Neuroprotective effects of apple-derived drinks in a mice model of inflammation

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Abbreviations:

AcJ: Carbonated acai juice;AD: Alzheimer's disease; AlJ: Carbonated aloe juice; ANOVA: Analysis of variance; ApJ: Carbonated apple juice; BC: Blackberry cider; CAT: Catalase; CJ: Carbonated coconut juice; DC: Dry cider; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GPx: Glutathione peroxidase; GSH: Glutathione; GSSG: Glutathione disulphide; iNOS: Inducible nitric oxide synthase; MDA: Malondialdehyde ;MTT: 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; NC: Natural cider; NFκB-p65:nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2: Nuclear factor (erythroid-derived 2)-like 2; PC: Pear cider; PD: Parkinson's disease; RM: Red must; ROS: Reactive oxygen species; S: Sangria; SC: Sweet cider; SOD: Superoxide dismutase; StC: Strawberry cider; TMRM: Tetramethylrhodamine methyl ester; TV: 'Tinto de verano'; TVl: 'Tinto de verano' with lemon; WM: White must; ΔΨm: Mitochondrial membrane potential

Abstract

Scope: Fruit-derived drinks consumption is considered beneficial due to the antioxidant and neuroprotective effects of polyphenols separately, but studies including their total constituents are scarce. In this work, we determined the antioxidant and anti-inflammatory neuroprotective effects of apple-derived beverages in a mouse model of LPS-induced inflammation.

Methods and results: Preliminary antioxidant and neuroinflammatory experiments were carried out with fifteen drink polyphenolic extracts in SH-SY5Y and BV2 cells, using H_2O_2 as pro-oxidant and LPS as pro-inflammatory stimulus, respectively. Extracts improved antioxidant systems functioning and presented neuroprotective mitochondrial-related effects. In microglia, extracts reduced reactive oxygen species and modulated cytokine release. To better mimic human consumption, four concentrated dealcoholized apple-derived drinks (three ciders and apple juice) were supplied to mice for seven days in substitution of drinking water. Mice treated with beverages presented reduced brain oxidative stress and inflammatory markers (lipid peroxidation, NO, iNOS, TNF-α) after LPS injection. Interestingly, genetic expression of antioxidant enzymes and glutathione levels were also greatly augmented after drink intake.

Conclusion: Our results confirm the antioxidant and anti-inflammatory-mediated neuroprotective properties of apple-derived drinks, suggesting that their consumption could be a good approach for prevention of neurodegenerative disorders. To our knowledge, this is the first description of cider neuroprotective effects.

1. Introduction

The progressive increase in life expectancy has led to an increment in the prevalence of neurodegenerative diseases such as Alzheimer`s disease (AD) or Parkinson's disease (PD), resulting in a major economic and social burden. These disorders are thought to begin many years before the appearance of the first symptoms and share common pathological features such as oxidative stress, neuroinflammation, protein aggregation and mitochondrial dysfunction[1]. Due to the lack of effective therapies against these pathologies, attention has been paid to prevention through the diet. Particularly, strategies based on fruit and vegetable consumption, characteristics of the Mediterranean diet, have been proposed to reduce inflammation and cognitive impairment[2]. Fruits contain great amounts of bioactive compounds, most of them polyphenols, with antioxidant and anti-inflammatory properties. Polyphenols such as quercetin, resveratrol, anthocyanins or catechins have presented promising *in vivo* results, and some of them have entered clinical trials[3]. Polyphenols interact when administered in combination, exerting synergistic effects, and their mixture could solve problems of low efficacy or side effects[4]. Although several works have determined the neuroprotective properties of polyphenols separately, data of direct fruit drink consumption are scarce. Particularly, few studies have been conducted to determine the neuroprotective properties of apple beverages [5-9], despite the promising results obtained with humans, in which apple juice intake was associated with an improvement in AD behavioural symptoms[10].

Oxidative stress is a critical hallmark of neurodegeneration, as aging produces mitochondrial failure and excessive generation of reactive oxygen species (ROS). The inability of endogenous antioxidant systems to neutralize these molecules leads to protein, lipid and nucleic acid damages, which produce cellular function deregulation and can even cause cell death[11]. Increased ROS levels also provoke the activation of microglia, the immune defence of the brain. Microglial function is altered in neurodegenerative diseases because the sustained presence of toxic molecules results in the chronic activation of these cells, which contribute to

49 the harmful surrounding environment by releasing pro-inflammatory cytokines, ROS and NO.
50 Therefore, neuroinflammation also generates oxidative stress through the production of
51 damaging mediators, creating a vicious cycle that leads to neurodegeneration[12].

52 In this work, the *in vitro* antioxidant and anti-neuroinflammatory effects of fifteen drink
53 polyphenolic extracts were determined. Four carbonated juices, three wine mixes, two musts
54 and six ciders produced by an industry of Northwest Spain were selected, and their
55 neuroprotective activity was evaluated in SH-SY5Y and BV2 cells. Then, four apple-derived
56 beverages were chosen to perform *in vivo* experiments and their beneficial effects were
57 assessed on a mice model of LPS-induced oxidative stress and inflammation.

58 ## 2. Material and methods

59 Information regarding chemicals and solutions can be found in Supporting Information

60 ### *2.1 Samples*

61 Fifteen drinks were used for the assays: four carbonated juices (coconut, aloe, acai, and apple)
62 two musts (white and red), three red wine mixes (sangria, 'tinto de verano', and 'tinto de
63 verano' with lemon), and six ciders (sweet, dry, natural, pear, strawberry, and blackberry).
64 Nutritional information is available in Supplementary Information (Tables S1, S2 and S3). All
65 the samples were produced and provided by a local industry of Galicia (Spain). Specifically,
66 apple-derived drinks were produced using the following local apple varieties: pero, verdeña,
67 perezosa, ollo mouro and rabiosa. All apples were harvested in September and October in the
68 region of Galicia (42º36'30"N 7º46'8"W).

