats were purchased from Charles River Laboratories (Barcelona, Spain), housed in animal quarters at 22 ºC with a 12-h light/ 12-h dark cycle and maintained for 1 week in quarantine. The animals were divided into the following three groups (8

63 animals/group): a control group fed with standard chow (Panlab, Barcelona, Spain)
64 (CON); a group fed with a cafeteria diet (bacon, biscuits with pâté, muffins, carrots and
65 milk with sugar) and water plus the standard diet and were vehicle-treated (CAF); and a
66 group fed with the cafeteria diet and water plus the standard diet and treated with 25 mg
67 GSPE/kg of body weight (bw) per day (CAF + GSPE). The treatments were
68 administered by voluntary feeding of the GSPE dose using sweetened condensed milk,
69 diluted 1:10 with water, as a vehicle. Every day, the food was withdrawn at 9 a.m. and
70 was replaced at 4 p.m.

71 At the 8th week of treatment, the rats were fasted overnight and an oral glucose
72 tolerance test (OGTT) was performed. Briefly, 2 g glucose/kg bw was dissolved in
73 water, administered by oral gavage, and tail blood samples were taken at 0, 15, 30 and
74 120 min after the administration of the glucose load. The plasma glucose and insulin
75 levels were measured.

76 At the 9th week of treatment, the rats were fasted overnight, tail blood samples were
77 taken and insulin levels were measured.

78 After 12 weeks of GSPE treatment, the overnight fasted animals, were anesthetized with
79 50 mg of pentobarbital/kg body weight and sacrificed by bleeding. The blood was
80 collected and treated with a commercial DPP4 inhibitor (Millipore, Madrid, Spain) and
81 a serine protease inhibitor, Pefabloc SC, (Roche, Barcelona, Spain) to prevent the
82 inactivation of active GLP-1 and ghrelin, respectively. The animal tissue specimens
83 were immediately frozen in liquid nitrogen and stored at -80 ºC until further analysis.

84 All of the procedures were approved by the Experimental Animal Ethics Committee of
85 the Universitat Rovira i Virgili (permission number: 4250).

Intestinal GLP-1 extraction

Active GLP-1 was extracted from colon as previously described [17]. Briefly, colon samples were homogenized using an ethanol-acid solution (100% ethanol-sterile water-12 M HCl, 74:25:1), placed for 24 h at 4 ºC and centrifuged at 2,000 x g for 20 min at 4 ºC. Supernatants were collected, diluted in 1X PBS and stored at -80 ºC until further analysis. Active GLP-1 was measured following the manufacturer's instructions.

Measurement of DPP4 activity

DPP4 was extracted from rat intestine as previously described [6]. Briefly, intestine samples were homogenized using lysis buffer (PBS containing 100 KIU/mL aprotinin and 1% Triton X-100), centrifuged at 1,000 x g at 4 ºC for 10 min to eliminate the cellular debris, and centrifuged twice at 20,000 x g at 4 ºC for 10 min. The supernatants were stored at -80º C until further analysis.

To determine the activity of DPP4 in the intestinal lysates and the rat plasma samples, the specimens were incubated with 0.2 mM H-glycylprolyl-pnitroanilide p-tosyalte in Tris-HCl buffer at 37 ºC, and the release of p-nitroanilide absorbance was measured every min at 405 nm for 30 min.

