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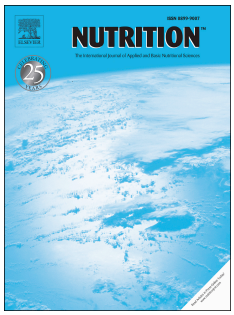

Dietary Fats Significantly Influence the Survival of Penumbral Neurons in a Permanent Isquemic Stroke Rat Model by Modifying Lipid Mediators, Inflammatory Biomarkers, Nos Production, and Redox-Dependent Apoptotic Signals

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PII: S0899-9007(15)00239-7

DOI: 10.1016/j.nut.2015.05.023

Reference: NUT 9548

To appear in: *Nutrition*

Received Date: 9 December 2014

Revised Date: 1 May 2015

Accepted Date: 21 May 2015

Please cite this article as: Lausada N, Arnal N, Astiz M, Marín MC, Lofeudo JM, Stringa . P, Tacconi de Alaniz MJ, Tacconi de Gómez Dumm N, Hurtado de Catalfo G, Cristalli de Piñero N, Pallanza de Stringa MC, Illara de Bozzolo EM, Bozzarello EG, Cristalli DO, Marra CA, Dietary Fats Significantly Influence the Survival of Penumbral Neurons in a Permanent Isquemic Stroke Rat Model by Modifying Lipid Mediators, Inflammatory Biomarkers, Nos Production, and Redox-Dependent Apoptotic Signals, *Nutrition* (2015), doi: 10.1016/j.nut.2015.05.023.

Type of paper: Basic nutritional investigation

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Abstract

Abstract

Objective: Brain stroke is the third most important cause of death in developed countries. We study the effect of different dietary lipids on the outcome of a permanent ischemic stroke rat model.

Research Methods & Procedures: Wistar rats were fed on diets containing 7 % commercial oils (S, soybean; O, olive; C, coconut; G, grape seed) for 35 days. Stroke was induced by permanent middle cerebral artery occlusion. Coronal slices from ischemic brains and sham-operated animals were supravitally stained. Penumbra and core volumes were calculated by image digitalization after 24, 48 and 72 h post stroke. Homogenates and mitochondrial fractions were prepared from different zones and analyzed by redox status, inflammatory markers, ceramide and arachidonate content, phospholipase A2, NOS, and proteases.

Results: S and G diets were mainly pro-oxidative and pro-inflammatory by increasing the liberation of arachidonate and its transformation into prostaglandins. O was protective in terms of redox homeostatic balance, minor increase in lipid and protein damage, conservation of reduced glutathione, protective activation of NOS in penumbra, and net ratio of anti- to pro-inflammatory cytokines. Apoptosis (caspase-3, milli- and micro-calpains) was less activated by O than by any other diet.

Conclusion: dietary lipids modulate NOS and PLA2 activities, ceramide production, and glutathione import into the mitochondrial matrix, finally determining the activation of the two main protease systems involved in programmed cell death. Olive oil appears to be a biological source for the isolation of protective agents that block the expansion of brain core at the expense of penumbral neurons.

Keywords: dietary lipids; ischemic stroke; neuronal death; lipid mediators; oxidative stress; inflammation

Introduction

Brain stroke is the third cause of death in developed countries, behind all types of cancers and hearth disease [1]. Cerebral ischemia generally results in immense distress and residual impairments to patients, and it is, therefore, viewed as one of the leading causes of decreased quality of life and living. In humans, ischemic stroke approximately accounts for 90 % of all strokes and specially affects the territory of the middle cerebral artery (MCA) [2]. Cerebral stroke is one of the diseases primarily linked to nutritional factors [1].