69 ### *2.2 Extract preparation*

70 Drink polyphenolic extracts were obtained as previously described[13]. Beverage foam and gas
71 were removed by ultrasonication and agitation. Ethanol was evaporated with nitrogen flow and
72 a solid-phase extraction was carried out. Oasis® MAX cartridges (Waters, Barcelona, Spain)
73 were activated with 1 mL of methanol and 1mL of sodium acetate 50mM, pH 7. After this, 1

74 mL of each drink acidified with 34 µL of 35% HCl was added to the cartridges. Next, 50mM
75 sodium acetate pH 7 with 5% methanol was added to each cartridge. Polyphenols were eluted
76 with 1.8 mL of methanol containing 2% formic acid. The eluted fractions were evaporated,
77 reconstituted with 100 µL of water and filtered.

78 *2.3 In vitro assays*

79 Oxidative stress and neuroinflammation assays were performed in SH-SY5Y and BV2 cells, as
80 previously described[14, 15]. Further details are presented in Supplementary Information.

81 *2.4 Experimental animals*

82 Female *Swiss albino* mice (Crl: CFW (SW), strain code: 024) were obtained from Charles River
83 Laboratories (Barcelona, Spain). The animals were four weeks old and not specific-pathogen
84 free. Mice were kept during all the experiment in the facilities of the animal care center of
85 Universidad de Santiago de Compostela in a controlled environment on a 12 h light/day cycle.
86 Animals were accommodated for 5 days before the beginning of the experiment, following the
87 center rules. All the mice received were used for the experiment and their average weight at the
88 beginning of the experiment was 21.4± 2.7 g. They were randomly distributed among groups
89 and singly housed to facilitate the measurement of daily drink intake. Animals were housed in
90 type III cages from Tecniplast (Buguggiate, Italy) (425x10.87x153 mm) with sterilized natural
91 wood fibers bedding (J. Rettenmaier & Söhne GmbH & Co KG, Barcelona, Spain). They were
92 fed with complete rodent diet (18% protein) (Envigo,Barcelona, Spain). Both, drinking water
93 and food, were sterilized before their use. Apple-derived drink concentrates were prepared
94 under laminar flow to avoid any contamination. Food and drink were supplied *ad libitum*.
95 Environmental conditions were controlled by Struxureware Building Operations software
96 (Schneider-Electric, A Coruña, Spain). Temperature and humidity variations during the
97 experiments were 22.0±0.8ºC and 52.0±10.3 %, respectively.
98 All protocols were approved by the Universidad de Santiago de Compostela Institutional animal
99 care and use committee (process number: AE-LU-002-011/14) and followed the *Guide for the*

100 *Care and Use of Laboratory Animals* published by the National Academy of Science (USA) and
101 the EU Directive 2010/63/EU for animal experiments.

102 *2.5 Dosage information/Dosage regimen*

103 Four drinks were selected to perform *in vivo* experiments: carbonated apple juice, and sweet,
104 dry and pear ciders. Mice were randomly divided in 10 groups: Control (n=6), LPS
105 (lipopolysaccharide, intraperitoneal (IP) injection) (n=6), ApJ (carbonated apple juice) (n=4),
106 PC (pear cider) (n=4), SC (sweet cider) (n=4), DC (dry cider) (n=4), ApJ+LPS (carbonated
107 apple juice and LPS, IP) (n=4), PC+LPS (pear cider and LPS, IP) (n=4), SC+LPS (sweet cider
108 and LPS, IP) (n=4),and DC+LPS (dry cider and LPS, IP) (n=4). Beverages were 6x
109 concentrated and cider ethanol was evaporated. Concentrated samples were administered to
110 treated mice in substitution of drinking water for 7 days and changed daily[5, 16, 17]. The last
111 day of treatment, 0.8 mg/kg LPS (HED: 0.07 mg/kg) dissolved in saline was administered by IP
112 injection to the corresponding mice[17]. Treated mice were singly housed and their drink
113 consumption was measured each day. The intake was 4.2±0.2 mL/d per mouse, without
114 significant differences between groups. Four hours after LPS injection, animals were sacrificed
115 in a dioxide carbon chamber, and brain and blood samples were collected. Serum was collected
116 after centrifuging the blood samples at 3000 rpm for 10 min. Brain and serum samples were
117 stored at -80ºC until use.

118 *2.6 Quantitative PCR*

119 Brain RNA was obtained using the HigherPurity™ Tissue Total RNA Purification Kit (Canvax
120 Biotech, Córdoba, Spain) following the manufacturer's instructions. RNA purity and
121 concentration were determined with Nanodrop™ 2000 spectrophotometer (Thermo Fisher
122 Scientific, Madrid, Spain). cDNA was synthetized with 0.5 µg of RNA, oligo-dT primers and
123 RevertAid Reverse Transcriptase (Thermo Fischer Scientific), following manufacturer's
124 instructions. Quantitative PCR was performed using PowerUp SYBR Green Master Mix in a
125 Step-One real-time PCR system (Applied Biosystems, Madrid, Spain). cDNA was amplified

with specific primers for catalase (CAT), superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPx1) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Table S4) [18]. Data were analysed with the Step-One software (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as normalization control[19]. Relative quantification was carried out using ΔΔCt method using the control group as calibrator. All experiments were carried out in triplicate.

