Effects of oral phytoextract intake on phenolic concentration and redox homeostasis in murine encephalic regions

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Vegetable infusions (VI) are one of the main phenolic sources for humans. They may act as antioxidants in the central nervous system, but data about their effect are insufficient. The main objective of the study was to determinate the effects of oral VI of Argentinean plants on phenolic concentration and redox homeostasis in different murine encephalic regions. Redox changes (peroxides -HP-, anion superoxide -SO- and γ-glutamyltranspeptidase activity) and tissue phenolics were assessed in Balb/c mice of both sexes treated with the following VI extracts: *Lantana grisebachii* Seckt. var. *grisebachii* (Verbenaceae) (LG), *Aspidosperma quebracho-blanco* Schltdl. (Apocynaceae) (AQB), and *Ilex paraguariensis* A. St.-Hil. (Aquifoliaceae) (IP). Brain (telencephalon and diencephalon), midbrain, brainstem, and cerebellum were studied (analysis of variance, $P < 0.05$). A redox homeostasis depending on an appropriate phenolic balance was detected after marker analysis. Under situations without exogenous challenges, the excessive or deficient levels were deleterious on each region. This finding was confirmed independently of the utilized phytoextracts. LG and AQB caused such phenolic imbalance and triggered oxidative stress. IP group showed region-specific differential redox effects. Overall, the last extract exhibited the best redox profile when the complete encephalon was analyzed. Since this plant has sanitary impact due to its high human intake, new studies about it are warranted.

Keywords: Central nervous system, *Ilex paraguariensis*, Infusion, Oxidative stress, Polyphenol

Introduction

Oxidative damage has been defined as 'the biomolecular damage caused by attack of reactive species upon the constituents of living organisms',[1] with the central nervous system (CNS) being particularly susceptible to this damage.[2–4] Regarding these reactive species, superoxides and hydroperoxides are important oxidizing compounds but have a deleterious effect on living tissues if they are over-produced.[5] This process is involved in the pathogenesis of several neurodegenerative illnesses, such as Parkinson's disease and Alzheimer's disease.[3–4,6–8]

The redox status of CNS may be regulated by diet ingredients.[2,6,9,10] In this sense, vegetable foods contain numerous antioxidant molecules, such as phenolic compounds, and other phytochemicals and nutrients.[5,11,12] These substances, referred to generally as phytonutrients, could exert protective effects through free radical inactivation, modulation of cellular processes as proliferation, apoptosis, and redox balance in CNS.[13,14] However, solid experimental data are not enough in order to establish the optimal phytochemical intake and other nutritional concerns to achieve those desirable health effects.[15]

In nature, there are many phenolic compounds, including phenolic acids, flavonoids, stilbenes, and lignans.[16,17] Their dietary sources are fruits, oil seeds, and different plants, which are consumed as infusions or teas.[4,17] In general, infusions are one of the main phenolic sources, with a strong antioxidant activity against free radicals, nitrite oxide, and lipid oxidation.[2,4] Nonetheless, their bioactivity may depend on the phytochemical capacity to reach the different CNS regions: the brain (telencephalon and diencephalon), midbrain, cerebellum, and the brainstem. For example, some tea catechins are able to penetrate the blood–brain barrier (BBB) in low levels, showing a good bioavailability after oral administration,[2,7,18] also reducing stress-related beta-amyloid peptides in neurons.[19]

Reports about Argentinean plants have identified species with antioxidant activity and ethnobotanical uses.[11,20] Among them, *Lantana grisebachii* Seckt.

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DOI 10.1179/1476830514Y.0000000130

var. *grisebachii* (Verbenaceae) (LG), *Aspidosperma quebracho-blanco* Schltdl. (Apocynaceae) (AQB), and *Ilex paraguariensis* A. St.-Hil. (Aquifoliaceae) (IP) were selected to be assayed as neuroprotective candidates. Moreover, the *in vivo* analysis in the CNS represents a new insight into the biomedical potential of these plants.

This study aimed to determine in mice the redox changes in the encephalic regions after oral phytoextract intake and their association with tissue phenolic availability.

Materials and methods

Materials and equipment

Folin solution and xylenol orange, as well as other chemicals, were obtained from Sigma Aldrich Inc. (USA). Solvents were from Cicarelli S.A (Argentina).