Occlusion of MCA (MCAO) is the most used worldwide experimental model to induce stroke in rats and it is has been proven as an effective and reproducible tool for the investigation of neuroprotective drugs as it closely resembles stroke injury in human patients [2]. Cerebral ischemia has been considered as untreatable pathology with no effective therapeutic protocols [1]. Human ischemic stroke is heterogeneous in its manifestations, causes, and anatomic sites of occurrence. Consequently, a wide variety of animal models have been developed to assess stroke-related pathologies in order to find better approaches to the study of neuronal injury, specially the recruitment of neurons from the surrounding tissues (penumbral zone or region) into ischemic core [1]. So far, it is known that there is a relative short window of opportunity during which a population of penumbral neurons remains viable for a variable number of hours following stroke onset [3]. Thus, penumbra is defined as a moderately hypoperfused region that retains structural integrity but has lost function [4,5]. Even with the implementation of therapeutic interventions this penumbra area will die and become recruited into the core ischemic zone [5]. Only 2 % of stroke patients receive tissue plasminogen activator (tPA) as thrombolytic agent [6]. The lack of effective therapeutic agents establishes a high unmet medical need for the development of stroke preventive strategies [7].

A number of previous studies has demonstrated that oxidative stress [8-14] and inflammation [15-17] play a crucial role in the pathogenesis of brain stroke [8,12,13]. These

factors strongly influence many other signals that converge into the regulation of pro-apoptotic cascade [8,10,12,13], in which phospholipase A2 [18], production of prostaglandins and cytokines [15-17], activation of nitric oxide synthethase activity [19-22], and ceramide overproduction [23,24] are the most crucial events affecting the final result.

Previous statistical evidence and experimental results clearly demonstrated that the quality and quantity of dietary fats and fatty acids significantly influence the incidence, prevalence, and outcome of brain stroke and ischemic heart disease [25,26]. Fatty acids may modulate inflammatory response [27,28], modify the antioxidant status of many tissues [29,30,31], and regulate ceramide generation [32]. In addition, several studies confirmed that other components of dietary fats such as polyphenols play an important role in the prevention of stroke and cardiovascular illnesses or they attenuate the detrimental effects caused by ischemia [33-41].

Based on previous evidence, we decided to investigate in detail the influence of different types of commercial oils as dietary lipid source in relation to the outcome of ischemic stroke in a MCAO rat model from 24 to 72 h post-stroke by determining: (1) the redox status (antioxidant enzymes and levels of lipid-soluble and water-soluble antioxidants), (2) the inflammatory response (prostaglandin and citokine production), (3) activity of phospholipase A2 and nitric oxide synthetase isoforms, (4) production of ceramide, and (5) activation of cellular death cascades (caspase-3 and milli- and micro-calpains) in order to contribute with experimental evidence that supports nutritional recommendation for the prevention of damages produced by brain stroke.

Research Methods and Procedures

Chemicals

All chemicals used were of analytical grade and obtained from Sigma Chem. Co. (Buenos Aires, Argentina or USA), Merck (Darmstadt, Germany), or Carlo Erba (Milan,

Italy). Dietary commercial oils were from Molinos Río de La Plata SAIC and Platafarm SA (La Plata, Argentina).

Animals and treatments

Certified pathogen-free male Wistar rats were used. The rats were maintained under controlled temperature conditions (25 º C) with relative humidity of 60 %, forced ventilation and a normal photoperiod of 12-h darkness and 12-h light. Animals were handled in accordance with the internationally recommended practices of the ILAR (*Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council* and the NIH regulations [42]. Solid food and drinking water were provided *ad libitum*. The experimental diets were prepared in our laboratory according to the recommendations for Wistar rats [43]. The experimental protocol was reviewed and approved by the Bioethics Committee of the School of Medical Sciences; UNLP (COBIMED, protocol code # 00382/11). Male Wistar pups (three weeks old, 48 ± 3 g/animal) were used. Animals were ramdomly asigned to four experimental groups (twelve animals for each experiment) and fed after weaning on specific diets for 35 d. The diets used were supplemented with a different oil as lipid source: soybean (S), olive (O), cocconut (C) or grape-seed (G). During the feeding period clinical examination, body weight, foot intake and water consumption were daily controlled [44]. All isocaloric diets were prepared in identical manner with the addition of one of the different commercial oils (70 g/ Kg diet) as detailed previously [30,31]. General composition and fatty acid composition of the diets were reported in detail in previuos papers [30,31].