Western Blot

Protein was extracted from the intestine using RIPA lysis buffer (15 mM Tris-HCl, 1% Triton X-100, 0,1% SDS, 167 mM NaCl and 0.5% Na-deoxycholate) with a protease inhibitor cocktail (diluted 1:1000, Sigma-Aldrich) and 1 mM PMSF. The total protein levels of the lysates were determined using the Bradford method [18]. The proteins were loaded and run on 10% SDS-polyacrylamide gels. The samples were transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA), blocked at room

temperature using 5% (w/v) non-fat milk in TBST buffer (Tris-buffered saline with 0.2% (v/v) Tween-20) and incubated overnight at 4 ºC with rabbit anti-β-actin antibody (Sigma-Aldrich) or anti-CD26 (Abcam, Cambridge, UK). After washing with TBST, the blots were incubated at room temperature with peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Buckinghamshire, UK). The blots were washed thoroughly in TBST, followed by TBS after immunoblotting, and the immunoreactive proteins were visualized using the ECL Plus Western blotting detection system (GE Healthcare). Densitometric analysis of the immunoblots was performed using ImageJ 1.44p software; all proteins were quantified relative to the loading control.

Quantitative RT-PCR

The total RNA from the hypothalamus was extracted using an RNAeasy Kit (Qiagen, Hilden, Germany), and the total RNA from the duodenum and colon was extracted using the TRIzol reagent following the manufacturers' protocols cDNA was generated using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain). Quantitative PCR amplification and detection were performed using the following TaqMan assay-on-demand probes (Applied Biosystems): Rn00562910_m1 for DPP4, Rn00571500_m1 for GIP, Rn00562293_m1 for proglucagon, Rn01460420_g1 for PYY, Rn00572200_m1 for Chromogranin A (CgA), and Rn00562406_m1 for GLP-1 receptor (GLP-1R). β-actin was used as the reference gene (Rn00667869_m1). The relative mRNA expression levels were calculated using the ΔΔCt method.

Data analyses

AUC and HOMA-IR were calculated as previously described by Ou et al. and Matthews et al., respectively [19,20].

The results are expressed as the mean values ± SEM. The effects were assessed by ANOVA and Student's t-test. All calculations were performed using SPSS software (SPSS, Chicago, USA).

Results

GSPE limits the cafeteria diet-induced decrease in intestinal GLP-1 biosynthesis

To analyze whether a preventive treatment with procyanidins modulates the incretin levels in cafeteria diet-fed rats, basal GIP and GLP-1 levels in plasma were assessed in rats fed a cafeteria diet for 12 weeks simultaneously with 25 mg of GSPE/kg of bw. As shown in **figure 1a**, cafeteria-fed rats exhibited reduced plasma levels of active GLP-1. GSPE did not alter the active GLP-1 levels compared to the vehicle-treated cafeteria-fed rats. Total GIP levels were neither modified by the cafeteria diet nor by the GSPE treatment (**figure 1b**).

To assess the effect of the cafeteria diet and GSPE treatment on incretin biosynthesis, we measured the GLP-1 levels and pro-glucagon gene expression in colon, as well as GIP gene expression in duodenum. As shown in **figure 2**, the colon levels of GLP-1 were decreased by the cafeteria diet by approximately 30% compared to the control diet (**figure 2a**), a reduction that was accompanied by a significant down-regulation of the pro-glucagon gene expression (**figure 2b**). The GSPE treatment prevented the decrease in colon levels of GLP-1 produced by cafeteria-diet, although it did not reach control levels (**figure 2a**). This effect was also observed for the expression of the pro-glucagon gene as shown in **figure 2b**. GIP gene expression in the duodenum was unaffected either by the cafeteria diet or the GSPE treatment (0.93 ± *0.17* and 0.89 ± *0.10*, respectively, versus control (1.03 ± *0.09*) ($P > 0.05$)).

GSPE prevents the cafeteria diet-induced decrease of enteroendocrine cells in the colon

To determine whether the reduction in GLP-1 is due to a modulation of GLP-1 gene expression or to a general effect on GLP-1 producing cells, we tested the effects of the cafeteria diet and GSPE on PYY, another hormone that is also secreted by intestinal L-cells. We evaluated PYY gene expression, and as shown in **figure 2c,** it was significantly down-regulated due to the cafeteria diet, whereas GSPE treatment partially blocked this effect.