2.7 Brain and serum cytokine measurement

Brain and serum cytokines were measured with a Mouse High Sensitivity T Cell Magnetic Bead Panel (Merck Millipore, Madrid, Spain). Fluorescence was read with Luminex 200 ™ instrument and xPONENT® software (LuminexCorp, Austin, USA). Brain samples were homogenized in extraction buffer containing 20 mM Tris HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, with protease and phosphatase inhibitor cocktails[20]. Samples were sonicated and centrifuged for 1 h at 14000g 4ºC. Cytokine signal was normalized with the protein concentration of each sample, measured with Direct Detect instrument (Merck Millipore).

2.8 Lipid peroxidation

Malondialdehyde (MDA) levels were measured with a Lipid Peroxidation Assay Kit (Sigma Aldrich, Madrid, Spain), following manufacturer's instructions. Briefly, 10 mg of brain were homogenized in lysis buffer containing butylated hydroxytoluene. Homogenates were centrifuged at 13000g 4ºC 10 min to remove insoluble fraction and thiobarbituric acid (TBA) was added to each vial. Samples were incubated for 1 h at 95 ºC and cooled for 10 min. Absorbance was measured at 532 nm. All the assays were performed in triplicate.

2.9 Determination of catalase and superoxide dismutases activity

The activity of antioxidant enzymes was evaluated in brain homogenates. Mouse tissue was lysed in ice-cold buffer containing 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1%

Triton x-100, and complete protease and phosphatase inhibitor cocktails. Samples were sonicated and centrifuged at 14000g 4ºC for 1 h. Protein concentration was determined with Direct Detect instrument (Merck Millipore).

CAT activity was measured with Amplex® Red Catalase Assay Kit, following manufacturer's instructions. Fluorescence was read at 530 nm excitation and 590 nm emission. Enzymatic activity was calculated by subtracting sample values to the no-CAT control.

SODs activity was determined with SOD determination Kit (Sigma-Aldrich) as described for cellular experiments. All the experiments were normalized by protein concentration.

2.10 Nitric oxide measurement

Griess Reagent Kit was used to evaluate NO levels in brain homogenates. Briefly, 150 µL of each sample were mixed with 20 µL of Griess reagent and incubated 30 min at room temperature. Next, the absorbance was measured at 548 nm. All experiments were performed in triplicate.

2.11 Brain glutathione determination

Glutathione (GSH) and oxidized glutathione (GSSG) levels were evaluated with a Glutathione Colorimetric Detection Kit, following manufacturer's instructions. Brain homogenates were deproteinized with 5% 5-sulfo-salicylic acid dehydrate and GSH/GSSG ratio was determined. For GSSG measurement, samples were treated with 2-vinylpyridine for 1 h. Absorbance was read at 405 nm in a spectrophotometer plate reader. Measurements were performed in triplicate and normalized by protein concentration.

2.12 Western Blotting

Electrophoresis was resolved in 4-20% sodium dodecyl sulphate polyacrylamide gels (Biorad, Madrid, Spain) with 15 µg of brain homogenates loaded by duplicate. Membrane blocking and antibody incubation were performed in Snap i.d. system (Merck Millipore). Anti- nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB-p65) (1:10000, Merck

Millipore), anti-inducible nitric oxide synthase (iNOS) (1:1000, Abcam, Cambridge, UK) were used for protein expression determination and signal was normalized with anti-β-actin (1:10000, Merck Millipore). Protein bands were detected with Supersignal West Pico Luminiscent Substrate and Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and Diversity GeneSnap system and software (Syngene, Cambridge, U.K.).

2.13 Statistical analysis

Data are presented as mean ± SEM. Differences were evaluated by one-way ANOVA and Dunnett's post hoc test with Graph Pad Prism 6 software. Statistical significance was considered at $p < 0.05$.

3. Results

3.1 Drink extracts protected SH-SY5Y cells from oxidative damage

SH-SY5Y cells have been established as an appropriate model for studying oxidative damage using H_2O_2 as oxidant stressor[21, 22]. In this study, we determined the antioxidant properties of fifteen drink polyphenolic extracts. Four carbonated juices: coconut juice (CJ), aloe juice (AlJ), acai juice (AcJ) and apple juice (ApJ); three wine mixes: sangria (S), 'tinto de verano' (TV) and 'tinto de verano' with lemon (TVl); two grape juices: white must (WM) and red must (RM); and six ciders: pear cider (PC), sweet cider (SC), dry cider (DC), strawberry cider (StC), blackberry cider (BC) and natural cider (NC) were used. Firstly, drink extracts cytotoxicity was determined. ApJ, TVl, PC, StC and BC were toxic at the highest concentration (1:100) (Figure S1), so those treatments were excluded in oxidative stress assays.

Next, cells were co-treated with extracts and 150 μM H_2O_2 and several parameters were analysed to determine their antioxidant potential. H_2O_2 addition produced a significant reduction in cell survival (73.0±2.5 %, $p<0.05$) (Figure 1a-c). Extracts protected cells from oxidative injury, being ApJ, RM and three ciders (PC, SC and DC) the most effective treatments, with levels about 120% of control cells. Mitochondrial state was determined through mitochondrial membrane potential ($\Delta\Psi_m$) assessment (Figure 1 d-f). Mitochondrial depolarization induced by

H_2O_2 was recovered by some of the extracts, producing AcJ, ApJ. TV, TVl, the musts and all the ciders a significant increase in $\Delta\Psi_m$.