Surgical procedure

To avoid individual differences among animals the experiments were run under equivalent conditions. On day 35 all rats were fasted overnight with free access to water, and

surgical procedure was implemented at 08:00 a.m. Anesthesia was conducted as recomended elsewhere [45]. Feedback-controlled heating lamp and pad were used to maintain animal temperature at 37 ºC. Stroke was induced by permanent middle cerebral artery occlusion model (MCAO) following the procedure of Longa et al. [46]. None of the animals experimented subarachnoid hemorraghe. This rat model of permanent ischemia is the most commonly used due of many reasons including up to two-week survival post-stroke, high reproducibility (involving frontoparietal cortex and lateral caudoputamen), and its resemblance to human in cerebrovascular anatomy and physiology [47-49]. Transient occlusion is considered inappropriate for the replication of spontaneous or thromboslysis-induced stroke [50,51].

Obtention of samples

We performed different sets of experiments. In one type, after various times post-stroke (24, 48 and 72 h) rats were killed by rapid decapitation withouth the use of anesthesia. To obtain plasma samples, blood was collected into heparinized (10 mUI/mL) sterile polystyrene test tubes and centrifuged at 2ºC, 1000 x g for 15 min. The brains were excised, cleaned, weighed and frozen for 5 min at -20 ºC. Coronal slices of 1 mm were performed beginning at 2 mm from the anterior tip of the frontal lobe. Sections were immersed in 2 % 2,3,5-triphenyltetrazolium chloride (TTZ) at 37 ºC for 20 min with gentle orbital agitation. Slices were photographed and analyzed using the Software Erdas Image v8.0 (Infosat Geomática, Buenos Aires, Argentina) that can digitalize and quantify infart area, penumbra, and undamage hemisphere (contralateral). After digitalizaction was complete, image analysis was compiled to obtain stroke core, penumbral zone and undamage tissue volumes in mm^3. Sham-operated animals were also run as reference. In all cases, the values obtained were corrected by edema using the fomula [52]: [total infarct volume – (volume of intact ipsilateral hemisphere – volume of intact contralateral hemipshere)]/[contralateral hemisphere

volume].100. In other types of experiments we dissected the core of the infarct zone from the penumbra using the methodology described elsewhere [24]. Tissues from contralateral hemipshere and sham-operated animals were also taken. From these biological materials we prepared whole homogenates using HEPES 50 mM pH 7.4 with CHAPS 5 mM, dithiothreitol 5 mM and aprotinin 10 mg/mL, in a proportion of 6 mL buffer to each 100 mg tissue. Also crude mitochondrial suspensions were prepared by two-step centrifugation (600g/11.000g) method following the procedure described elsewhere [5]. For lipid analysis and glutathione content mitochondrial suspensions were previously sonicated on ice (three 45-sec bursts at 50 % output in a Heat Systems Ultrasonic sonicator model W-220F from Plainview, NY).

Analytical methods

Biomarkers of ROS production

Markers of ROS-induced damage were determined according to classical methodologies. Thiobarbituric acid-reactive substances (TBARS) were measured in brain homogenates as previously described [53]. TBARS (mainly malondialdehyde, MDA) were reacted with 2-thiobarbituric acid (TBA) to yield TBA-MDA adducts and quantified at 532 nm. The concentration of the chromophore was calculated from a calibration curve prepared with fresh tetramethoxypropane (TMP) solutions (TMP was purchased from Sigma Chem. Co., Buenos Aires, Argentina). Small aliquots of total lipids extracts (see below) were used to determine the spectral register of the conjugate diene formation at 234 nm [54]. Nitrate and nitrite [NOx] concentrations were measured using the method by Griess on samples previously reduced with vanadium chlorohydrate [55]. Quantification was performed after calibration with standard solutions of sodium nitrate from Merck Co. (Darmstadt, Germany). Protein carbonyls (PCs) were determined by the method by Reznick and Packer [56]. PC concentrations were calculated from a calibration curve prepared with a stock solution of sodium pyruvate (Sigma Chem. Co.). DNA damage was assessed by the classical

electrophoretic methodology using 1 % agarose gels and DAPI staining. After examination under UV light (435 nm) gels were photographed and scanned with a CCD-L4 densitometer (Kinetic Imaging Ltd., Liverpool, UK) [57].

