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Epicatechin and quercetin exhibit *in vitro* antioxidant effect, improve biochemical parameters related to metabolic syndrome, and decrease cellular genotoxicity in humans

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ABSTRACT

Metabolic syndrome is a condition whose incidence has been increasing around the world. It promotes a metabolic state of chronic systemic inflammation, correlated to cellular stress and genetic mutations, and subsequently with deadly chronic diseases, such as type 2 diabetes mellitus, cardiovascular diseases, and cancer. A randomized placebo-controlled study (n = 156) was conducted to determine the effects of consuming an enriched bread with 0.05% of a 1:1 mixture of (−)-epicatechin and quercetin on anthropometric and biochemical parameters of the participants. As a result, total cholesterol, LDL-cholesterol, total triglycerides, and fasting plasma glucose significantly decreased after three months of daily enriched bread consumption. Nuclear abnormalities in buccal epithelium cells also decreased (15.8 ± 3.2 down to 8.3 ± 1.0), showing a genoprotective effect. The antioxidant properties of these compounds were observed by monitoring changes in the cytoplasmic redox tone of intact Caco-2 cells expressing HyPer, a fluorescent redox biosensor. The combination of (–)-epicatechin and quercetin changes the cytoplasmic redox ambient in living cells and significantly improves biochemical parameters related to metabolic syndrome, and decreases the number of cell abnormalities in buccal epithelium cells of patients.

1. Introduction

Metabolic syndrome (MS) is a condition whose incidence worldwide has been increasing in recent years (Saklayen, 2018). It is diagnosed by the presence of at least three of the following risk factors in patients: excessive visceral fat storage, dyslipidemia, hypertension, and hyperglycemia (Mohamed, 2014). Moreover, diet is one of the leading lifestyle factors that could significantly affect, either positively or negatively, the progression of MS (Mohamed, 2014). Patients with MS present chronic systemic inflammation, a process correlated with cellular oxidative stress and genetic mutations, both pathogenic mechanisms involved in deadly chronic diseases, such as type 2 diabetes mellitus (T2DM), cardiovascular diseases (CVD), and cancer (Esser et al., 2014, Deng et al., 2016). Furthermore, obesity and diabetes are metabolic diseases commonly associated with systemic genetic damage (Erol, 2010, Leyva-Soto et al., 2018). DNA integrity might be evaluated in oral epithelium cells by the micronucleus test, a non-invasive technique that analyzes nuclear morphology to determine the genotoxic effect for a specific compound or condition (Bolognesi et al., 2013).

Antioxidant compounds exert their beneficial properties, such as

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regulating glucose metabolism and improving lipid profile by lowering inflammatory cytokine levels and regulating oxidative stress in animal models and preliminary human trials (Shi et al., 2017). Real-time monitoring of HyPer, a redox biosensor, allows the possibility to observe if the interaction of compounds with cellular components leads to a redox response. Additionally, the redox properties of cytoplasm in living cells become exposed by challenging the cells to an exogenous H_2O_2 stimulus, an experimental strategy useful to assign biologically relevant antioxidant impact (Hernández et al., 2018, Hidalgo et al., 2020). Accordingly, a compound that induces an antioxidant response in intact cells might improve oxidative stress, condition implicated in β-cell dysfunction, impaired glucose tolerance, insulin resistance, and eventually, T2DM (Gunathilaka et al., 2019).

Flavonoids have been studied due to their anti-inflammatory and antioxidant properties (Stahl et al., 2009, Belguith-Hadriche et al., 2010, Bladé, Arola & Salvadó, 2010). Although most fruits and some legumes contain flavonoids, the reported levels in eatable parts are low, varying from 4.5 mg/kg in kiwifruit to 610 mg/kg in black chocolate (Leyva-Soto et al., 2018; Stahl et al., 2009). Even though the concentration, characterization, and biological effects of flavonoids in fresh vegetables and fruits have been studied, little is known about their interactions in bakery products such as bread, which is an essential component in the daily diet of European and American populations. Besides, its properties as a food matrix allow it to be used to supplement bioactive compounds to potentially produce a beneficial effect on the health of consumers (Jing, et al., 2018).

In the present work, we evaluated the effect of consuming bread enriched with (-)-epicatechin and quercetin for three months on the anthropometric and biochemical parameters and the genoprotective effect in buccal epithelium cells of Mexican adult participants with at least three risk factors for MS. Finally, the biological antioxidant impact of (-)-epicatechin, quercetin, and the combination of both (1:1) was addressed by analyzing the biosensor HyPer response in living Caco-2 cells.

2. Materials and methods

2.1. Breadmaking

The bread was made as previously reported; the pup loaf straight-dough bread micro-baking method 10–10.03 (Lazo-Vélez, Chuck-Hernadez & Serna-Saldivar, 2015). The formulations included composite flours enriched with 0.05% or 0% of a mixture 1:1 of (−)-epicatechin (purity ≥ 90%, from a local market, Tempe, Arizona, USA) and quercetin (purity ≥ 95%, Sigma-Aldrich, Toluca, Mexico) further addressed as bread with flavonoid mixture (BF) and control bread (CN), respectively. All ingredients were incorporated in the initial mixing step, and the doughs were mixed with the predetermined amount of 25 °C distilled water using a 100–200 g dough mixer (National Manufacturing Co., Lincoln, Nebraska). Optimum water absorption and mixing times were determined by observing dough gluten development (film formation, gloss, and dough stickiness). The resulting doughs were weighed and then cut into two identical pieces before fermentation. Fermentation and baking conditions were conducted as previously reported (Lazo-Vélez, Chuck-Hernadez & Serna-Saldivar, 2015, Secretaria de Salud, 1994). Doughs were proofed for approximately 45 min before baking for 35 min in an oven (Electrolux EOG Gas single oven × 601) set at 180 °C. Upon 30 min cooling at room temperature, BF and CN bread were cut into 15 mm thick slices, packaged in sealed polyethylene bags, and stored at room temperature for 24 h for flavonoid concentration measurement.

