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Original Article

Fruit polyphenols can upregulate the expression of opioid receptors (OPRD1) in brain cells, a molecular in vitro and in silico study.

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ABSTRACT

Abstract: Anecdotic evidence suggests fruit ingestion may be followed by an inner sensation of wellbeing, but biological basis for this phenomenon is not fully explained. Our aim was to investigate if there was a plausible biological mechanism that could explain if ingestion of fruit bioactives (polyphenols) could make us "feel good". NS20Y cells were cultured according to procedures and increasing concentrations of FruitOx (proprietary fruit-polyphenols blend extract) added to the media. Viability at 24h using Presto BlueTM showed no statistically significant differences at increasing concentrations (0-50 ppm). Quantitative real-time PCR analysis showed slight (1.5) but statistically significant ($p<0.009$) upregulation of delta opioid receptor 1 (OPRD1). Quantification of the protein by the Analysis of protein expression by western blot showed no statistically significant difference between FruitOx treated and control cells. *In silico* evaluation of the bioactives contained in the fruit blend revealed that chlorogenic acid (polar surface area 164, predicted bioactivity: G-coupled protein receptor, nuclear receptor) was the most likely candidate for eliciting such effect, suggesting a biological plausibility. We conclude that bioactive substances in fruits maybe able to stimulate neural pathways that may reinforce healthy eating habits. Further in vivo work is necessary to validate this theory.

1. Introduction

Anecdotic evidence suggests fruit ingestion may be followed by an inner sensation of wellbeing. However, a placebo effect cannot be ruled out and this phenomenon is not well understood. Although epidemiological data indicate regular consumption of fruit polyphenols and other bioactives is good for our health there seems to be a tendency to consume unhealthy foods (i.e. rich in fats) which is one of the culprits of the current obesity epidemic [1, 2]. Indeed this is a contradictory behavior. Discovering a positive feedback, reinforcement mechanism (i.e. feeling good after ingestion will make one seek the rewarding behavior) could be regarded upon to be advantageous in promoting healthy over unhealthy foods. Our aim was to investigate whether there was a plausible biological mechanism that could explain if ingestion of fruit bioactives (polyphenols) could make us feel good using *in vitro* nutrigenomics. FruitOx is a proprietary blend of different fruit extracts (prune, blueberry, pomegranate, apple, white cherry and grape leaf) which has shown to induce normalization of blood lipid profiles in obese individuals coupled with decreased levels of oxidative stress biomarkers in a clinical study [3]. A typical high-performance liquid chromatography profile is depicted in figure 1 and includes key active phytochemicals such as chlorogenic acid, phloridzin, punicalagins, hydroxycinnamic acid and dihydroquercetin-3-o-rhamnoside (Astilbin) (Table 1). Most of the fruit polyphenols contained in high concentrations in the blend are commonly found in other fruits and plants. Hence, this blend was regarded a reasonable representation of the bioactives that could be available when eating different fruits regularly. We decided to study whether fruit polyphenols could induce gene regulation changes in neurons.

2. MATERIALS AND METHODS

Cell culture

A mouse neuroblastoma cell line, NS20Y (ATCC 08062517), was cultured in Dulbecco´s Modified Eagle´s Medium (HyClone DMEM/High Glucose) supplemented with 5% fetal bovine serum 5% calf serum, 1% L-Glutamine 200mM (PAA) and 1% penicillin/streptomycin. Cells were maintained at 37°C in a humidified 5% CO_2 incubator and medium was changed every day.

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Cell Viability

Cell viability was determined using the Presto Blue cell viability kit (Spain) following the manufacturer´s instructions. The cells were seeded into 96-well plates at a density of $6x10^4$ cells/ml. The culture medium was replaced with DMEM containing various concentrations of FruitOx for 24 h. Control cells were cultured in DMEM without extract. Before Presto Blue kit reagents were added, the medium was removed. The cells were incubated with Presto Blue for 1h at 37°C. Fluorescence was measured on a MX3005P Q-PCR System (Agilent Technologies) using a Cy3 filter set on plate read mode.

RNA and Protein extraction

NS20Y cells were incubated with 10ppm FruitOx for 24 h. RNA and proteins were extracted using All Prep RNA/Protein Kit (Spain). RNA was stored at -80°C and protein at -20°C until further use.