Next, we analyzed the gene expression of CgA, a marker of endocrine differentiation. **Figure 2d** shows that the expression of CgA gene in colon was significantly decreased in the CAF group, whereas GSPE treatment prevented the effect of the cafeteria diet, similar to the effect observed for GLP-1 and PYY. The expression of CgA gene in the duodenum was also assessed, but it was unaffected by the cafeteria diet or by the GSPE treatment (0.88 ± *0.11* and 1.13 ± *0.27*, respectively, versus control (1.16 ± *0.22*) ($P > 0.05$)).

GSPE modulates intestinal DPP4

To assess whether the cafeteria diet and the GSPE treatment could also modify the enzyme responsible for incretin degradation, DPP4, the plasma and intestinal levels of DPP4 activity were measured at the end of the treatment. As shown in **figure 3a**, plasma DPP4 activity was slightly but significantly decreased by the cafeteria diet, whereas it was unaffected by the GSPE treatment. Intestinal DPP4 activity was also reduced by the cafeteria diet, whereas in contrast to the effects in plasma, the simultaneous GSPE treatment prevented this decrease (**figure 3b**). To analyze whether the preventive effect elicited by GSPE was due to a modulation of DPP4 production, its gene and protein expression were also evaluated in the intestine. As shown in **figures 3c**

and **3d**, DPP4 gene expression and protein levels were reduced by the cafeteria diet, which is consistent with what was observed in terms of its activity. GSPE prevented the cafeteria-induced decrease in DPP4 protein expression, but elicited no effect at the gene expression level.

GSPE treatment modulates the expression of GLP-1 and GLP-1 receptor genes in the hypothalamus

To evaluate the systemic preventive effects of GSPE on GLP-1 production, pro-glucagon gene expression was also assessed in the hypothalamus. As shown in **figure 4a**, the cafeteria diet tended to up-regulate pro-glucagon expression and GSPE treatment further enhanced this effect. We also evaluated the sensitivity to GLP-1 by assaying GLP-1 receptor (GLP-1R) gene expression, and we found that its expression was unaffected by the cafeteria diet, whereas it was down-regulated by the GSPE treatment compared to cafeteria-fed rats treated with the vehicle (**figure 4b**).

GSPE modulates insulinemia after 9 weeks of treatment

To assess whether a preventive GSPE treatment can modulate insulinemia, an OGTT of 2 h was performed at the 8^{th} week of treatment. As shown in **figure 5**, insulin AUC (area under the curve) was increased in cafeteria diet-fed rats, whereas a preventive GSPE treatment prevented this effect.

Moreover, fasting plasma insulin and glucose levels were measured after 9 and 12 weeks to test whether preventive GSPE treatment affects insulin resistance induced by the cafeteria diet. As shown in **figure 6a**, HOMA-IR was significantly increased after 9 weeks of cafeteria diet, whereas a simultaneous treatment with 25 mg of GSPE/kg of bw prevented this effect, resulting in a healthier HOMA-IR. However, after 12 weeks,

GSPE treatment was unable to prevent the increase in HOMA-IR induced by the cafeteria diet (**figure 6b**).

Discussion

Procyanidins from grape seed have been shown to improve insulin resistance, eliciting a corrective effect when the glucose homeostasis of the animal has been slightly disturbed by a cafeteria diet [1,2,5,21]. However, whether GSPE can act in a preventive way has not yet been described. Moreover, GSPE has been shown to elicit an incretin-like effect in healthy rats [6]. However, our understanding of the effects of GSPE on the incretin system in animals with disturbed glucose homeostasis remains limited. In this study, we developed a 12-week cafeteria diet-fed model that caused a decrease in GLP-1 plasma levels and production in the intestine, with no effects on GLP-1 production and detection in the hypothalamus. The cafeteria diet also caused a decrease in the amount of DPP4 enzyme at the plasma level and in the intestine, and the HOMA-IR clearly revealed the induction of insulin-resistance. We found that procyanidins were able to counteract several of these effects, which were predominantly found in the intestine.