To confirm the antioxidant properties of extracts, ROS levels were evaluated. The addition of H_2O_2 augmented ROS until 126.7±3.7% (p <0.05) and, once again, all the extracts presented positive results. Treatment with juice extracts diminished ROS levels to percentages between 50-60% (Figure 1g). Grape-derived drink extracts were less potent, but their effect was also significant (Figure 1h). Ciders also displayed antioxidant effects, being DC, StC, BC and NC especially effective (Figure 1f).

Next, the effect of drink extracts on cellular antioxidant systems was determined. For these assays, the most effective concentrations were selected. GSH levels were increased when most of the extracts were present (Figure 2 a-c), showing CJ, S, RM. PC and StC the best results, with percentages about 120% of control cells. Regarding SODs activity (Figure 2 d-f), the addition of three juice extracts (AlJ, AcJ and ApJ), all the grape-derived drinks and the ciders (except StC) produced a recovery on enzymatic activities.

3.2 Drink extracts reduced neuroinflammation in vitro

BV2 microglial cells were used to determine the anti-neuroinflammatory properties of polyphenolic extracts. Cells were activated with LPS, a component of bacterial membrane known to induce inflammation[23]. First, the cytotoxicity was determined, but none of the extracts affected to cell survival (Figure S2). Therefore, we analysed their effect on microglial ROS release (Figure 3a-c). Stimulation with LPS significantly increased ROS levels (146.1±3.7%, p<0.05), and juice extracts (55-73%), wine mixes (54-98%) and PC (95-102%) were the most effective treatments. To clarify the effect of extracts on microglia, we assessed the levels of an anti-inflammatory (IL-10) and a pro-inflammatory cytokine (TNF-α). As Figure 3d-f shows, cells treated with juice extracts, S, PC, SC, and DC presented the best results in IL-10 release, increasing its levels until 130-135%. Regarding TNF-α, juice extracts, wine mixes and BC reduced its release about a 20% of LPS-activated cells (Figure 3g-i).

3.3 Drink concentrates diminished oxidative stress and neuroinflammation in vivo

Four beverages were selected to perform *in vivo* experiments based on their good results in cellular experiments: ApJ, PC, SC and DC. Despite the good results obtained with red wine mixes and musts, we chose apple-derived drinks due to the lower number of studies with this kind of beverages. Mice brain RNA was isolated, and the relative expression of antioxidant genes was analysed. Treatment with the drinks alone significantly upregulated the expression of SOD1, CAT, GPx1 and Nrf2 (except SC) compared to control ($p<0.001$)(Figure 4 a-d). As expected, mice treated with LPS presented increased antioxidant gene expression, except for CAT. Concentrates consumption provokes an increment in SOD1, CAT and GPx1 expression in LPS-treated mice. Interestingly, Nrf2 is also upregulated in animals treated with ApJ and LPS ($p<0.05$) *versus* LPS group.

To confirm these results, SODs and CAT activity and GSH levels were evaluated in brain homogenates. SODs activity is increased in LPS-treated mice with respect to the control group (165.4±14.9%, $p<0.01$), whereas the addition of ApJ reduced the enzyme activity (91.1±11.4%, $p<0.05$) (Figure 4e). Otherwise, DC+LPS group presented an elevation in SODs activity ($p<0.05$). DC seems to affect the antioxidant systems, as CAT activity is greatly increased when mice drink that concentrate (Figure 4f). The oxidative stress produced by LPS injection also augmented CAT activity (139.6±8.5%, $p<0.001$). Consumption of ApJ, PC and SC reduced that increase, reaching a 42.6±1.5% in the case of ApJ ($p<0.001$). GSH levels were decreased in LPS-treated mice compared to control animals (Figure 4g). Surprisingly, PC intake alone also reduced GSH levels. However, when ApJ, PC and DC were supplied to LPS-injected animals, an increase in the levels of the antioxidant is observed.

To better characterise the fruit drink effects on the brain, MDA and NO levels were evaluated. MDA is a by-product of lipid peroxidation and its levels can be used to determine the oxidation state of the brain. This assay presents some limitations due to the reactivity of TBA with other compounds not related to lipid peroxidation[24], and therefore, it must be supported by other

oxidative stress markers measurement, such as antioxidant enzyme activity. As Figure 5a shows, treatment with ApJ and SC alone reduced MDA levels to percentages among 55-73%, compared to control animals. Otherwise, DC increased MDA until levels of LPS-injected animals (≈208 %). Consumption of ApJ, PC and SC also protected mice brains from the damage produced by LPS ($p<0.05$).

Regarding NO, DC produced an increase in the release of the molecule ($p<0.01$) (Figure 5b). LPS-injection also augmented NO levels (17.2 μmol/mg, $p<0.001$), but pre-treatment with ApJ, PC and SC reduced the release of this toxic mediator, confirming the neuroprotective properties of these drinks.

The expression of two proteins related to the inflammatory cascade, iNOS and NfkB-p65, was also determined (Figure 5c-d). LPS injection produced an increase in iNOS levels (75.5±1.9%) *versus* control, which ApJ, PC and SC significantly reduced. NFκB-p65 expression was also augmented by LPS (53.8±5.5%) but it was reduced only by ApJ($p<0.05$).