Quantitative real-time RT-PCR analysis

Total RNA was quantified using a fluorometric method with Quant-iT kit (Spain). cDNA was reverse-transcribed from the RNA extract using RT^2 First Stand cDNA kit and we used a RT^2 Profiler PCR Array to analyze a panel of 84 genes representative of and involved in G-Protein Coupled Receptor-mediated signal transduction pathways (Qiagen, Spain). Quantitative real-time RT-PCR was carried out using a SYBR-Green/ROX detection in a MX3005P Q-PCR System. Samples were heated at 95°C for 10 min, followed by a second stage composed of 15 sec at 95°C, 1 min at 60°C which was repeated 40 times and third stage for dissociation curve composed of 1 min at 95°C, 30 sec at 55°C and 30 sec at 95°C.

To analyze the PCR-array data, an MS-Excel sheet with macros was downloaded from the manufacturer's website (http://www.sabiosciences.com). This program calculated relative gene expression and statistical significance.

Western Blotting

Total protein was quantified using a fluorometric method with Quant-iT kit (Spain). The samples (40µg of protein) were separated on 10% SDS- polyacrylamide gel (BioRad, Spain), transferred to a nitrocellulose membrane (Invitrogen, Spain) and subsequently subjected to inmunoblot analysis using primary antibodies including OPRD-1 and β-Actin (Abcam, U.K). After incubation during 24h at room temperature with primary antibody, the membranes were incubated with horseradish peroxidise (HRP)-conjugated secondary antibody (Abcam, U.K) for 24 h. The blots were visualized with chemiluminescent detection kit on a Kodak Image Station 4000MM (Carestream Health, USA). In silico evaluation of bioactives

In silico evaluation of FruitOx bioactives was performed using Molinspiration Chemoinformatics 2012©. Chemical structures were uploaded (http://www.molinspiration.com/cgi-bin/properties) using the Simplified Molecular-Input Line-Entry System (SMILES). Properties and predicted bioactivities were then calculated and tabulated.

Results

There was no loss of cell viability using Pesto Blue cell kit within the dose range of 0,1 to 50 ppm at 24h (figure 2). We found a weak (1.5-fold), but highly statistically significant ($p<0.00865$) upregulation of opioid receptor delta-1 (OPRD1) (figure 3). Quantification of the protein expression at 24h by Western-blot did not show a statistically significant difference between control and FruitOx treatments (not shown).

In silico evaluation of FruitOx bioactives is summarized in table 2. Comparison of calculated properties and predicted bioactivies showed that chlorogenic acid was the compound to be most likely responsible for the neuro-biological effect.

Table 1. Bioactive compounds in Fruitox provided by the different ingredients.

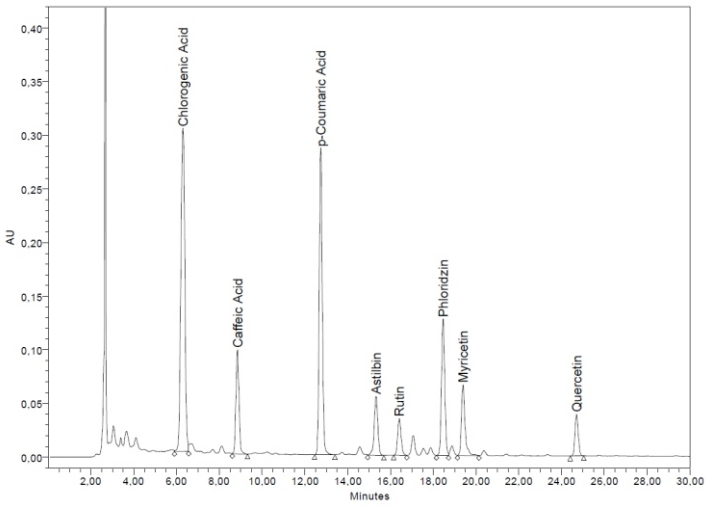
Source	Species	Main active compounds
Prune extract	*Prunus domestica*	Chlorogenic Acid
Blueberry extract	*Vaccinium angustifolium*	Chlorogenic acid, Caffeic acid
Pomegranate extract	*Punica granatum*	Punicalagins (A & B), Punicalins, Ellagic acid glycoside, Ellagic acid
Apple extract	*Malus domestica*	Phloridzin
White Cherry	*Prunus Avium*	Hydroxycinnamic Acid, Neocholorogenic acid, Chlorogenic acid, Cyanidin 3-rutenoside, Rutin, Quercetin 3-glucoside
Grape leaf extract	*Vitis vinifera*	Dihydroquercetin-3-o-Rhamnoside (Astilbin)

Table 2. In silico evaluation of the main bio-actives found in the FruitOx.