Spectrophotometric readings were performed on a GloMax®-Multi microplate multimode reader (Promega Corp., USA).

Plant extracts

After the corresponding governmental consent (Cordoba MINCYT, Argentina), plant samples of LG and AQB were collected during the summer from the mountain zone of the Chaquenian phytogeographic region in Central Argentina (31°15′40″ S, 64°27′50″ W). The identified specimens were stored in the RIOC Herbarium (UNRC, Argentina). IP was obtained from commercial organic cultures. Ten grams of pulverized air-dried samples were extracted by adding 50 ml of initially heated water at 95°C. These infusions were allowed to cool at room temperature for 1 hour reaching a final temperature of 35–40°C (in darkness, with constant agitation). After the blends were centrifuged, extracts were recovered from each supernatant by filtration and lyophilized for 24 hours at $<-$ 50°C under vacuum. Finally, the phytonutritional profiles of the extracts are as follows:

- LG: 10.23 ± 0.43 gallic equivalent mg/g of dry extract (total phenolics, TP), with 58.46% of total flavonoids (5.98 ± 0.12 quercetin equivalent mg/g).
- AQB: 2.57 ± 0.14 mg/g of TP, with 37.74% of total flavonoids (0.97 ± 0.06 mg/g).
- IP: 21.77 ± 1.37 mg/g of TP, with 24.48% of total flavonoids (5.33 ± 0.13 mg/g).[21]

Extract antioxidant activities have been established *in vitro* by FRAP (Fe + +μmole/g of plant).[5] LG = 351.30 ± 10.89; AQB = 234.30 ± 8.63; and IP = 1653.57 ± 24.86. Other specific phytochemical analyses of these plants are available.[22–24]

Experimental conditions

This study was carried out according to the ethical and technical US guidelines; 2-month-old Balb/c mice of both sexes were treated and sacrificed. Animals were bred under standard laboratory conditions and fed *ad libitum* with commercial diet (200 ± 13 g/kg/day; Cargill SACI, Argentina) and potable water (150 ± 10 mL/kg/day; Aguas Cordobesas SA, Argentina). They were separated into four experimental groups of six animals each one to be treated *in vivo* (oral intake) for 1 month: C (water without extract), AQB, LG, and IP (100 mg/kg/day of each water-dissolved extract, equivalent to the consumption of 2 liters of infusion in humans approximately). General status, weight, food, and water consumption were controlled weekly. After the treatment, the mice were sacrificed, the organs were weighed, and divided into different regions: *brain* (*telencephalon* and *diencephalon*) *midbrain, brainstem*, and *cerebellum*. These tissues were mechanically homogenized to assay proteins (using the Bradford method) and γ-glutamyltranspeptidase (GGT) activity, and then extracted (0.1 g of tissue in 1 ml of 60% methanol and 2.5% trichloroacetic acid, for 30 minutes at 50°C) to assay the other biochemical parameters.

Determinations

Total phenols (TP) were measured in murine tissue extracts according to the Folin–Ciocalteu technique.[25] Briefly, the samples were mixed with 2 N Folin reactant, water, and saturated sodium bicarbonate solution (1:1:6:2 v/v/v/v), and incubated for 30 minutes in darkness at 37°C. Results at 750 nm were calculated from a standard curve of gallic acid (0–0.1 mg/ml) and expressed per protein mg.

Aqueous and lipid peroxides (AHP and LHP, respectively) were analyzed in murine tissue homogenates. Samples were mixed with a xylenol orange-based reactant (1:10 v/v), and incubated for 30 minutes. Then, hydroperoxide (HP) concentrations were standardized by the protein content and then expressed as percentages of absorbance (560 nm) with respect to control (%).[11]

Superoxide anion (SO) was assessed spectrophotometrically in murine tissue extracts by nitroblue tetrazolium (1 mg/ml) staining. Samples were mixed with the colorant (9:1 v/v), and incubated for 30 minutes in darkness at 37°C. Then, this mix was added with dimethylsulfoxide and potassium hydroxide (2:1:1 v/v/v) before reading at 600 nm. SO absorbance were divided per protein content and then expressed as percentage with respect to control (%).