Finally, IL-10 and TNF-α levels were assessed in mice brain and blood. Only SC consumption had effect on brain IL-10, increasing the anti-inflammatory cytokine levels about three times compared to LPS group ($p<0.001$) (Figure 5e). As expected, TNF-α is increased in LPS-injected mice brains ($p<0.001$), and ApJ and PC were able to greatly reduce the cytokine level (Figure 5f). Regarding blood levels, IL-10 is augmented in LPS-injected animals (3273.9 pg/mL, $p<0.001$), and treatment with ApJ alone produced a slight increase in the molecule (Figure 5g). Interestingly, all the drinks reduced TNF-α blood levels (300-750 pg/mL) (Figure 5h).

4. Discussion

Epidemiological studies have evidenced the relationship between fruit-rich diets and a lower risk of suffering chronic diseases such as cancer, diabetes, and neurological pathologies. These beneficial effects are, at least in part, attributable to the presence of polyphenols. In the case of neurodegeneration, the blood brain barrier is an important obstacle to face its treatment and polyphenolic compounds have been reported to cross it through specific transporters[25]. Fruit

juices, ciders and wines contain high amounts of phenolic compounds with neuroprotective properties, such as anthocyanins, catechins or resveratrol[26-28], so fruit-derived drinks intake will have a protective effect. Nevertheless, diabetic people should be careful with sugar content, especially with added glucose[29]. Due to the extensive research carried out with resveratrol[30], most studies have been focused on grape-derived beverages[31]. However, pomegranate, cocoa or apple juice positive properties have been previously determined, with effects such as improvement of cardiovascular system, lower risk of Alzheimer's disease, and diabetes prevention in humans[32-34]. In this work, we demonstrated the neuroprotective ability of apple-derived drinks, being remarkable the results obtained with ciders, as this is the first neuroprotection study with this kind of beverage.

Firstly, we evaluated the *in vitro* neuroprotective effects of fifteen polyphenolic extracts obtained from fruit-derived beverages produced in Northwest Spain. The extracts protected cells from oxidative injury, being noteworthy their reduction of ROS levels and the mitochondrial-related effect, which agrees with the well-known antioxidant properties of polyphenols and their ability to accumulate in mitochondria[25]. Extracts also improved antioxidant systems functioning, suggesting an indirect antioxidant effect that has also been reported for polyphenolic compounds[25]. Moreover, the results obtained with microglia point to anti-inflammatory properties of drink extracts, as evidenced by ROS and TNF-α decrease, and by the augmentation of the anti-inflammatory cytokine IL-10.

The promising results obtained in cellular models, along with the shortage of studies with apple-derived drinks, lead us to select three ciders and the apple juice to evaluate their neuroprotective activity *in vivo*. All these drinks were produced from the whole apples, including pulp and peel, as the later is known to contain higher polyphenol concentrations[35, 36]. In this case, all the beverage content was concentrated to better simulate human consumption. Our results confirmed the drinks ability to upregulate antioxidant gene expression, acting as indirect antioxidants. However, this increase was not reflected in the enzymatic activity, suggesting also a direct antioxidant effect of beverages. Some polyphenols have been described as both direct

and indirect antioxidants, being able to directly reduce ROS and to activate Nrf2[4], a transcription factor that regulates the expression of antioxidant enzymes[37]. Drink intake decreased lipid peroxidation, probably through directly ROS scavenging, so the activation of antioxidant enzymes activity would not be necessary. Regarding LPS-injected mice, MDA levels were increased and, as a defensive response, SOD and CAT enzymatic activity was incremented and GSH levels reduced, agreeing with previous works[17, 38]. The reduction in NO, iNOS and TNF-α brain levels produced by drink consumption suggests an anti-inflammatory activity of beverages. The differences observed in the drinks potency could be explained by their composition: ApJ, composed by 100% of juice, presented the best results, followed by PC, which is a mixture of apple cider and pear juice (10%). Apple fermentation seems to be affecting the drinks antioxidant potency, and juice addition maybe a good strategy to improve their neuroprotective ability. Nevertheless, SC also displayed neuroprotective effects, being the only beverage able to increase IL-10. Regarding DC, its effects at transcriptional level were like the other drinks, but this beverage increased lipid peroxidation and NO, leading to augmented CAT and SOD activity. However, GSH brain levels were recovered and TNF-α blood release was decreased. Further experiments are needed to clarify the effect of this drink.

This study provides preliminary evidences of the antioxidant and anti-inflammatory-mediated neuroprotective effects of apple-derived drinks, suggesting that their consumption would be a good preventive approach against neurodegeneration. Apple juice presented the best results, agreeing with previous works [6, 8, 9] and, to our knowledge, this is the first description of cider neuroprotective properties, although the antioxidant effects of cider vinegar have been previously determined[39]. Therefore, this work is a starting point for further experiments to better determine the effects of beverages on different brain regions and other pathological markers of neurodegeneration.