2.2. Concentration of (−)-epicatechin and quercetin in bread samples and antioxidant determination in bread

The flavonoid content was determined in an extract by a colorimetric method using a Beckman Coulter DU 520 Spectrophotometer (Brea, CA, USA) at 510 nm, according to previous reports (Leyva-Soto et al., 2018). The extract was obtained through an 80% methanol in water (v/v) solution with A standard curve developed with catechin (Sigma-Aldrich, MI, USA). Assays were carried out in triplicate.

The capacity of bread (CN and BF) to scavenge the free radical cation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was conducted as reported before (Yu, Nanguet & Beta, 2013). Briefly, a 60 μM of DPPH in methanol solution was made. DPPH solution was added to each sample in a 1:40 proportion. Every measurement was made by triplicate. Absorbance (Abs) was measured using a Coulter DU 520 Spectrophotometer at 515 nm immediately (t = 0) and after 60 min of reaction (t = 60). The antioxidant capacity is calculated as:

$$DPPH = 1 - [Abs_{t=60}/Abs_{t=o}]$$

A Trolox standard curve was used to measure the capacity to diminish free radicals of DPPH. Based on the curve, the concentrations of samples were expressed as μmol equivalent of Trolox per gram. Assays were carried out in triplicate.

2.3. Biochemical and anthropometric parameters measurement (dependent variables) in participants and study design

A randomized, placebo-controlled, double-blind study was undertaken in Rosarito, Baja California. The Ethics Committee of Hospital General de Baja California approved the study (CONBIOETICA 2CEI01120180619), and written consent was signed in agreement for all subjects prior to enrollment in the study. Briefly, random consumers were recruited between November 2017 and May 2019. The inclusion criteria for the enrollment in the study were: Mexican nationality and of Mexican parents, being adults from 30 to 50 years old, and have at least 3 of the following risk factors for MS: triglycerides greater than 160 mg/dL, glucose higher than 100 mg/dL, HDL less than 45 mg/dL, LDL greater than 130 mg/dL, abdominal obesity (waist circumference greater than 102 cm for men or >88 cm for women), elevated blood pressure ≥ 130/≥85 mmHg, and body mass index (BMI) greater than 29. Exclusion and elimination criteria for participation in the study included the consumption of hypocholesterolemic medication and weight-loss or antihypertensive medications.

Trained research staff gave participants detailed instructions of the study, including bread storage recommendations (store at 4 °C for one week at most). They also assisted them in completing questions on dietary information. Table 1 describes the characteristics of participants.

The dietary intervention consisted of 30 g of BF or CN daily consumption for three months with a weekly follow-up. During the weekly interview, the Diet History Questionnaire II (National Institutes of Health, 2010) was applied to each participant, and the patients were checked for possible elimination criteria. Every four weeks of daily consumption of BF or CN, a blood sample was obtained from each participant to measure Total cholesterol (mg/dl), LDL-cholesterol (mg/dl), HDL-cholesterol (mg/dl), Triglycerides (mg/dl), Fasting plasma glucose (mg/dl), and Glycosylated hemoglobin HbA1c (%) using IDEXX Catalyst Dx® equipment (Madrid, Spain). Body Mass Index -BMI- (Kg/m^2) and waist circumference (cm) were also measured every four weeks with a digital weighing scale and a measuring tape. The study began with 156 participants after data cleaning (particularly for poorly completed dietary data), and all participants finished the study (n = 156).

2.4. Genotoxicity in buccal epithelium cells.

Genotoxicity in buccal epithelium cells was measured in each participant by micronuclei assay, as reported previously (Leyva-Soto et al., 2018). Briefly, buccal cavity cells were obtained during weekly interviews by scraping the cheeks with a tongue depressor. Then, the samples were transferred dropwise to pre-cleaned slides. Next, the slides

Table 1
Characteristics of the study population at recruitment.

Characteristics	n	(%)	CN	BF	Observations
Participants	156	(100)	78	78	
Sex					
Men	72	(46.16)			
Women	84	(53.84)			
Education Level (completed or in progress)					According to the information provided in the surveys conducted.
Primary School	2	(1.28)	2	0	
High School	15	(9.61)	10	5	
University	139	(89.10)	66	73	
Current smokers	58	(37.17)	35	23	According to the information provided in the surveys conducted.
Physical activity					According to the information
Inactive	47	(30.12)	27	20	provided in the surveys
Moderate active	81	(51.92)	41	40	conducted.
Active	28	(17.94)	14	14	
Obesity (BMI range)					The classification was made
Underweight (Below 18.5)	0	(0.00)	0	0	according to the world health organization (WHO).
Normal weight (18.5–24.9)	21	(13.46)	10	11	
Pre-obesity (25.0–29.9)	52	(33.33)	26	26	
Obesity class I (30.0–34.9)	64	(41.03)	32	32	
Obesity class II (35.0–39.9)	19	(12.17)	10	9	
Obesity class III (Above 40)	0	(0.00)	0	0	
Risk Factors					
Hypertension	27	(17.30)	14	13	Defined as systolic blood pressure ≥ 140 mmHg and/ or diastolic blood pressure ≥ 90 mmHg.
Diabetes	32	(20.51)	16	16	Having fasting plasma glucose ≥ 120 mg/dl (≥7 mmol/l).
Dyslipidemia	77	(49.35)	39	38	Defined as having at least one of the following anomalies: total cholesterol ≥ 190 mg/dl (≥4.9 mmol/l), Triglycerides ≥ 150 mg/dl (≥1.7 mmol/l), LDL-cholesterol ≥ 115 mg/dl (≥3.0 mmol/l), HDL-cholesterol < 40 mg/dl for men and < 46 mg/dl for women.