Antioxidant defense enzymes

The activity of catalase (CAT) was determined using the method by Aebi [58]. *K* values from the curves were electronically calculated using the equation $k = (2.30/t).\log (DO_f - DO_i)$. The specific activity of the enzyme was obtained considering the concentration of proteins corresponding to each sample. Superoxide dismutase (SOD) was determined using the method by Misra and Fridovich [59]. To calculate the activity of SOD in the samples we performed a reaction without sample homogenate in order to obtain the rate of the auto-oxidation of epinephrine. An enzymatic unit was defined as the amount which inhibits 50 % of auto-oxidation. To obtain the specific activity of SOD the ratios between the values of enzymatic units of each sample and the corresponding values of protein concentration (mg/mL) were calculated. The activity of glutathione transferase (GST) was determined by the method by Habig et al. [60]. The specific activity of the enzyme was calculated for each sample in terms of nanomoles of product/min.mg of total cellular protein, with $\varepsilon = 9.6\ mM^{-1}.\ cm^{-1}$ (340 nm). Glutathione peroxidase (GPx) was determined by the method of Flohé and Gunzler [61]. To calculate the enzymatic units (in terms of nmoles of NADPH /min), $\varepsilon = 6.22\ mM^{-1}.\ cm^{-1}$ (340 nm) was considered. The specific activity was obtained as units of enzyme activity/corresponding value of protein content (mg/mL). Glutathione reductase (GR) was determined by the method by Carlberg and Mannervik [62]. The specific activity of the enzyme was calculated for each sample in terms of micromoles of product/min.mg protein, with $\varepsilon = 6.22\ mM\text{-}^{1}.\ cm^{-1}$ (340 nm).

Antioxidant molecules

The levels of reduced (GSH) and oxidized (GSSG) glutathione were determined by the glutathione reductase/dithio-nitrobenzoic (DTNB) method [63]. To calculate the ratio GSH/GSSG samples were re-analyzed after derivatization with divinyl-pyridine (3 mM final concentration). Aliquots for glutathione analyses were immediately treated with equal volume of $HClO_4$ (12 % W/V) and N-ethylmaleimide (40 mM final concentration) [64] in order to preserve the proportion between oxidized and reduced glutathione. To calculate the ratio GSH/GSSG, samples were re-analyzed after derivatization with divinyl-pyridine (3 mM final concentration). Vitamin E (α-tocopherol) and retinol concentrations were measured after extraction with the Buttriss and Diplock method [65] using an HPLC technique [66] with UV detection (results in pmol/mg protein and nmoles/mg prot., respectively). Ascorbate was determined in deferoxamine mesylate-treated samples by the method of Benzie [67]. Total antioxidant reducing ability (FRAP assay) was measured according to Benzie and Strain [68]. FRAP values were obtained by comparing the change in absorbance at 593 nm (slope of the initial velocity reaction from 0 to 2 min) in the test sample mixtures with standards containing known concentrations of ferrous ions and expressed as μM of equivalent TROLOX® or α-tocopheryldiacetate.

Lipid analysis

Total lipids were extracted [69] and quantified gravimetrically. The phospholipid fraction was separated from the extracts by a micro-column chromatography method [70]. Aliquots of the organic extracts were evaporated and the residue was dissolved in 50 mM sodium phosphate buffer (pH 7.4) containing digitonin 1 %. These solutions were used to measure cholesterol (Cho) and phospholipids (PL) using commercial kits from Wienner Lab. (Rosario, Argentina). Ceramide content was determined according to the method described by Ohtani et al. [21] using the diacylglycerol-kinase assay that converts ceramide into ceramide-1-phosphate by incubation with the enzyme and γ^{32}-ATP. The incubation mixture was seeded