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Conflict of interest

The authors declare no conflict of interest

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Figures

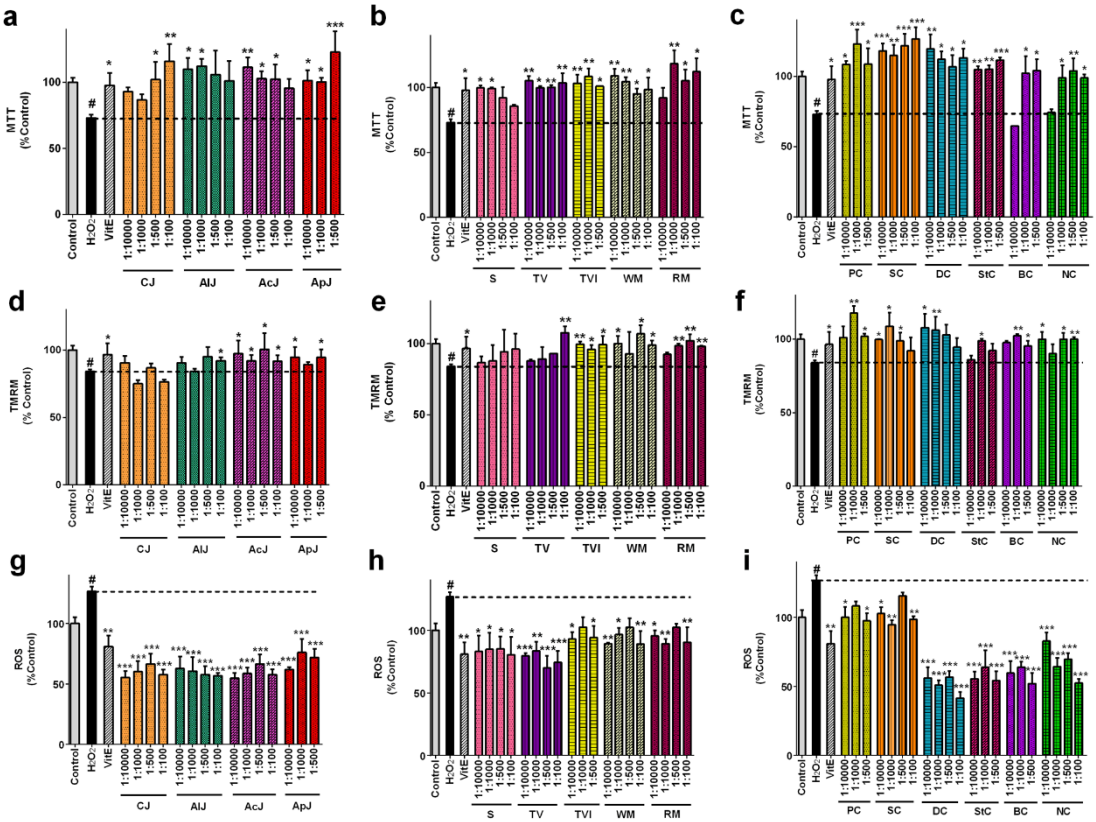
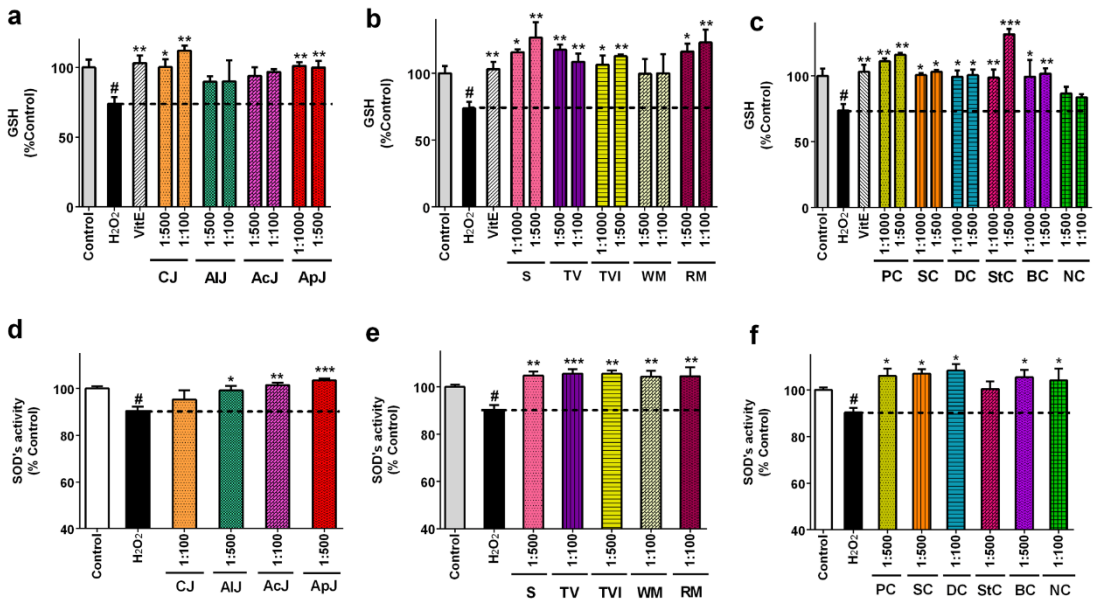


Figure 1. Effect of fruit drink polyphenolic extracts on an oxidative stress model in SH-SY5Y cells. Neuroprotective ability of carbonated juices (a), wine mixes and musts (b) and cider extracts (c) on cell viability. Effect on $\Delta\Psi_m$ (d-f). Measurement of ROS levels (g-i). Vitamin E (Vit E) at 25 µM was used as positive control. Mean±SEM of three replicates. *p<0.05, ** p<0.01, *** p<0.001 compared to H_2O_2 control. #p<0.05 *versus* untreated cells



458

459 **Figure 2**. Effect of beverage extracts on antioxidant systems in neuroblastoma cells. (a-c) GSH

460 levels determination. (d-f) SODs activity. Mean±SEM of three experiments. *p<0.05, **

461 p<0.01, *** p<0.001 compared to H_2O_2 control. #p<0.05 *versus* untreated cells