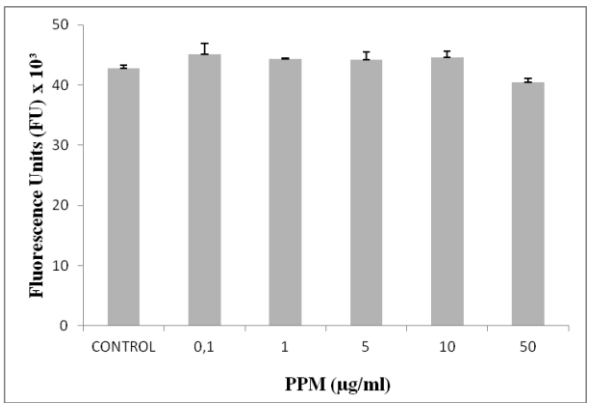
Compound	Properties						Predicted bioactivity	
	Mol. weight	Log P	Polar surface area	Volume	No. Atoms	No. Violations	GCPR ligand	Nuclear receptor ligand
Astilbin	450.40	0.013	186.37	370.19	32	2	0.11	0.12
Caffeic acid	180.16	0.94	77.75	154.50	13	0	-0.48	-0.10
Chlorogenic acid	354.31	-0.45	164.74	296.27	25	1	0.29	0.74
Cyanidin 3-rutenoside	595.53	-3.49	250.39	490.79	42	3	-0.02	-0.24
Dihydro-quercetin	304.25	0.71	127.45	246.32	22	0	0.09	0.29
Ellagic acid	302.19	0.94	141.33	221.78	22	0	-0.29	0.11
Ferulic acid	194.19	1.25	66.76	172.03	14	0	-0.47	-0.14
Myricetin	318.24	1.39	151.58	248.10	23	1	-0.06	0.32
Neochlorogenic acid	595.53	-3.49	250.39	490.79	42	3	-0.02	-0.24
p-coumaric acid	164.16	1.43	57.53	146.48	12	0	-0.56	-0.12
Phloridizin	436.41	0.40	177.14	372.23	31	1	0.17	0.26
Punicalagin	1084.72	2.05	526.59	804.93	78	3	-4.48	-4.69
Quercetin	302.24	1.69	131.35	240.08	22	0	-0.06	0.36
Quercetin 3-glucoside	464.38	-0.36	210.50	372.21	33	2	0.06	0.20
Rutin	610.52	-1.06	269.43	496.07	43	3	-0.05	-0.23

GCPR: G-coupled protein receptor

Figure 1. High performance liquid chromatography of FuitOx



AU: Absorbance Units.



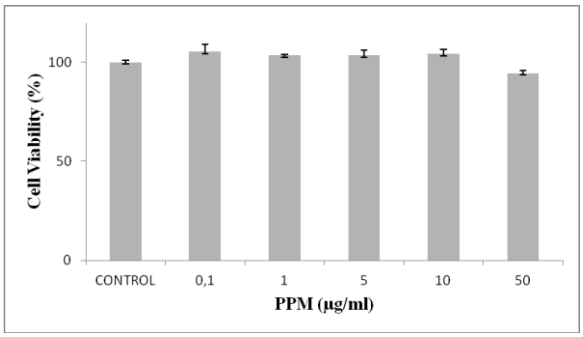
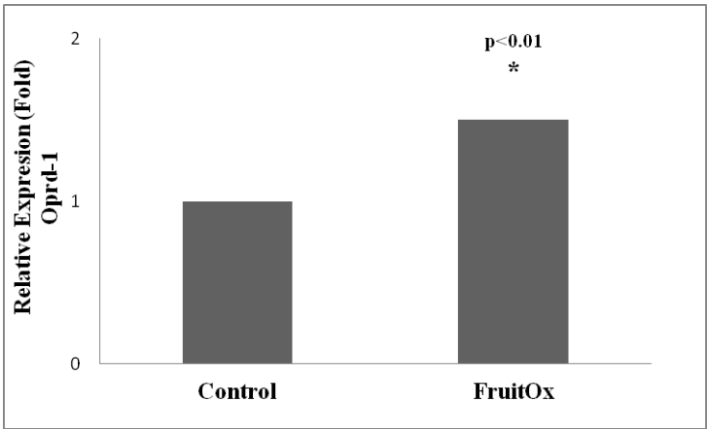