on Silica Gel 60 TLP plates (Merck, Darmstadt, Germany) and developed with a solvent system including chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, by volume). Ceramide was individualized by comparison with a pure standard (Santa Cruz Product Block Biotechnol. Inc., Heidelberg, Germany) and radio-densitometrically quantified (in a BAS-2000TM equip, Fuji, Japan). Results were expressed as µmoles of palmitoyl-ceramide (MW: 537.9)/mg total lipids. Araquidonic acid content was determined by capillary gas-liquid chromatography (c-GLC) of the fatty acid methyl esters (FAME). Analyses were performed as indicated in a previous paper [71] using a capillary column (Omegawax 250, from Bellefonte, Supelco, PA) mounted on a Hewlett Packard HP 6890 Series GC System Plus (Avondale, PA) equipped with a terminal computer integrator and data station. The FAMEs were identified by comparison of their relative retention times with authentic standards, and mass distribution was calculated electronically by quantification of peak areas. Eicosamonoenoic acid (21:0) was used as the internal standard.

Programmed cell death biomarkers

Caspase-3 activity was measured by a colorimetric assay kit (CASP-3-C), based on the hydrolysis of the synthetic peptide substrate acetyl-Asp-Glu-Val-Asp-p-nitroaniline (Ac-DEVD-pNA) by caspase-3 (Sigma Chem. Co., Buenos Aires, Argentina). The released p-nitroaniline (p-NA) was monitored at 405 nm. Each assay was run in parallel with inhibitor-treated cell lysate (to measure the non-specific hydrolysis of the substrate) and caspase-3 positive control (using commercial caspase-3, 5 mg/mL provided by the kit manufacturer). A calibration curve using a standard solution of p-nitroaniline (p-NA) was also run for each assay to calculate the protease activity expressed as µmol p-NA released/min.mL of sample. The activities of milli- (m) and micro- (µ) calpains were also measured. The technique involves the hydrolysis of ultra-pure casein (Sigma, Chem. Co., CA, USA) by calpain(s) and the subsequent detection of trichloroacetic acid (TCA)-soluble peptidic fragments at 280 nm

[72]. To select the activity of each calpain isoform, the level of calcium in the medium was regulated (5 mM or 500 µM of $CaCl_2$ for m- or µ-calpain, respectively). The activities of calpains were calculated considering a unit of calpain to be the amount of enzyme that produces a change of absorbance of 0.01 at 280 nm. Results were expressed as units/min.mg of protein.

Inflammatory biomarkers and phospholipase A2 (PLA2) activities

Interleukins 1β (IL-1), 6 (IL-6) and 10 (IL-10) were measured using ELISA kits from Abcam®, IL-1beta Mouse ELISA Kit (ab108866), IL-6 Mouse ELISA Kit (ab108868), and IL-10 Mouse ELISA Kit (ab108870), respectively (Abcam®, Cambridge, MA, USA). Range of detection for the cytokines were 0.12 to 20.3, 0.15 to 19.2, and 0.90 to 29.7 pg/mL, for IL-1, -6, and -10; respectively (inter- and intra-assay coefficient of variations were 9 %). Prostaglandins E2 and F2α were determined by ELISA method using two commercial kits, Prostaglandin E2 ELISA Kit (ab133021) (Abcam®, MA), and Prostaglandin F2α EIA Kit #516011 (Cayman Chemical, Michigan, USA) with a range of detection of 11.1 to 345.5, and 0.15 to 366.8 ng/mg serum proteins (inter- and intra-assay coefficient of variations were < 11 %). Phospholipase A2 (PLA2) activities were determined as described by Hirata et al. [73] with the modifications detailed in our previous paper [74]. The assays were conducted in the presence of Ca (total PLA2 activity) and with EGTA 3 mM (i-inducible-PLA2). The isoform activity c-PLA2 was obtained by subtraction. Results were expressed as nmoles of released fatty acid/min.mg prot with a linear range of detection between 0.5 to 74.0 µmoles arachidonate released/mg protein ((inter- and intra-assay coefficient of variations were < 8 %).