BF = Consumers grouped of bread enriched with 0.05% of a mixture of (−)-epicatechin and quercetin (1:1).
CN = Consumers grouped of control bread.

were air-dried and fixed with 80% methanol, stained with hematoxylin for 3–5 min, and finally with eosin for 5–8 min. Genotoxic damage was determined by observing the slides in a Zeiss Primo Star microscope (Zeiss Mexico, NL, Mexico); the cellular malformations were observed and counted. Assays were carried out in triplicate.

2.5. Real-time measurements of antioxidant effect of (-)-epicatechin and quercetin in Caco-2 cells

The cell line from human colorectal adenocarcinoma epithelial cells (Caco-2) was purchased from the American Type Culture Collection (ATCC ® HTB-37 ™), cultured in Minimum Essential Media (Sigma-Aldrich, MO, USA) with 10% fetal bovine serum (Sigma-Aldrich, MO, USA). The cells were maintained under a humidified atmosphere with 5% CO_2/air, and the culture medium was changed every 48 or 72 h. When the confluence was observed between 70 and 80%, the cultured cells were recultivated on glass coverslips. The Caco-2 cells were infected with adenoviral particles by adding a 1:100 virus dilution. Two days later, cells were ready for HyPer imaging (Belousov et al., 2006). The coverslips were mounted in an open recording chamber; the culture medium was replaced with KRH Buffer (in mM: 140 NaCl, 4.7 KCl, 20 HEPES, 1.25 $MgSO_4$, and 1.25 $CaCl_2$, pH 7.4) and supplemented with 5 mM glucose. Imaging took place in an inverted Nikon Ti microscope (Tokyo, Japan) equipped with a 40× oil objective (numerical aperture, N.A. 1.3). A xenon lamp was coupled to the monochromator device (Cairn Research Ltd., Faversham, UK), which allowed dual excitation at 420 and 490 nm. Emission over 520 nm was collected by a long-pass filter, fluorescence was digitalized by a cooled charge-coupled device (CCD) camera (Hamamatsu ORCA 03, Hamamatsu, Japan), and image analysis was performed using free micromanager software (San Francisco, CA, USA) (Edelstein et al., 2014). Data sampling was acquired every 10 s and expressed as a 490/420 ratio. For every recording, an initial lapse of 20 min was done to ensure a reliable and stable baseline, which functions as the basal value. A concentration of 5–10 μM of the flavonoid of interest (quercetin and (−)-epicatechin) was added and recorded for 15–20 min. Subsequently, two consecutive pulses of H_2O_2 were applied. The first was 50 μM, enough to evoke a moderate and transient increase in the HyPer signal, and the second was a saturating pulse of 500 μM H_2O_2, useful to determine the maximum response of the biosensor. Assays were carried out in triplicate.

2.6. Statistical analyses

Data were analyzed using MiniTab 18 and GraphPad Prism 9 (GraphPad Software Inc., CA, USA) software. The replicates of each experiment are detailed in each section. Statistical differences for mean data obtained from the present study were analyzed by two-way ANOVA and Tukey's range test. The correlation was made using MiniTab (MiniTab18 General Linear Model).

3. Results

3.1. Effect of adding (−)-epicatechin and quercetin (1:1) on flavonoid content and antioxidant capacity of bread

Bread enriched with 0.05% of a mixture of (−)-epicatechin and quercetin in 1:1 proportion to whole wheat flour (BF) was made. Flavonoid content was measured in both bread samples (CN and BF), and the results are presented in Table 2. Flavonoid and antioxidant capacity were significantly greater in BF bread than in CN bread, and the retention of added flavonoids after the baking process was 88% (Table 2). There was a color difference (appearance) of the bread containing the mixture of flavonoids (BF) compared with the control bread (CN) (Fig. 1). The information obtained on the characterization, the shelf life of the bread, and the stability of the flavonoids in the bread, has been included in a separate report dataset (Leyva-Soto, et al. under review). It was observed that flavonoid content does not vary significantly during the first seven days at 4 °C. With those results, the storage instructions for the participants were determined, where participants had to store the bread at 4 °C for a time not greater than seven days.

Table 2
Flavonoid Content in bread.

Treatment	Flavonoid content (μmol)/g	Antioxidant Capacity (μmol Trolox Equivalents/g)	Percentage of retention[¥]
BF	33.1 ± 0.11*	5.83 ± 0.07*	88%
CN	11.2 ± 0.02	3.01 ± 0.05	NA

[¥] Percentage of compounds retained, compared to those added before making the bread.
* Significative different ($p < 0.005$)/

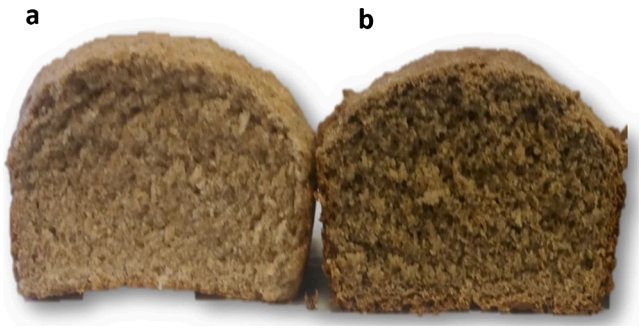

Fig. 1. Appearance of used bread for dietary intervention. (a) Control whole wheat bread (CN); (b) bread enriched with 0.05% of a mixture of (-)-epicatechin and quercetin in a proportion of 1:1 (BF).