Specific GGT activity (EC 2.3.2.2) was measured in homogenates using the kinetic Szasz method, given its significant role in glutathione metabolism.[26] Briefly, the samples were mixed with kit reactants (pH 8.25, 100 mmole/l Tris buffer, 2.9 mmole/l L-γ-glutamyl-3-carboxy-4-nitroanilide, and 100 mmole/l glycylglycine) at 25°C. Catalytic international units were calculated by multiplying 1158 times (a constant) the

absorbance difference/minute, divided by per protein content (mIU/mg). Then, the GGT activity was expressed as positive or negative (using technical precision criteria as cut point) under conditions of initial velocity and linearity ranges.

Statistical analysis

Data were expressed as mean ± standard error (SE) from three separate experiments performed in triplicate. Age and body weight-fixed analysis of variance models were used to evaluate differences of tissue phenolic content, oxidation, and GGT activity (effects) among treatments (C, AQB, LG, and IP). Then, the Tukey test was used for mean comparisons, considering a significant level of $P < 0.05$. Analytical probes were performed using the InfoStat 2012 software (InfoStat Group, Argentina).

Results

Encephalic TP were higher in male mice treated with LG and IP and lower with AQB extract in females ($P < 0.05$) with respect to the control (C) (Fig. 1).

Moreover, the intake of the different plant extracts was associated with a general oxidative status of the CNS (Fig. 2).

Brain

Telencephalon

TP were increased by LG and IP treatments (157 and 161%, respectively) ($P < 0.05$) in males, whereas TP were decreased to 42% by LG in females ($P < 0.05$) (Fig. 3). AHP were increased by LG and IP treatments (270% in both cases) in males, and these extracts also increased them (256%) in females ($P < 0.05$). In males, LHP were reduced by the AQB treatment (43% with respect to controls; $P < 0.05$).

In female mice, SO determinations were increased significantly ($P < 0.05$) by all treatments. The GGT concentrations were elevated only in male mice treatments with the IP extract (0.30 ± 0.04) with respect to the control group (0.07 ± 0.03) ($P < 0.05$) (Table 1).

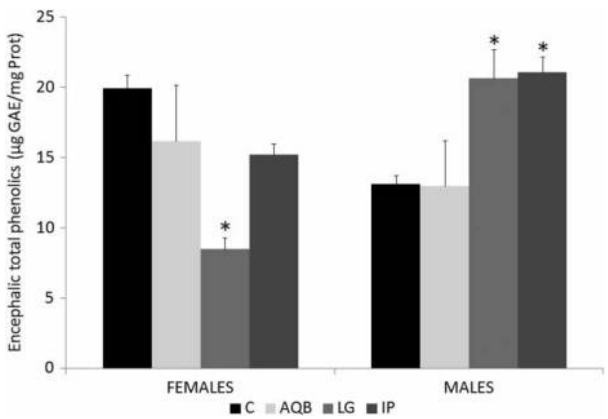

Figure 1 Encephalic total phenolics in Balb/c mice treated for a month with 0 mg/kg/day (control) or 100 mg/kg/day of each plant extract. C: control, AQB: *A. quebracho-blanco*, LG: *L. grisebachii*, IP: *I. paraguariensis*. Mean ± SE are expressed as µg GAE (gallic acid equivalents) per mg proteins (*$P < 0.05$).

Diencephalon

The levels of TP were elevated 11 times in females treated with AQB ($P < 0.05$) (Fig. 3). In this experimental group, AHP and LHP were augmented (500 and 1600%, respectively) ($P < 0.05$). On the other hand, no significant modifications with respect to C of these parameters were observed in males with any phytoextract treatments.

Female mice treated with AQB showed a decrease of SO ($P < 0.05$). GGT determinations were reduced by all treatments in females and were greater due to AQB in males (1.12 ± 0.00) with respect to the control group (0.07 ± 0.03) ($P < 0.05$) (Table 1).