Nitric oxide synthetase activities

NOS activities were determined as described by Zhu et al [24] using a commercial available kit (Juli Bioengineering Co., Nanjig, China) that is based on the oxidation of oxyhemoglobin to metahemoglobin by NO. Ca-independent (eNOS plus nNOS) and dependent (iNOS) were determined by subtracting Ca-independent NOS activity from the total activity (the assay was run in the presence of Ca or EGTA 3 mM). Results were expressed as pmoles/min.mg prot. (range of linear detection between 2.2 to 77.3 pmoles/mg protein ((inter- and intra-assay coefficient of variations were < 12 %).

Other determinations

Protein content was determined by the method by Lowry et al. [75] Total polyphenol content in oils was measured using the Folin-Ciocalteu reagent following the method described by Ali et al. [76]. Results were expressed as caffeic acid (mg/Kg oil).

Statistical analysis

All values represent the mean of 4 rats assayed in duplicate expressed as mean ± standard deviation (SD). Data were analyzed by ANOVA plus the Tukey test with the aid of SPSS 12.0.1 software (SPSS Inc., Chicago, IL). Differences in the variations of a parameter over time were analyzed by two-way ANOVA including time as a measure. Results were also plotted and analyzed using Sigma Scientific Graphing Software (version 12.0) from Sigma Chem. Co. (St. Louis, MO). The statistical significance ($p \leq 0.01$) of differences is indicated by asterisks or letters, as appropriate.

Results

Stroke outcome

We obtained highly reproducible infarct zones with a clear definition of core and penumbral zones. Fig. 1 showed a representative TTZ-stained coronal slide before and after

the digitalization procedure (panel A) and also comparative ischemic zones for the same slide of brains obtained from the four experimental diets (panel B). It is evident that the extension of the damage decreases in the order G>S>C>O. Fig. 2 clearly demonstrated that the core volume (Cv, panel A) was significantly minor for O and increased with time after stroke (24 to 72 h) for all treatments; however, the increment was lesser in the case of O-feeding rats. Penumbral volume (Pv, panel B) was also of lesser extension for O. Pv remained constant for S after 24-h post stroke and then decreased. For the rest of the diets Pv decreased with time. Pv/Pc ratio (Fig. 3) showed a linear decrease as a function of time (regressions values $0.97 < r^2 < 0.99$), being the velocities of decrease (slope of the regression lines.10^{-3}) significantly high for C (-2.91) followed for S (-1.67) and G (-1.46) and finally by O (-1.25). The ratio Pv/Pc for O was the lowest one observed.

Redox status, biomakers of damage, and NOS activities

Experimental diets profoundly influence both the levels of antioxidants and the markers of oxidative dame in whole homogenates obtained from non ischemic brains (Table 1, basal status). Home-made diets were prepared with a constant amount of water- and lipid-soluble antioxidants (ascorbate, tocopherol); however, the extra amount incorporated by the dietary oils was significantly different. Tocopherol contents in oils were (mg/100 g): 2.1 ± 0.1 (C) < 3.5 ± 0.2 (S) = 3.3 ± 0.3 (G) < 13.8 ± 0.3 (O) and the content of total polyphenols (expressed as caffeic acid) were (mg/100 g): 1.7 ± 0.1 (S) < 3.1 ± 0.2 (C) < 4.0 ± 0.2 (G) < 19.6 ± 0.4 (O). While ascorbate was higher in S and G than in O and C, the content of tocopherol was higher (and similar) in O and C. Retinol was more abundant in O-supplemented diet. The lowest value for GSSG content was observed for O group and the highest GSSG/GSH ratio was measured for G group. FRAP assay -that reflects the total antioxidant capacity- decreased in the order O = C > S > G. The biomarkers for lipid damage (peroxidation of fatty acyl chains) TBARS and conjugated dienes were elevated -and almost

similar- in S and G groups, intermediate for O, and lower for C (enriched in saturated fatty acids). The behavior of protein carbonyls (PCs) and nitrate plus nitrite production (NOx) that estimates oxidative damage to proteins and nitrative stress, respectively, was very similar to that previously described.