Regarding GLP-1 production, the CAF+GSPE animals exhibited an increased amount of GLP-1 in colonic cells, with a simultaneous increase in GLP-1 mRNA. Similarly, Dao et al. reported that resveratrol increased the colon levels of active GLP-1, together with an increase in pro-glucagon gene expression, in mice fed with a HFD [10]. Dao et al. also suggested that the increase in GLP-1 production by resveratrol might explain its preventive effects on plasma levels of GLP-1, which are reduced in the vehicle-treated HFD-fed mice after the administration of an oral glucose load but are increased in the resveratrol-treated HFD-fed animals [10]. Actually, an acute dose of GSPE has also been found to effectively improve glucose-stimulated GLP-1 secretion in healthy rats

(submitted results). Besides, some other proanthocyanidins (i.e., berry purees [22] and the tetrameric procyanidin cinnamtannin A2 [23]) have been shown to elicit this effect.

Our results showed that treatment with GSPE also up-regulated the colon expression of the PYY gene, a hormone that has been reported to be co-expressed with GLP-1 in enteroendocrine L-cells [24]. Enteroendocrine cells actively self-renew and differentiate throughout the life of an animal [25], and procyanidins are known to modify cell proliferation [21,26,27]. Therefore, we assessed whether GSPE modulated CgA levels, a marker of endocrine cells [28,29]. We found that GSPE again counteracted the effects of the cafeteria diet. Our results suggest that the modulation on GLP-1 production is not only due to a direct modulation of its gene expression but also to changes in the amount of enteroendocrine cells, which were reduced by the cafeteria diet. There are no previous studies that have analyzed the effects of a cafeteria diet on the number of enteroendocrine cells. A high-fat diet was suggested to increase the proliferation of L-cells, causing a dysfunction and reducing GLP-1 secretion [30]. The different results might be attributed to differences in experimental conditions (type of diet, animal gender, length of the study) between studies. Moreover, that study analyzed the effects within the duodenum, whereas we found effects that were limited to the colon, the predominant location for GLP-1 production [31]. In contrast, we found that the expression of CgA and GIP genes in the duodenum, where GIP-producing cells are predominantly found [32], was unchanged in our experiment, which is consistent with the unaltered plasma levels of GIP.

The effects of GSPE on DPP4 are consistent with our previous results, which demonstrated the sensitivity of intestinal DPP4 to GSPE and a lack of GSPE effects on plasma DPP4 [6]. In this study, we found that GSPE prevents the decrease in intestinal

DPP4 activity and protein caused by a cafeteria diet. These results differ from previous observations because we had shown an inhibitory effect of 25 mg of GSPE/kg of bw after 45 days of treatment in healthy animals or after 30 days in animals with cafeteria diet-induced insulin resistance [6]. The effect that a cafeteria diet elicited on DPP4 is unclear. Several authors have reported an increase in circulating DPP4 activity in humans with obesity or type 2 diabetes [33-35] and in rats with type 1 diabetes or impaired glucose tolerance [36,37]. However, limited studies in humans have shown decreased plasma DPP4 activity in diabetic subjects [38] and unchanged activity in obese, diabetic and impaired-glucose tolerance subjects [34,39]. Taking together, these data indicate that a severe degree of hyperglycemia is required to induce an increase on circulating DPP4 activity, whereas mild hyperglycemia is insufficient to induce its increase [34,39]. Our model supports this conclusion because the cafeteria diet did not affect glycemia, which is consistent with the lack of increased DPP4. In contrast, we found a decrease that is partially prevented by GSPE. In a completely different context, the flavonoid apigenin has been reported to counteract the decrease in DPP4 activity caused by cancer in a colorectal carcinoma cell lines, an observation that is in line with the effects on DPP4 observed in our study [40]. However, there is limited information regarding DPP4 activity in the intestine. Thus, it is difficult to make further conclusions. Considering that this is the first preventive study on GSPE effects, further studies are warranted to fully assess the implications of the DPP4 inhibition in the cafeteria diet-fed model and of the opposing effects elicited by GSPE.