3.2. Changes in measured parameters in the participants who consumed the enriched bread

Dietary, anthropometric, and biochemical variables measured in the present study are reported in Table 3. During the follow-up of the participants, no changes were detected in their lifestyle. Also, dietary and anthropometric variables did not significantly change by the consumption of BF in the participants. However, total cholesterol, LDL-cholesterol, total triglycerides, and fasting plasma glucose significantly improve ($p < 0.005$) after three months of daily consumption of the enriched bread compared to the beginning of the study.

3.3. Frequency in nuclear abnormalities in buccal epithelial cells.

Next, we evaluated if the enriched bread (BF) consumption diminishes genotoxicity in buccal epithelium cells. At the beginning of the study, nuclei abnormalities in the buccal epithelial cells were 15.8 ± 3.2, without statistical differences between groups consuming BF (Fig. 2a) or CN (Fig. 2b). Participants showed abnormalities of broken egg nucleus, micronucleus, and binucleus (Fig. 2c). After the dietary intervention with BF, participants significantly decreased nuclei abnormalities in buccal epithelial cells (8.3 ± 1.0, $p < 0.05$). Interestingly, fasting plasma glucose (mg/dl) and LDL-cholesterol (mg/dl) showed a significant correlation ($p < 0.0001$) with nuclear abnormalities in buccal epithelial cells (Fig. 3b and 3c).

3.4. Antioxidant effect of (-)-epicatechin and quercetin in Caco-2 cells

Finally, to determine a possible cause of the decreased genotoxicity, we evaluated the antioxidant capacity of cytoplasm in living HyPer-expressing Caco-2 cells; HyPer has a pair of cysteines that form a disulfide bond upon oxidation by H_2O_2 that changes the fluorescence spectra (Belousov et al., 2006). At steady-state, the biosensor signal reports a redox equilibrium between endogenous H_2O_2 production and disulfide reduction activity. Upon exposure with quercetin (Que), (-)-epicatechin (Epi), and the mixture of both flavonoids in 1:1 proportion (Epi + Que), the baseline signal elicited a remarkable decrease (Fig. 4a), indicating that the flavonoids affect the redox tone of cytoplasm towards a more reducing environment in a matter of seconds. In the summary of the ratio signal values, it is also observed that in the cells treated either with only quercetin or epicatechin, the addition of 50 μM H_2O_2, shifted the HyPer signal towards baseline values, whereas 500 μM H_2O_2 increase the biosensor signal significantly (Fig. 4b). When both flavonoids were simultaneously used, no significant changes were observed at 500 μM H_2O_2 (Fig. 4b). After flavonoids removal, only in cells treated with epicatechin or quercetin alone, a slight recovery in the signal could be noted. Treatment with both flavonoids induced a redox change that showed no reversibility in the temporal window observed (Fig. 5). Observations suggested that the addition of both flavonoids can modify the redox environment, making the cytoplasmic proteins more resistant to exogenous oxidation in Caco-2 cells. Moreover, the combination of both flavonoids shown to have a more antioxidant effect than each one in separated treatments.

Table 3
Dietary, anthropometric, and biochemical variables before and after 3 months of consumption of control bread or bread enriched with (-)-epicatechin and quercetin.

Variables	Intervention group	Beginning of the study Mean ± SD	End of the Study Mean ± SD
Age	BF	35.2 ± 3.4	35.6 ± 3.1
	CN	33.6 ± 3.5	34.1 ± 2.6
Participants	BF	78	78
	CN	78	78
Dietary variables			
Fruit and vegetable intake (g/d)	BF	373.9 ± 178.2	378.2 ± 187.2
	CN	375.8 ± 126.5	377.2 ± 153.2
Total energy intake (kJ/d)	BF	2210 ± 331	2220 ± 316
	CN	2208 ± 351	2210 ± 321
Total carbohydrate (%E)	BF	48.9 ± 7.2	47.8 ± 8.9
	CN	46.8 ± 9.3	51.1 ± 4.2
Added sugar	BF	8.3 ± 3.2	7.9 ± 3.5
	CN	8.2 ± 4.1	8.8 ± 3.5
Total Fat (%E)	BF	32.3 ± 6.1	35.3 ± 6.7
	CN	34.6 ± 6.3	36.2 ± 7.3
Saturated fat (%E)	BF	18.8 ± 6.1	16.9 ± 5.9
	CN	19.2 ± 5.1	20.3 ± 3.4
Unsaturated fat (%E)	BF	17.6 ± 5.8	22.8 ± 5.6
	CN	18.5 ± 2.9	20.1 ± 3.9
Anthropometric variables			
BMI (Kg/m^2)	BF	32.9 ± 2.8	31.1 ± 2.2
	CN	31.4 ± 3.2	31.2 ± 2.5
Waist circumference (cm)	BF	101.7 ± 3.5	99.4 ± 3.2
	CN	104.2 ± 4.1	103.8 ± 3.1
Biochemical variables			
Total cholesterol (mg/dl)	BF	223.3 ± 16.7	**206.2 ± 15.7***
	CN	224.1 ± 19.3	220.4 ± 15.4
LDL-cholesterol (mg/dl)	BF	149.72 ± 14.4	**123.5 ± 21.1***
	CN	148.23 ± 18.1	144.9 ± 23.4
HDL-cholesterol (mg/dl)	BF	40.5 ± 12.8	45.2 ± 8.9
	CN	40.4 ± 10.1	42.1 ± 10.2
Triglycerides (mg/dl)	BF	218.34 ± 21.9	**164.73 ± 19.25***
	CN	213.7 ± 23.6	214.91 ± 20.1
Fasting plasma glucose (mg/dl)	BF	118.03 ± 12.71	**101.23 ± 13.25***
	CN	119.31 ± 10.73	120.67 ± 10.9
HbA1c (%)	BF	5.1 ± 1.0	4.4 ± 1.1
	CN	5.7 ± 1.0	5.7 ± 0.9
Systolic blood pressure (mmHg)	BF	139.2 ± 12.3	127.8 ± 12.3
	CN	139.3 ± 21.5	138.9 ± 17.1
Diastolic blood pressure (mmHg)	BF	87.26 ± 12.8	84.31 ± 10.14
	CN	87.13 ± 10.13	86.02 ± 9.49

BF = bread enriched with 0.05% of a mixture of (−)-epicatechin and quercetin (1:1), CN = control bread. *p values for testing the differences among variables across two groups (before and after dietary intervention) by using $\chi 2$ test ($p < 0.05$).