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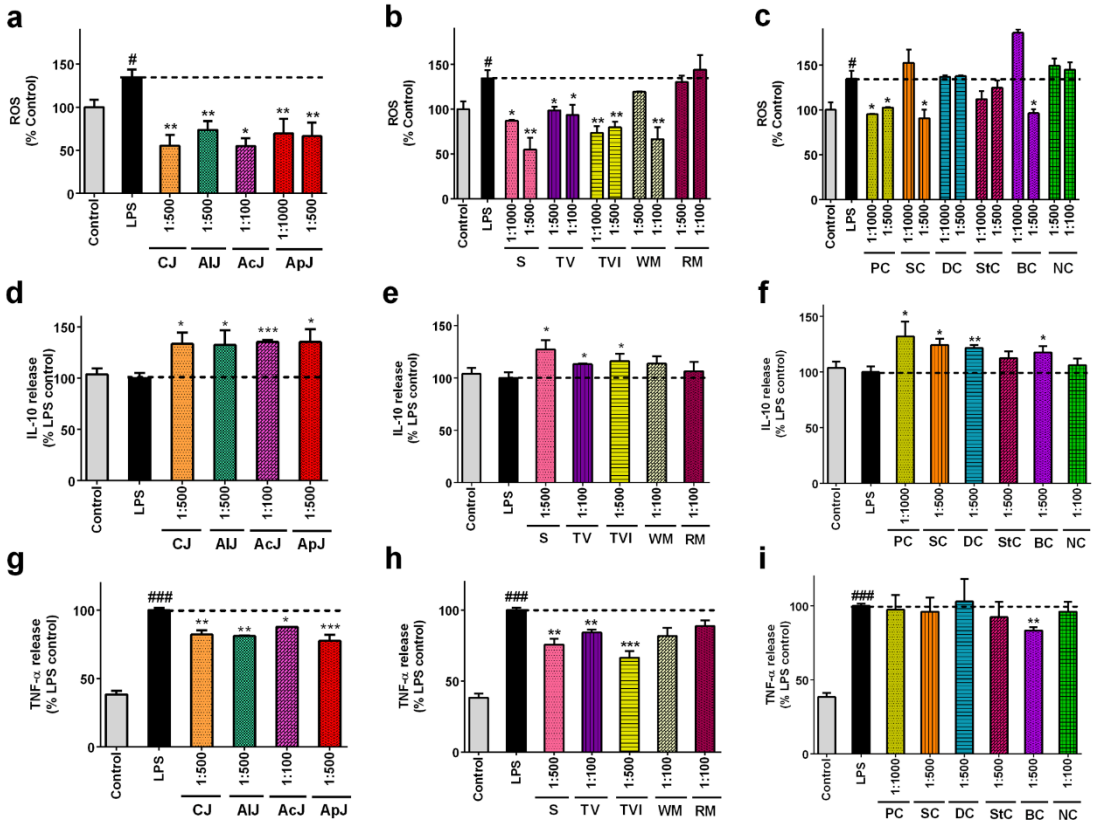


463

464 **Figure 3.** Evaluation of anti-neuroinflammatory properties of fruit drink extracts. (a-c)

465 Determination of ROS levels on BV2 cells. (d-f) The release to the medium of IL-10 and (g-i)

466 TNF-α was also determined. Mean±SEM of three experiments. *p<0.05, ** p<0.01, ***

467 p<0.001 *versus* LPS control cells. #p<0.05, ### p<0.001 compared to control cells