To evaluate the systemic preventive effects of GSPE on GLP-1, we analyzed GLP-1 and its receptor in the hypothalamus, where they are also expressed [41,42]. Previous studies have shown that brain pro-glucagon gene expression is up-regulated by HFD [43,44]. We observed a tendency to increase hypothalamic pro-glucagon gene expression.

276 However, we found that GLP-1 expression was stimulated in CAF+GSPE rats. This
277 stimulation is consistent with the reduced expression of the GLP-1R gene. Knauf et al.
278 found that blockade of brain GLP-1Rs in HF-fed mice improves insulin resistance and
279 enhances glucose utilization by muscle [43]. The available evidence suggests that enteric
280 glucose absorption activates GLP-1R-sensitive CNS networks that promote enhanced
281 glucose disposal. Furthermore, brain GLP-1R signaling controls peripheral blood flow
282 and insulin sensitivity predominantly under hyperinsulinemic/hyperglycemic
283 conditions. The relative importance of central versus peripheral GLP-1 action for the
284 control of glucose homeostasis remains unclear [42].

285 All of these results demonstrate that GSPE interacts with the GLP-1 system,
286 predominantly preventing the negative effects induced by a cafeteria diet. Therefore, the
287 CAF+GSPE animals were expected to show an improved glucose metabolism compared
288 to the CAF group. At the 8th week, we performed an OGTT and found that the GSPE
289 treatment corrected the glucose-induced increase in insulin AUC as expected. Indeed,
290 these results are consistent with the action of a GSPE corrective treatment of 4 weeks,
291 which was previously shown to improve peripheral insulin resistance at the same dose [2].
292 This effect was maintained at 9 weeks, when we observed an improved HOMA-IR due
293 to GSPE pretreatment. However, surprisingly, after 12 weeks, our results do not show a
294 clear improvement in the HOMA-IR by the GSPE treatment. This might be due to the
295 dose of GSPE, since we previously described a high variability on the insulin levels
296 after different GSPE doses. Working with healthy animals, we have previously shown
297 that this variability is not dose-dependent [45]. In fact higher GSPE doses for a longer
298 time showed an improved insulinemia and glycemia in type 2 diabetic rats [46,47]. This
299 was accompanied by an improved functionality of β-cells related to the GSPE
300 treatment, as we have previously found with lower doses [5,27].

In conclusion, we have found that a cafeteria diet induces a decrease in active GLP-1 levels by decreasing GLP-1 production in the colon, which might be attributed to a loss of enteroendocrine cells, an effect that is prevented by simultaneous treatment with GPSE. GSPE treatment also increased hypothalamic GLP-1 production, and down-regulated GLP-1Rs, opposing the effects of the cafeteria diet. This preventive action also impacts intestinal DPP4, predominantly by preventing the decrease in its activity and protein levels.

Abbreviations used

DPP4, dipeptidyl-peptidase 4; GSPE, grape seed procyanidin extract; bw body weight; OGTT oral glucose tolerance test; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; CgA, chromogranin A.

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The authors declare that they have no conflict of interest.

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Footnote:

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FIGURE CAPTIONS

Figure 1. Effect of cafeteria diet and GSPE on incretin plasma levels. Effects on plasma active GLP-1 levels (**A**), and total GIP levels (**B**) were assayed after 12 weeks of cafeteria diet and preventive treatment with 25 mg of GSPE/kg of bw. The data are displayed as the mean ± *SEM*. **a** and **b**, statistically significant differences at $P < 0.05$.