Midbrain

In both sexes, TP were not detected under the different treatments, except for the AQB extract ($P < 0.05$) (Fig. 3). AHP tissue levels were elevated by all treatments: AQB, LG, and IP (714, 952, and 476%, respectively) in females ($P < 0.05$), whereas only AQB extract increased AHP by three-fold in males ($P < 0.05$). In both sexes, LHP concentrations were augmented by AQB and LG treatments (278% in males and 313% in females, with similar amounts with both extracts; $P < 0.05$).

SO concentrations were decreased by IP/LG and AQB/LG in female and male mice, respectively ($P < 0.05$). Only female mice GGT concentrations were decreased by LG and IP extracts (0.00 ± 0.00) with respect to the control group (0.07 ± 0.02) ($P < 0.05$) (Table 1).

Brainstem

TP contents were higher in AQB and LG treatments (1.8 and 2.5%, respectively, $P < 0.05$) than C females and males, respectively (Fig. 3).

AHP was increased by LG (250 and 300% in females and males, $P < 0.05$) in both sexes while the LHP levels were elevated by LG and IP (322 and 1240%, respectively, $P < 0.05$) only in females.

SO was decreased in the female mice treated with LG and increased in male mice that received the AQB extract ($P < 0.05$). The GGT activity was diminished by IP (0.04 ± 0.01) and LG treatments (0.02 ± 0.00) with respect to the control group (0.09 ± 0.02) in female mice and only by IP extracts (0.00 ± 0.00) with respect to the control group in male mice (0.15 ± 0.02; $P < 0.05$) (Table 1).

Cerebellum

A decrease of TP in male mice treated with AQB and IP was observed in relation to the C group (48 and 36%, respectively, $P < 0.05$; Fig. 3). Moreover, AHP

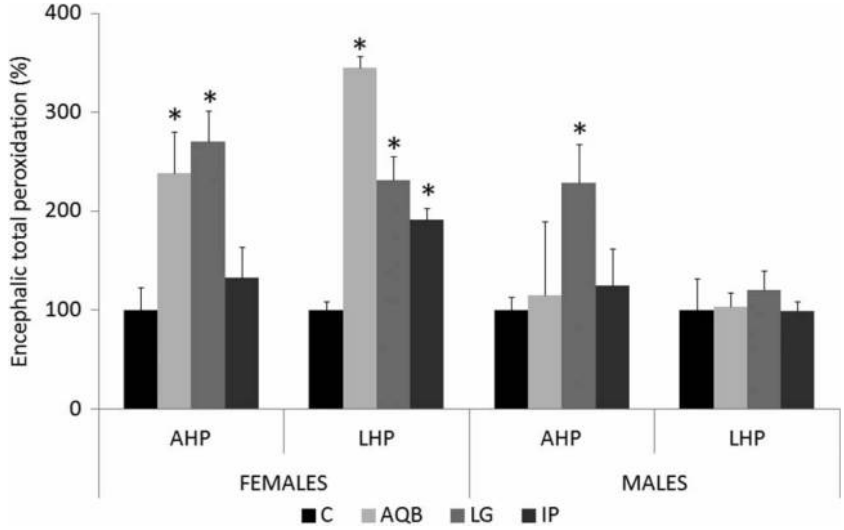

Figure 2 Encephalic total peroxidation: aqueous and lipid peroxides (AHP and LHP) in Balb/c mice treated for a month with 0 mg/kg/day (control) or 100 mg/kg/day of each plant extract. C: control, AQB: *A. quebracho-blanco*, LG: *L. grisebachii*, IP: *I. paraguariensis*. Mean ± SE are expressed as percentages of absorbance with respect to the control after standardizing by the protein content (%) (*$P < 0.05$).