4. Discussion

4.1. Effect of flavonoids in its consumption in enriched bread and on in-vitro bioactivity

Participants who consumed a whole wheat bread enriched with

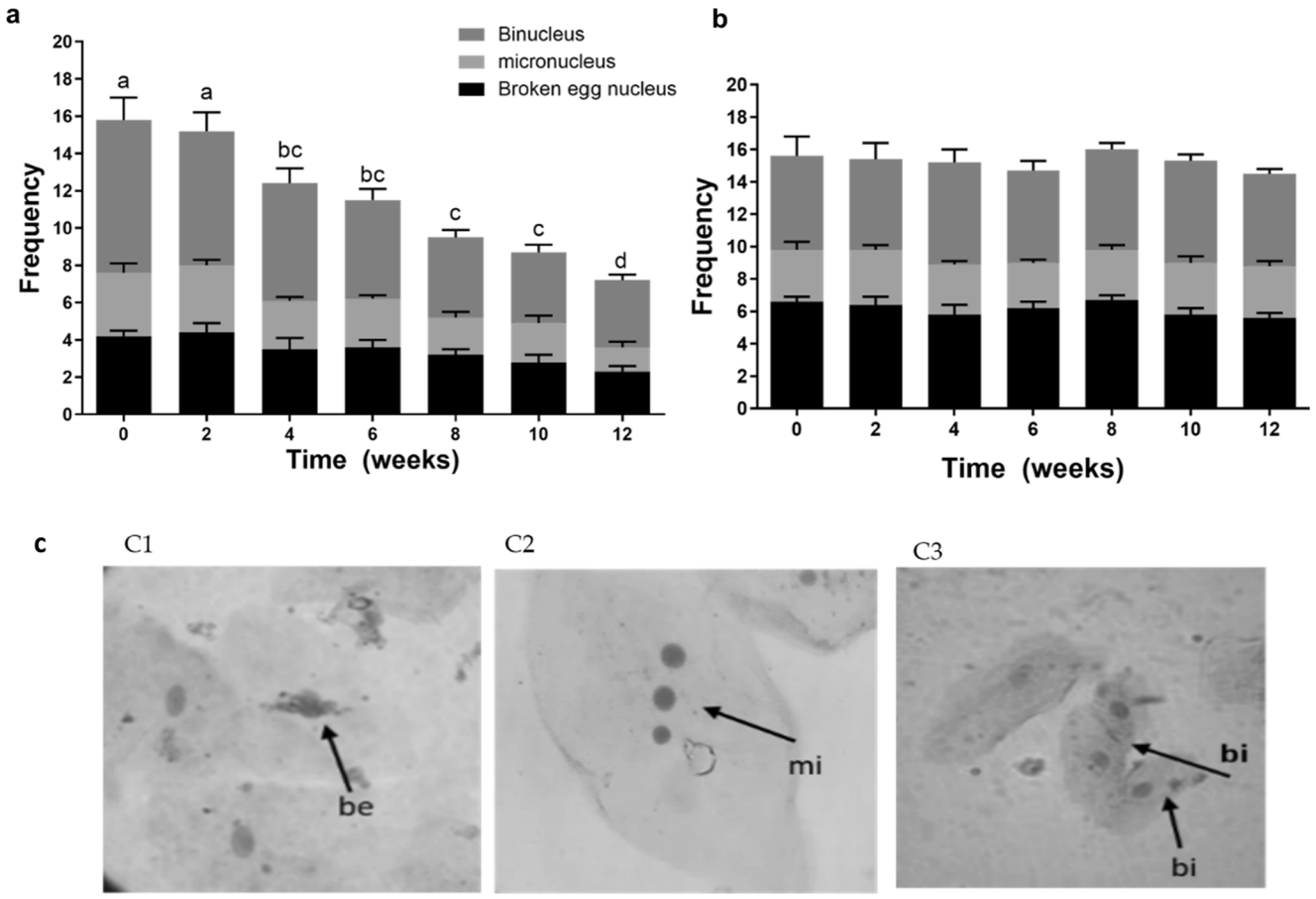

Fig. 2. The Frequency in nuclear abnormalities in buccal epithelial cells. (a) The frequency of nuclear abnormalities in participants consuming bread enriched with 0.05% of (−)-epicatechin and quercetin (1:1) measured every week, for twelve weeks; (b) The frequency of nuclear abnormalities in participants consuming control bread measured every week, for twelve weeks; (c) The most observed abnormalities were broken egg (be), micronucleus (mi) and binuclear cells (bi). Objective 40x. Significant ranges in the decrease of abnormalities in buccal epithelial cells of participants ($P < 0.005$).