Figure 2. Effect of cafeteria diet and GSPE on hormone production and CgA gene expression in colon. Effects on colon active GLP-1 content (**A**), and gene expression of proglucagon (**B**), PYY (**C**), and CgA (**D**) were determined after 12 weeks of cafeteria diet and simultaneous treatment with 25 mg of GSPE/kg of bw. The data are displayed as the mean ± *SEM*. **a**, **b** and **c**, statistically significant differences at $P < 0.05$.

Figure 3. Effect of cafeteria diet and GSPE on DPP4. After 12 weeks of cafeteria diet and simultaneous treatment with 25 mg of GSPE/kg of bw, DPP4 activity was determined in plasma (**A**), and intestine (**B**). Intestinal DPP4 gene expression (**C**) and protein expression (**D**) were also assessed by RT-PCR and Western Blot, respectively. The data are displayed as the mean ± *SEM*. **a**, **b** and **c**, statistically significant differences at $P < 0.05$.

Figure 4. Effect of cafeteria diet and GSPE on hypothalamic GLP-1 and GLP-1R. Effects on hypothalamic gene expression of pro-glucagon (**A**), and GLP-1R (**B**) were assessed after 12 weeks of cafeteria diet and preventive treatment with 25 mg of GSPE/kg of bw. The data are displayed as the mean ± *SEM*. **a** and **b**, statistically significant differences at $P < 0.05$.

Figure 5. Effect of cafeteria diet and GSPE on insulinemia. After 8 weeks of cafeteria diet and a preventive treatment with 25 mg of GSPE/kg of bw, a 2 h-OGTT

504 was performed, insulin was analyzed at time 0, 30 and 120 min (**A**), and the AUC was
505 calculated (**B**). The data are displayed as the mean ± *SEM*. **a** and **b**, statistically
506 significant differences at $P < 0.05$.

507 **Figure 6. Effect of cafeteria diet and GSPE on insulin resistance.** Plasma glucose
508 and insulin levels were measured and HOMA-IR was calculated after 9 weeks (**A**), and
509 12 weeks (**B**) of cafeteria diet and preventive treatment with 25 mg of GSPE/kg of bw.
510 The data are displayed as the mean ± *SEM*. **a** and **b**, statistically significant differences
511 at $P < 0.05$.

Figure 1

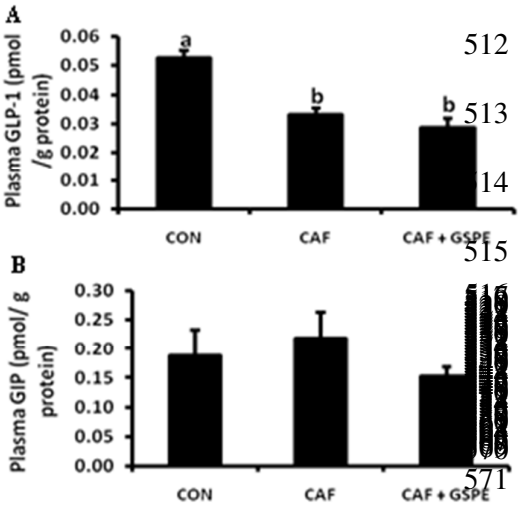
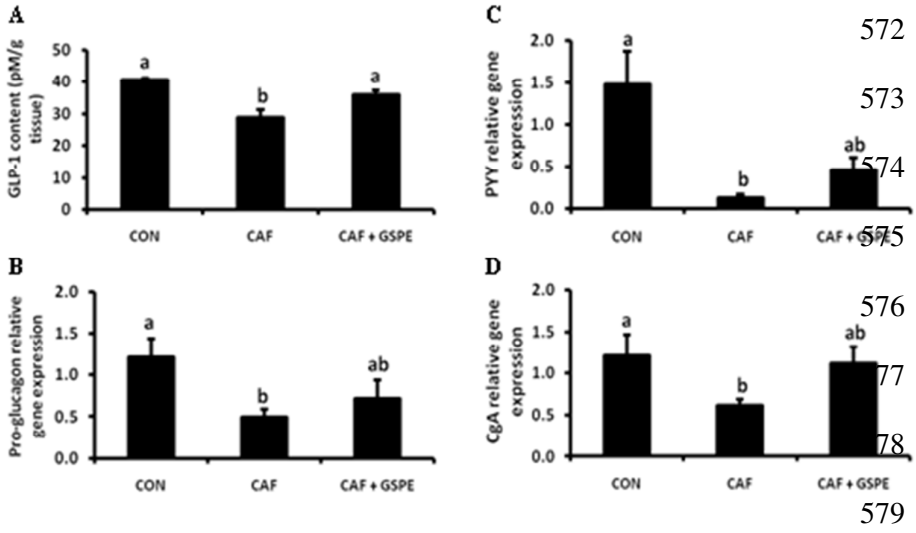