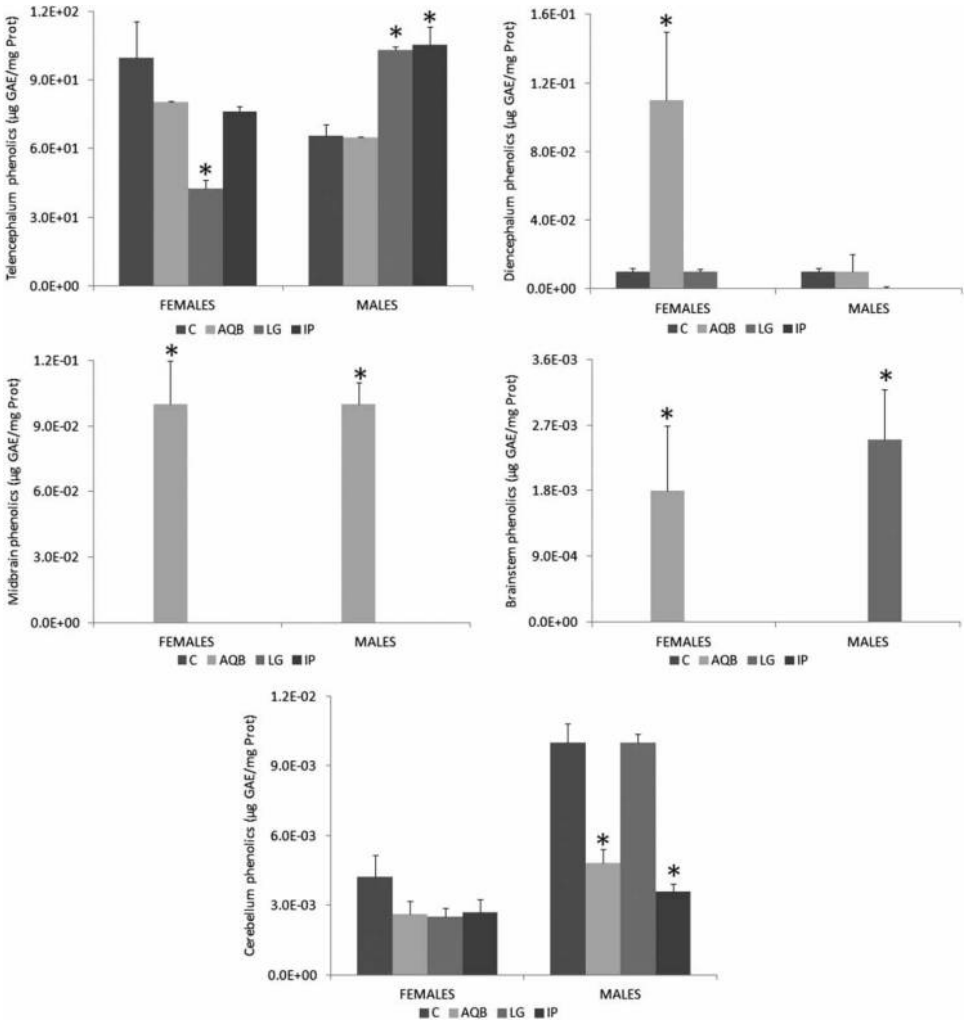

Figure 3 Total phenolics in different regions of CNS in Balb/c mice treated for a month with 0 mg/kg/day (control) or 100 mg/kg/day of each plant extract. C: control, AQB: *A. quebracho-blanco*, LG: *L. grisebachii*, IP: *I. paraguariensis*. Mean ± SE are expressed as µg GAE (gallic acid equivalents) per mg proteins (*$P < 0.05$).

Table 1 Redox biomarkers in different encephalic regions of Balb/C mice. Mean ± SE are expressed as percentages (%) (AHP, LHP, and SO) and IU/mg (GGT). C: Control, AQB: *A. quebracho-blanco*, LG: *L. grisebachii*, IP: *I. paraguariensis* (*$P < 0.05$).