Figure 2. Cytotoxicity of FruitOx in mouse neuroblastoma cell line, NS20Y. Cells were cultured with media containing FruitOx (0.1-50 ppm) for 24 hr. Results are expressed in fluorescence units (FU) and percentage of viability, calculated using the following equation: (FU treated/FU control) x 100. Results are expressed as (cell viability %) compared to the control cell cultures for each time group.

Figure 3. Relative expresion of protein (OPRD1) between control and test (p<0,01). Total protein was quantified using a fluorometric method with Quant-iT kit (Spain) after incubation with 10ppm FruitOx for 24 h.



Discussion

Opioid receptors are expressed primarily in the cortex, limbic system and brain stem. There is overlap in their binding sites for the 3 different receptors (delta, kappa and mu) in most structures but some of them exhibit higher expression of a particular receptor than others. In particular, the delta receptors are the most abundant in the olfactory tract and the cortices (whole neocortex and regions of the amygdala) and the striatum [4]. Opioid receptors are present in most regions of the complex widespread network that regulates hedonic food intake. Delta opioid receptors are expressed (densely or moderately) specifically in the nucleus accumbens, ventral pallidum, ventral tegmental area and amygdala, all in control of regulating food intake [5-7]. Dysregulation of food intake neural pathways (both homeostatic and hedonic) have been proposed as key players in the genesis of obesity. In fact, functional studies with nuclear magnetic resonance (fNMR) have reveled interesting findings in the way food interacts with neural pathways. Specifically, obese individuals have shown greater activation of food reward brain areas when looking at high calorie foods than normal weight volunteers. These areas with differential activation included the amygdala where delta opioid receptors are densely expressed [8-10]. These data support the existence of a hyperactive reward system to high-calorie food cues that is involved in the pathophysiology of obesity. Other studies have shown also that obese individuals respond differently to satiety, which may contribute to overeating [11, 12]. Since it is clear that high-calorie, fattening food may induce rewarding mechanisms to support a positive feedback sustaining obesity it would be interesting to use the same types of mechanisms with non-fattening foodstuffs to promote and reinforce healthier eating habits. Our pilot study sought to find biological plausibility to this approach. FruitOx is a blend of different fruit extracts with widely distributed antioxidant polyphenols (figure 1). Neurons treated with the test substance expressed mild upregulation of OPRD1 (1.5 fold, p<0,00865). However we could not find statistically significant expression of such protein at 24h. One possible explanation is that the time frame was not adequate. Cells may take longer to produce enough quantities or the protein could be degraded sooner than that. Also, we did not perform a dose-response study and perhaps greater concentrations of FruitOx may elicit a proportional response with greater amounts of protein being detected.

Another limitation of our study is the fact that we did not test for the individual bioactives. While it is an academically interesting approach, it does not represent real life. People are more likely to ingest different fruits and vegetables thus incorporating many bioactives at once rather than a specific compound. Also, we cannot exclude an interaction between these bioactive natural substances as it may happen in real life. However in silico evaluation of the natural antioxidants was deemed a valuable alternative to better understand the potential for a specific compound to have such neuro-biological effect. In this regard chlorogenic acid has 25 atoms, 354.311 molecular weight, 164.744 polar surface area, one violation of the Lipinski´s rule, and a predicted bioactivity of being a G-protein coupled receptor ligand all compatible with required physicochemical properties for central nervous system drugs [13]. Further work is required to validate our findings and translate them into *in vivo* studies.

In conclusion our pilot study showed that fruit polyphenols may have the potential to modulate central pathways that may be useful in reinforcing healthy food reward patterns. Although more research is needed to support these preliminary data, they open an interesting field in using neuroscience to fight against obesity.

CONFLICT OF INTEREST

José M Zubeldia, Miguel Jimenez-del-Rio, Verónica Pérez-López and Aarón Hernández-Santana work for Polinat SL, the company that manufactures FruitOx.

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