A
Plasma GLP-1 (pmol /g protein)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
a
b
b
CON
CAF
CAF + GSPE
B
Plasma GIP (pmol/ g protein)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
CON
CAF
CAF + GSPE

Figure 2



572
573
574
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578
579

Figure 3

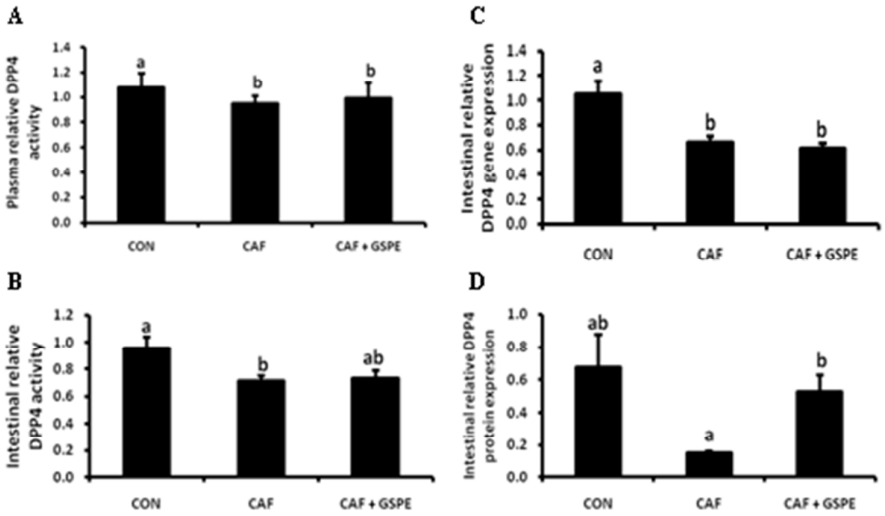

A
Plasma relative DPP4 activity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
CON
CAF
CAF + GSPE
C
Intestinal relative DPP4 gene expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
CON
CAF
CAF + GSPE
B
Intestinal relative DPP4 activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
ab
CON
CAF
CAF + GSPE
D
Intestinal relative DPP4 protein expression
1.0
0.8
0.6
0.4
0.2
0.0
ab
a
b
CON
CAF
CAF + GSPE

Figure 4

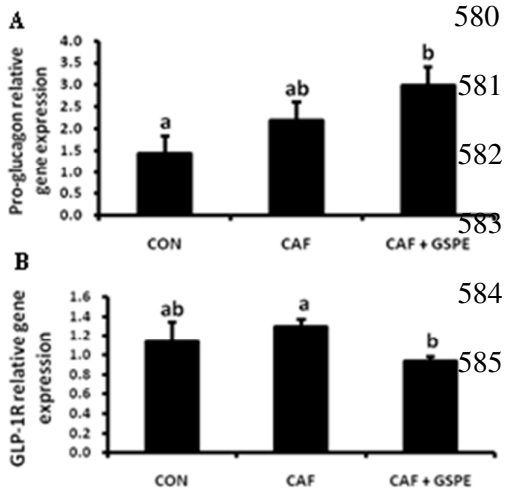

A
Pro-glucagon relative gene expression
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
b
CON
CAF
CAF + GSPE
B
GLP-1R relative gene expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ab
a
b
CON
CAF
CAF + GSPE