Treatments	Aqueous peroxides (%)		Lipid peroxides (%)		Superoxide anion (%)		GGT activity (IU/mg)	
	Females	Males	Females	Males	Females	Males	Females	Males
Telencephalon								
C	100.00 ± 12.82	100.00 ± 06.21	100.00 ± 00.80	100.00 ± 08.80	100.03 ± 08.72	100.00 ± 12.47	0.19 ± 0.04	0.07 ± 0.03
AQB	115.38 ± 43.58	089.18 ± 08.10	100.00 ± 15.00	043.00 ± 11.00*	175.89 ± 06.95*	076.15 ± 06.15	0.18 ± 0.05	0.12 ± 0.00
LG	256.41 ± 12.81*	270.27 ± 06.21*	100.00 ± 13.00	100.00 ± 13.00	137.96 ± 05.29*	079.00 ± 14.75	0.30 ± 0.04	0.00 ± 0.00
IP	256.41 ± 35.89*	270.27 ± 08.64*	100.00 ± 05.00	100.00 ± 05.80	177.28 ± 21.47*	093.99 ± 03.73	0.10 ± 0.02	0.30 ± 0.04*
Diencephalon								
C	100.00 ± 04.20	100.00 ± 15.00	100.00 ± 18.80	100.00 ± 50.00	099.97 ± 05.39	100.00 ± 04.94	2.11 ± 0.93	0.07 ± 0.03
AQB	500.00 ± 100.0*	034.00 ± 06.50	1600 ± 400.00*	123.07 ± 19.23	070.74 ± 09.70*	090.65 ± 09.35	0.00 ± 0.00*	1.12 ± 0.00*
LG	200.00 ± 48.00	100.00 ± 16.00	148.00 ± 18.00	130.76 ± 24.61	075.18 ± 13.14	087.68 ± 04.75	0.00 ± 0.00*	0.25 ± 0.04
IP	037.00 ± 19.00	100.00 ± 16.00	064.00 ± 19.60	103.84 ± 08.46	094.72 ± 10.07	110.14 ± 07.79	0.00 ± 0.00*	0.29 ± 0.20
Midbrain								
C	100.00 ± 80.95	100.00 ± 14.00	100.00 ± 21.87	100.00 ± 44.40	100.00 ± 05.29	100.03 ± 15.73	0.07 ± 0.02	0.20 ± 0.03
AQB	714.28 ± 42.85*	300.00 ± 22.00*	312.50 ± 65.62*	277.77 ± 36.11*	070.10 ± 15.49	052.57 ± 21.11*	0.09 ± 0.01	0.17 ± 0.00
LG	952.38 ± 23.80*	200.00 ± 25.00	312.50 ± 75.00*	277.77 ± 18.80*	069.76 ± 09.99*	051.80 ± 03.84*	0.01 ± 0.01*	0.12 ± 0.01
IP	476.19 ± 97.61*	100.00 ± 12.00	084.37 ± 20.00	086.10 ± 15.27	078.01 ± 03.77*	081.73 ± 03.66	0.00 ± 0.00*	0.23 ± 0.12
Brainstem								
C	100.00 ± 06.50	100.00 ± 00.33	100.00 ± 18.38	100.00 ± 46.00	099.97 ± 05.25	100.00 ± 04.50	0.09 ± 0.02	0.15 ± 0.02
AQB	100.00 ± 20.50	035.00 ± 05.50	322.58 ± 26.12*	100.00 ± 01.00	106.53 ± 03.26	106.53 ± 03.26*	0.09 ± 0.01	0.16 ± 0.04
LG	250.00 ± 50.00*	300.00 ± 39.00*	1240.3 ± 54.83*	100.00 ± 39.00	077.16 ± 01.80*	077.16 ± 01.80	0.02 ± 0.00*	0.09 ± 0.02
IP	100.00 ± 12.50	046.00 ± 00.78	695.16 ± 100.0	100.00 ± 07.80	105.44 ± 19.26	105.44 ± 19.26	0.04 ± 0.01*	0.00 ± 0.00*
Cerebellum								
C	100.00 ± 08.70	100.00 ± 08.40	100.00 ± 04.54	100.00 ± 12.00	100.04 ± 15.36	099.95 ± 16.21	0.00 ± 0.00	0.00 ± 0.00
AQB	100.00 ± 03.80	100.00 ± 01.50	227.27 ± 88.63	100.00 ± 14.00	080.07 ± 15.24	084.56 ± 32.27	0.03 ± 0.01	0.19 ± 0.07
LG	100.00 ± 06.40	300.00 ± 28.00*	227.27 ± 59.09	100.00 ± 07.00	121.59 ± 14.50	106.48 ± 30.56	0.00 ± 0.00	0.09 ± 0.01
IP	100.00 ± 18.00	200.00 ± 32.00*	227.27 ± 52.27	100.00 ± 09.50	080.07 ± 15.24	107.57 ± 09.22	0.14 ± 0.05*	0.07 ± 0.01

was raised by LG and IP treatments (200 and 300%, respectively, $P < 0.05$) in males although extract effects were not statistically significant on LHP concentration with respect to C. SO was unaffected, whereas the GGT concentration was increased by IP (0.14 ± 0.05) with respect to the control group (0.00 ± 0.00) in female mice ($P < 0.05$; Table 1).