0.05% of (−)-epicatechin and quercetin (1:1) (BF) for three months significantly decreased the biochemical parameters of total cholesterol, LDL-cholesterol, triglycerides, and fasting plasma glucose ($p < 0.005$), compared with participants that consumed the control bread (CN). It has been reported that flavonoids could modulate lipid homeostasis through the regulation of Liver X Receptor (LXR) via AMPK phosphorylation (Chavez-Santoscoy et al., 2014). Flavonoids participate in various biological processes, such as antioxidant and anti-inflammatory activity, inhibition of lipid peroxidation, maintains capillary integrity, and generates hepatoprotective effects (Yao et al., 2004), which in turn renders beneficial health features as observed in the biochemical parameters in our participants. Data in the present work suggested that the improvement in biochemical parameters could also be related to the genoprotective effect of flavonoids. The frequency of nuclear abnormalities in buccal epithelial cells decreased significantly (Fig. 2a), from 15.8 ± 3.2 to 8.3 ± 1.0 ($p < 0.005$) in the group that consumed the enriched bread. Interestingly, the decreasing frequency of nuclear abnormalities was significantly correlated to LDL-Cholesterol levels ($r = 0.761$, $p < 0.0001$) and fasting plasma glucose levels ($r = 0.713$, $p < 0.0001$) as shown in Fig. 3. Previous studies have also demonstrated the correlation between the frequency of nuclear abnormalities and the levels of LDL-cholesterol and fasting plasma glucose (Leyva-Soto et al., 2018, Ojelabi et al., 2018). The increase of the intracellular calcium signal is closely linked with ROS production (Higashi et al., 2017). Authors suggested that calcium ions could accumulate in the nuclei of cells, leading to the formation of micronuclei and other damages in the DNA (Higashi et al., 2017). Also, increased levels of calcium in the nucleus could cause inhibition of the DNA repair function, which is also related to the formation of micronuclei and the induction of mitotic alterations (Harding et al., 2017).

By comparing the effects of the selected compounds on the redox biosensor, it was noted that quercetin induced a faster decline in the HyPer signal than epicatechin. This cellular effect agreed with the superior chemical scavenger capacity reported for quercetin compared with epicatechin (Dueñas et al., 2010). In humans, oral administration of quercetin (100 mg/day), but not epicatechin (160 mg/day), for four weeks was effective to diminish circulating methylglyoxal levels, a precursor of advanced glycation process observed in patients diagnosed with diabetes type II and hypertension (Van den Eynde et al., 2018). A decline in the biosensor signal needs the action of cytoplasmic disulfide reduction system, since the recovery of the oxidized biosensor is sensitive to thioredoxin inhibitor and auranofin incubation, a selenium chelator (Hernández et al., 2018), suggesting that the redox effect induced by the flavonoids includes the stimulation of the antioxidant capacity of cells.

At the plasmatic membrane, ABC cassette transporters and facilitative glucose carriers have been reported as mediators of absorption of flavonoids in the epithelial intestine; these carriers are expressed in Caco-2 cells (Ai et al., 2019) that according to differential affinities (Rozanski, Studzian & Pulaski, 2019), would determine how fast the cytoplasm will be loaded with flavonoids upon a transient exposure (Omidian, Rafiei & Bandy, 2020). Inside the cells, flavonoids modify the redox state in minutes affecting the mitochondria functioning (Omidian, Rafiei & Bandy, 2020), thiol-reducing cellular system, or playing as ROS scavenging molecule (Dueñas et al., 2010, Yousefian et al., 2019), all of them are potential mechanisms of action for flavonoid beneficial effects.