Figure 5

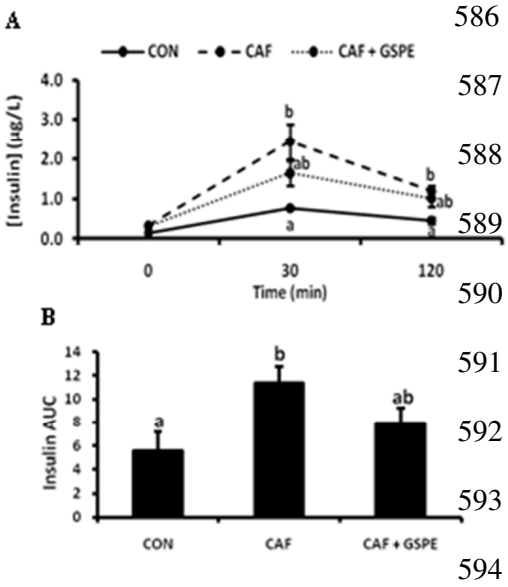
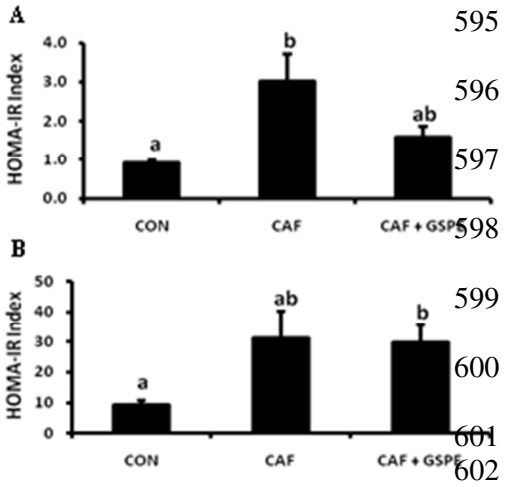
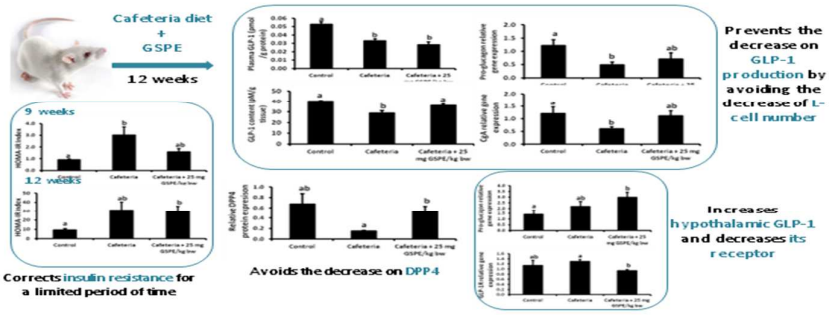

A
CON
CAF
CAF + GSPE
[Insulin] (μg/L)
4.0
3.0
2.0
1.0
0.0
b
ab
b
ab
a
a
0
30
120
Time (min)
B
Insulin AUC
14
12
10
8
6
4
2
0
a
b
ab
CON
CAF
CAF + GSPE

Figure 6


A
HOMA-IR Index
4.0
3.0
2.0
1.0
0.0
a
b
ab
CON
CAF
CAF + GSPE
B
HOMA-IR Index
50
40
30
20
10
0
a
ab
b
CON
CAF
CAF + GSPE

TOC


Cafeteria diet
+
GSPE
12 weeks
9 weeks
12 weeks
Corrects insulin resistance for a limited period of time
Avoids the decrease on DPP4
Prevents the decrease on GLP-1 production by avoiding the decrease of L-cell number
Increases hypothalamic GLP-1 and decreases its receptor