Discussion

In the present work, wide variations were observed in the redox state of the studied encephalic regions of mice after each phytoextract intake.

The first plant considered was IP because it is the main species consumed by humans in South America with approximate values of 2 liters per day.[22] Concerning the redox markers used, SO is a transient precursor of different HP (initial aqueous forms, such as hydrogen peroxide) and lipoperoxides (later organic derivatives). This chain reaction was induced by IP consumption in the murine cerebellum and brain of both sexes, with telencephalon being affected, whereas initiation of this oxidative pathway was prevented subcortically. This differential redox susceptibility of the brain cortex has been established previously in phytonutritional approaches.[7] Moreover, the tissue GGT activity was consistent with these changes, thus confirming the cellular stress response.[26]

The phenolic augmentation in male telencephalon was due to their high systemic bioavailability,[21] which favored their passage through BBB.[6,8] This finding is similar to other studies that demonstrate the capacity of infusion-derived phenolics to concentrate in the CNS after oral intake.[2] However, this effect was not linked to the observed redox modifications. Thus, these outcomes might respond to other BBB-permeable excitatory compounds present in IP.[22] In fact, this experimental group showed increased diurnal wakefulness,[21] which in turn may predispose to cortical oxidation without affecting the other brain regions.[27]

The sexual differences in phenolic concentration were seen in the telencephalon and brainstem of animals that consumed LG, leading to an imbalance in both cases: deficiency in female mice and accumulation in male ones, which in turn might cause redox disturbances.[28] Since the oral bioavailability and phytochemical profile were the same for both sexes,[21] it was inferred that active transport processes were opposed in males (influx) and females (efflux/clearance). However, specific mechanisms are unknown. Nevertheless, the encephalic GGT inhibition in females could be involved. This enzyme is a structural and functional marker of the BBB,[29,30] since it promotes barrier permeability and water fluxes,[31] key for infusion-derived compounds. Thus, its lesser catalytic activity, usually observed in female tissues,[32] might explain the phenolic reduction found here. Furthermore, LG consumption has proved to inhibit GGT in rats.[24] The intake of this plant extract was associated with a general oxidative status of the CNS, which was expected in accordance with previous results.[24]

The AQB treatment induced phenolic redistribution in female encephalon leading to subcortical increases (brain, midbrain, and brainstem), which was related to peroxyl triggering.[28] Moreover, females showed a residual oxidative effect in the telencephalon. Otherwise, males responded to this extract to less extent, with redox imbalance being only mesencephalic. The sex-related pharmacokinetic features of phenolics may explain their increase in female encephalon with respect to males (with high metabolism and clearance).[33]

On the other hand, the cerebellum was less responsive to the different extract treatments than the supratentorial organs of both sexes.

Although plant polyphenols have been proposed as a panacea for human health,[34] the appropriate phenolic concentration in CNS tissues is a critical factor in order to maintain redox homeostasis in mice, given that deficient or excessive levels are deleterious (mainly under situations without exogenous challenges), leading to oxidative stress independently of the utilized phytoextracts. Among them, LG and AQB caused this phenolic imbalance and exhibited oxidizing effects. Then again, IP showed differential region-specific redox effects; however, this extract showed the best redox profile when complete encephalon was analyzed. In accordance to these results and given the massive IP consumption by humans,[22] this plant could be a source of chemopreventive agents for oxidative stress-related neurodegenerative pathologies with high sanitary impact. Thus, new studies about it are warranted.

Disclaimer statements

Contributors

The four authors participated in the design, experiments, redaction and critical revision of the manuscript.

Funding

None.

Conflicts of interest

None.

Ethics approval

None.

Acknowledgements

This study was financially supported by the SECYT (National University of Cordoba, Argentina) and the

INC (National Ministry of Health), with the authors declaring that there were no conflicts of interest. Authors thank Garrett Gardner, a native English speaker, and Daniela Defagó PhD (Harvard School of Public Health, USA) for critical revision of the manuscript.

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