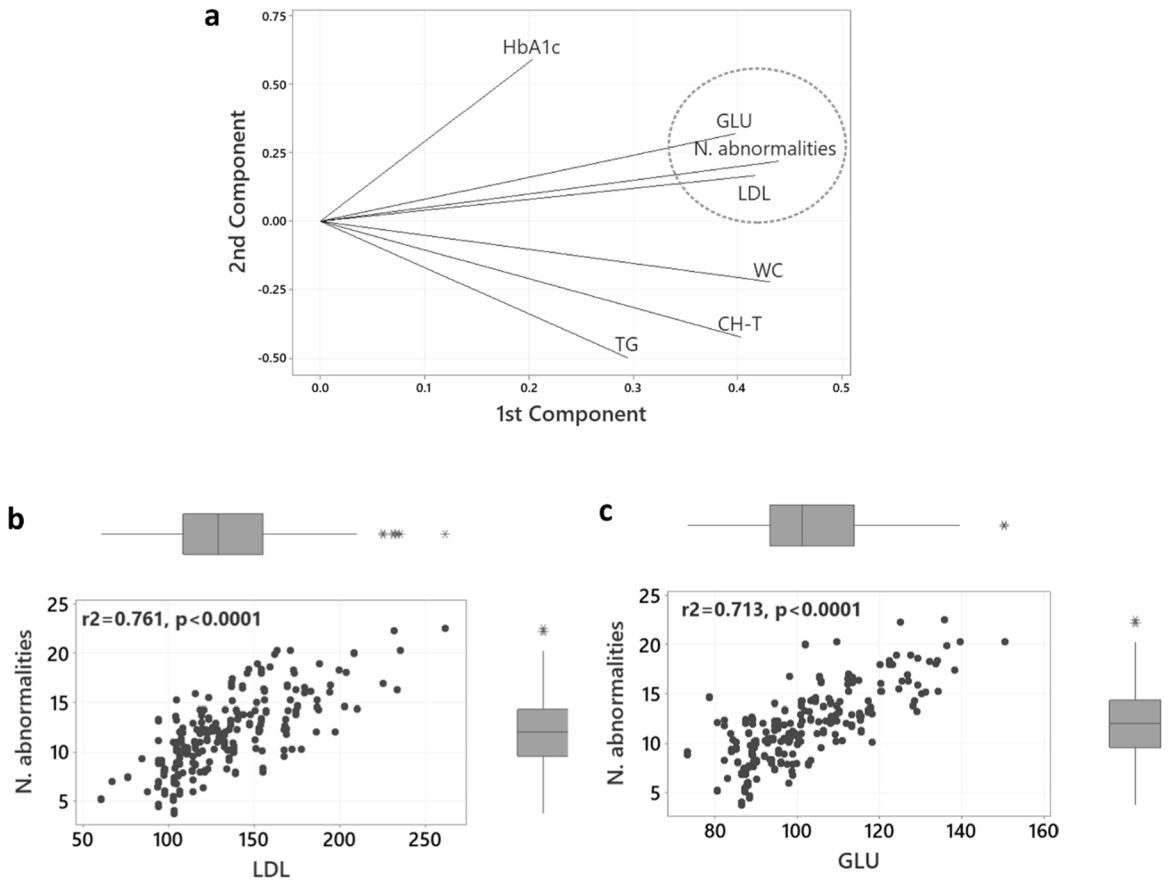

Fig. 3. Multivariate component analysis of nuclear abnormalities with biochemical parameters. (a) Complete component analysis. The variable most correlated with nuclear abnormalities were (b) LDL-cholesterol (LDL) $r^2 = 0.761$, ($p < 0.0001$), and (c) Fasting plasma glucose (GLU) $r^2 = 0.713$, ($p < 0.0001$).

A few minutes of exposure with the flavonoids induced changes in the redox properties of the cytoplasm of Caco-2 cells that last after flavonoids removal. The addition of exogenous H_2O_2 serves as an oxidative challenge to know how resistant this environment is to protein oxidation. The present work indicates that cell treatment with quercetin and epicatechin induced protection to H_2O_2-induced protein oxidation. Moreover, the combination of these two compounds showed an additive effect, suggesting that each flavonoid recruits different mechanisms to modify the antioxidant capacity of cells. Alternatively, Chung et al. (2018) demonstrated that a 2 h preincubation with quercetin or fisetin in Caco-2 cells, at similar concentrations used in this work, increased epicatechin absorption competing for catechol-O-methyl-transferase, an enzyme responsible for excretion of catechol-containing flavonoids (Salucci et al., 2002). This interaction between both flavonoids could explain the higher levels of circulating epicatechin observed in rats being co-administered with quercetin compared to epicatechin alone (Van den Eynde et al., 2018).

Differential interactions of diet flavonoids with carriers located at the plasma membrane of intestinal epithelial cells have physiological relevance and offer rational design to food technology.

4.2. *Effect of added flavonoids on flavonoid content and antioxidant capacity.*

Whole wheat bread enriched with 0.05% of (−)-epicatechin and quercetin (1:1) (BF) was further evaluated for its features and bioactivity. Baking properties, sensory evaluation, shelf life, and color tests were conducted and presented in a supplementary journal publication in Data in Brief (Leyva-Soto, under review). However, Fig. 1 showed changes in bread color obtained for the addition of flavonoids (BF) compared with control bread (CN). It has been previously reported that the addition of polyphenols and flavonoids in bread significantly decreases these values producing darker crumbs (Gunathilaka et al., 2019).

The antioxidant capacity of enriched bread was significantly greater compared with the control bread. Jin and collaborators determined that incorporation of quercetin into steamed bread (0.05–0.2%) significantly increased the antioxidant capacity and reduced the formation of advanced glycation endproducts (AGEs) in bread (Jing et al., 2018). Also, cookies fortified with quercetin or epicatechin significantly reduce their total fluorescent AGEs formation, and the formation of reactive carbonyl species (Zhang et al., 2014). Furthermore, green tea catechins added in baked and steamed bread significantly lower glucose release in an *in vitro* digestibility test (Goh et al., 2015).

The retention percentage of added flavonoids (88%) after the baking process was similar to the retention percentage reported before (Chavez-Santoscoy et al., 2016). Moreover, in the present work, flavonoid content was also monitored during storage for 15 days at three temperatures. Those results are presented in a supplementary dataset in Data in Brief (Leyva-Soto, under review). Flavonoid content significantly decreased during storage, and the higher the storage temperature, the greater the loss of flavonoid content over time ($p < 0.005$). Similarly, it has been reported that the content of tocopherols and the antioxidant capacity during storage decreases in cereal grains and that it could be an indicator of changes induced by oxidation (Dziki et al., 2014). However, information on the stability of phytochemicals during storage in processed cereal-based food is limited.

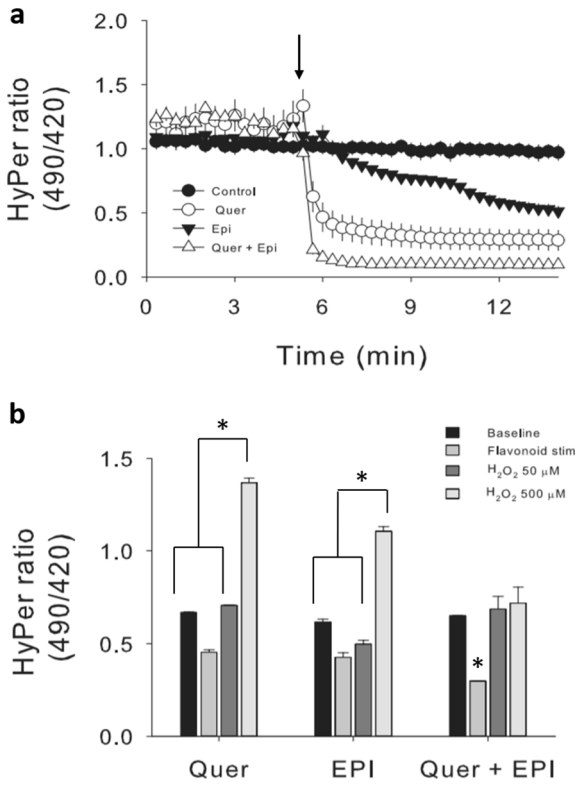

Fig. 4. Acute redox impact of quercetin, epicatechin, and both combined in intact Caco-2 cells expressing HyPer. (a) Time-course of the effect of 10 μM of quercetin (Quer), (−)-epicatechin (Epi), or the mixture of both flavonoids in 1:1 proportion (Quer + Epi). For comparison, untreated cells were added to the plot (control, filled circles), flavonoids stimuli were added to the cells at the moment indicated by the arrow. Data correspond to the average ± SE of more than 15 cells for each group from at least three independent experiments. (b) Summary of the ratio signal values before and after the exposure of cells to of 10 μM of quercetin (Quer), (−)-epicatechin (Epi), or the mixture of both flavonoids in 1:1 proportion (Quer + EPI); and the response of the biosensor upon application of exogenous pulses of 50 and 500 μM H_2O_2. Data correspond to the average ± SE of the same number of cells used in graph a. *Significant differences $p < 0.005$.