Table 2 shows the results of the enzyme components of the antioxidant defense system. Total SOD activity was elevated compared to O and C groups. Olive fed rats showed the lowest values for the mitochondrial and cytosolic isoforms of SOD. Glutathione reductase (G-Re) was very active in G group followed by S > C > O. A similar pattern was observed for glutathione peroxidase and catalase. Glutathione transferase showed no significant changes among groups.

We also studied the activities of the two main isoforms of the nitric oxide synthethase (NOS) (Ca-independent and -dependent forms, Table 3) after different post-stroke times (24, 48 and 72 h). In both isoforms basal values varied significantly among the experimental groups; however, results for sham-operated rats remain almost constant and very similar to those of the contralateral hemisphere. In core and penumbra of S and G groups there was a sustained increment with time of the Ca-independent isoform (iNOS). On the contrary, Ca-dependent isoforms (eNOS and nNOS) decreased in all groups and in both regions (core and penumbra). There was a slight increase at 48-h post stroke for S group and a significant time-dependent rise for the O feeding rats. Due to the fact that 48-h post stroke seems to experiment particular changes in the behavior of NOS, we focus our study on that time post stroke. Tables 4 and 5 show the results obtained for the non-enzymatic and enzymatic antioxidant defense systems and biomarkers of oxidative damage (in core and penumbra) after 48-h post stroke, respectively. We found a reduction of antioxidants (ascorbate, tocopherol and retinol) in all groups (Table 4) which was more noticeable in penumbra compared to core. Concomitantly, the proportion of GSSG increased in core and penumbra in all groups except O. We also observed an increment of the biomarkers of damage in core and penumbra for all

groups with the exception of NOx and PCs for O. The activity of total SOD (Table 5) increased in all groups in core and penumbra with the exception of C group. Interestingly, in O and C groups the mitochondrial (Mn) SOD remains unaltered with respect to reference values. The cytosolic form (Cu, Zn) of SOD was also indistinguishable from control data. Except for C group, the glutathione reductase was strongly stimulated in both zones, especially in penumbra of O group. G-Px was only increased in S and G groups, while G-Tr did not modify its activity in any group and/or zone. Catalase was increased in S and G groups, had no changes in C and was stimulated in penumbra of O.

Analysis of DNA after 48-h post stroke demonstrated that S and G diets generated extensive damage whereas C produced an intermediate effect, and there is no evidence of damage in O group (Fig. 4).

Lipid derived mediators

Oxidative stress is intimately linked to alterations in lipid parameters (cytokines, prostaglandins, arachidonate, ratio cholesterol/phospholipids, etc.) and enzymes of lipid metabolism (mainly phospholipase A2) that finally converge into pro-apoptotic signals. These parameters are therefore considered to be “lipid mediators” of programmed cell death. For example, there is a connection between the cholesterol ratio (Cho)/phospholipids (PL) in mitochondria and the entry of GSH from the cytosol into the matrix. Table 6 shows that despite the differences in the basal values among groups, the Cho/PL ratio increased in core and even more so in penumbra with the exception of O rats. This was reflected in a drastic increase in the GSSG/GSH ratio in mitochondria from the core zone and an even more significant increase in penumbra for all groups except O (an approx. 50 % change in O with respect to control *vs* 93 to 162 % for the other groups).

Phospholipase A2 (PLA2) is activated under pro-oxidative conditions and is involved in inflammatory response (via araquidonate release) and programmed cell death. We

determine the activities of the c- (Ca-dependent) and i- (Ca-independent) PLA2 isoforms in core and penumbra (Fig. 5). c-PLA2 (Fig. 5-A) increased in both zones for S and G groups, remaining unaltered for the other experimental diets, whereas c-PLA2 (Fig. 5-B) increased significantly in all groups, especially in the core of C. The lowest observed values were those for O. i-PLA2 did not change significantly in penumbra from the O group.