468

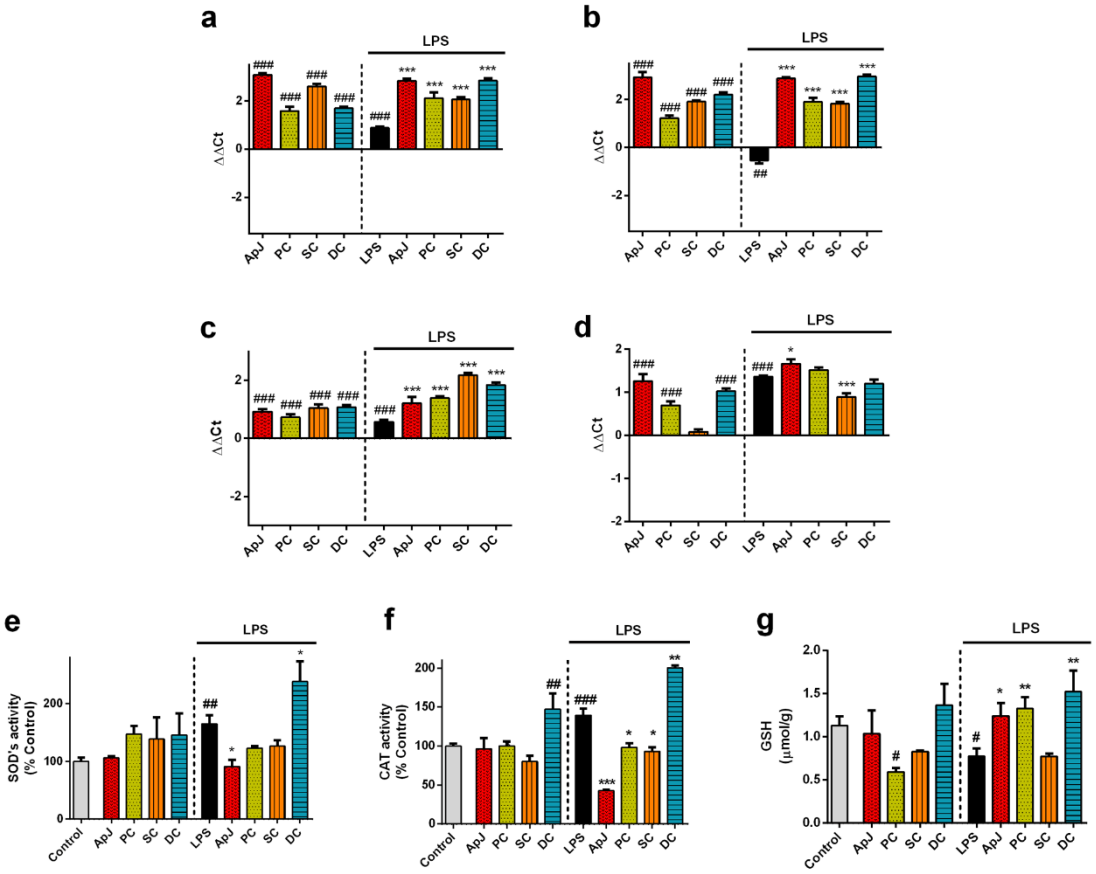
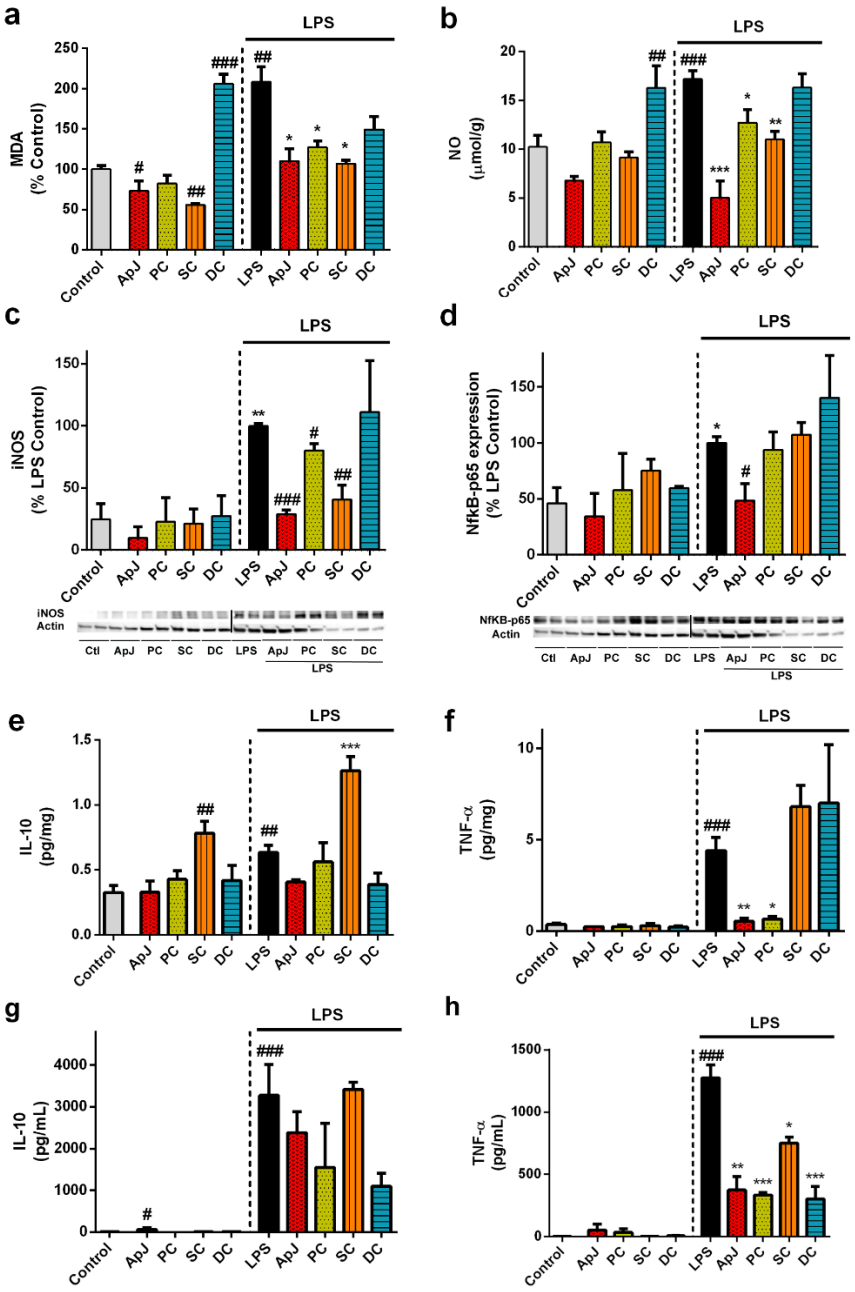


Figure 4. Antioxidant properties of concentrated drinks on mice brains. Relative gene expression of SOD1 (a), CAT (b), GPx1 (c) and Nrf2 (d) after treatment with selected beverages. Activity of SODs (e) and CAT (f), and GSH levels (g) in brain homogenates. **p*<0.05, ** *p*<0.01, *** *p*<0.001 compared to LPS-injected mice. #*p*<0.05, ## *p*<0.01, ### *p*<0.001 *versus* control mice



476

477 **Figure 5.** Determination of oxidative stress and inflammatory markers in mice. (a) Levels of
478 MDA and (b) NO in mice brains. (c) Expression levels of iNOS and (d) NFκB-p65 in brain.
479 Representative blots of two gels run and incubated in parallel, separated by a dividing line.
480 Controls were loaded in both gels, and samples were quantified with respect to their
481 corresponding control, although they were cropped to avoid repetitions. (e-f) Cytokine levels in
482 brain. (g-h) IL-10 and TNF-α levels in blood. *p<0.05, ** p<0.01, *** p<0.001 *versus* LPS-
483 injected mice. #p<0.05, ## p<0.01, ### p<0.001 compared to control.