5. Concluding remarks

Participants that consumed bread enriched with flavonoids (0.05%) for three months significantly decreased several biochemical parameters (total cholesterol, LDL-cholesterol, triglycerides, and fasting plasma glucose) related to metabolic syndrome, compared with participants that consumed control bread. Data in the present work also includes real-time measurements of the antioxidant impact of flavonoids in Caco-2 Cells, conferring biological relevance to the antioxidant effect. Moreover, the antioxidant effect of these flavonoids could be related to their genoprotective effects observed in the frequency of nuclear abnormalities in buccal epithelial cells. The consumption of enriched bread with (−)-epicatechin and quercetin could provide health benefits in patients with altered biochemical parameters related to metabolic syndrome.

CRediT authorship contribution statement

Aldo Leyva-Soto: Methodology, Investigation, Writing - original draft. **Rocío Alejandra Chavez-Santoscoy:** Conceptualization, Methodology, Supervision, Writing - review & editing. **Omar Porras:** Conceptualization, Methodology, Supervision, Writing - review & editing. **Miltha Hidalgo-Ledesma:** Methodology. **Aracely Serrano-Medina:** Methodology. **Ana Alejandra Ramírez-Rodríguez:** Investigation, Writing - review & editing. **Nydia Alejandra Castillo-Martinez:** Methodology.

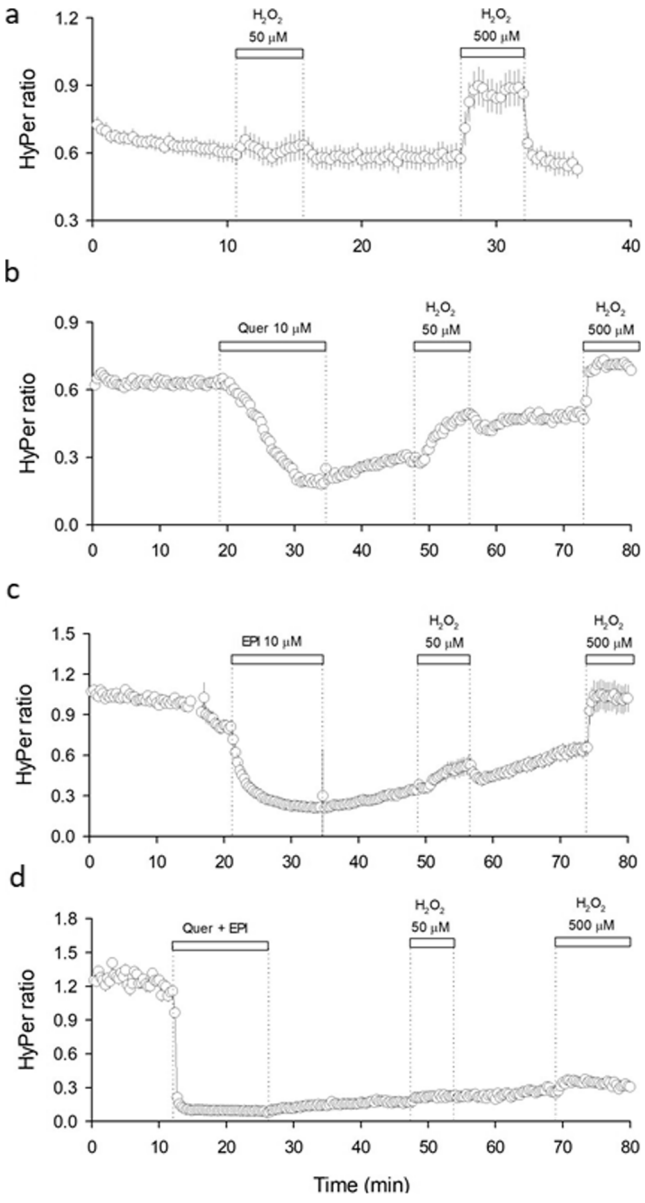

Fig. 5. Whole recordings of HyPer biosensor from untreated and flavonoid-treated Caco-2 cells. a) Untreated Caco-2 cells were exposed sequentially to 50 and 500 μM H_2O_2 as indicated by dotted lines and white bars on the plot. **b)** After more than ten minutes of baseline record, 10 μM of quercetin (Quer) was added to extracellular medium as indicated by dotted lines and white bar, after washing out two sequential pulses of 50 and 500 μM H_2O_2 were applied. **c)** As described in b but for cells were exposed to 10 μM (−)-epicatechin (EPI). **d)** Cells were exposed to a mixture of both flavonoids in 1:1 proportion (Quer + EPI). For each graph, data correspond to average ± SE of a representative experiment composed by more than 5 cells each one.

Methodology.

Declaration of Competing Interest

None.

The authors ensure that the work has been carried out in accordance with The Code of Ethics of the World Medical Association for experiments involving humans. The Ethics Committee of Hospital General de Baja California approved the study (CONBIOETICA 2CEI01120180619), and written consent was obtained from all subjects prior to enrollment in the study. The privacy rights of human subjects have been always observed.

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