Activation of PLA2 is likely associated with araquidonate release. Contrary to our expectation (derived from the results obtained for the PLA2 activities), after 48-h post stroke arachidonic acid content was reduced in core and penumbra from all groups (especially S, C and G) (Fig. 6-A). This reduction could be associated with arachidonate transformation into prostaglandins, since PGE2 and PGF2α increased in core and penumbra in accordance with the previously observed reductions in arachidonate content (Fig. 6-B and C vs. Fig. 6-A). PGE2 showed similar increases in core and penumbra for S, C, and G groups, but it was only augmented in core from the O group. PGF2α characteristically showed more pronounced increments in penumbra compared to core, except for the O group, in which the increases were negligible (Fig. 6-C).

Ceramide levels and programmed cell death

Liberation of arachidonate stimulates sphingomyelinases causing production of ceramide. Also, oxidative stress induces ceramide production. We observed that the levels of ceramide in core remain similar to those of sham-operated rats or contralateral hemisphere. However, they increased significantly -in very different magnitudes- in penumbra from all experimental groups. In percent change -compared to contralateral values- results were: S, 113 ± 9.9; O, 31 ± 2.8; C, 193 ± 11.1; G, 243 ± 15.4.

Caspase-3 activity (Fig. 7-A) increased in penumbra and in more noticeable way in core of S, C, and G groups. In O group the protease was elevated only in penumbra. The activities of milli- (Fig. 7-B) and micro- (Fig. 7-C) calpains were increased in core and

penumbra following a similar pattern for all groups, being the increments of the O group the lowest observed.

Cytokine levels in peripheral plasma

Modification of the quality of the fatty acyl chains in the dietary lipids profoundly influenced the concentration of cytokines in peripheral plasma (Table 8). In sham-operated rats olive oil supplementation produced a significant increase in the ratio between anti- and pro-inflammatory interleukins. After 48 h ischemia the changes observed in the group fed with olive oil also have the best ratio among all groups studied (Table 8).

Discussion

We demonstrated a profound influence of dietary lipids (commercial oils) on the evolution of brain stroke in a worldwide accepted model of focal ischemia. Other authors have previously reported the beneficial effects of Mediterranean diet (enriched in olive oil) for the prevention of brain and cardiovascular diseases [25,26,40]. Moreover, it was reported the improvement of brain stroke evolution with the administration of curcumin [34,35], tyrosol and hydroxytyrosol [36, 38], red wine polyphenols [37], resveratrol [41], and other phenolic compounds [39]. The biological effect of the polyphenols is heterogeneous. For example, caffeinol confers cortical but not sub-cortical protection after stroke [6]. The elevated concentration of polyphenols in the virgin olive oil employed compared to the other commercial oils assayed has led us to assume that it is in this unsaponifiable fraction where the protective effects resides. In addition, if we consider the fatty acid composition of the oils, we and other researchers have demonstrated that oxidative stress is highly dependent on the unsaturation of the fatty acyl chains [31]. The fatty acids of soybean and grape seed oils are strong pro-oxidative inducers [29-31]. It is well recognized that fatty acids (especially from the n-6 series) are cellular promoters of oxidative stress and pro-inflammatory effects and that

docosahexenoic and docosapentaenoic acids of the n-3 series could be the only fatty acids with recognized antioxidant and neuroprotective properties [29,77]. Fatty acids can increase free radical production through many mechanisms [29] and influence significantly on the pro-inflammatory routes [27,28]. In addition, saturated fatty acids such as those present in coconut oil are inducers of apoptosis by stimulation of ceramide generation and activation of programmed death signals [32]. Olive fed rats have the best performance in the post stroke evolution, they also showed maintenance of the redox homeostasis (antioxidant levels and activities of enzymes of the antioxidant defense system), with a simultaneous activation of the calcium-dependent NOS (eNOS plus nNOs) activity in penumbra which is neuroprotective in opposition to iNOS (that actively generates peroxinitrite and cellular damage) [23, 24]. Olive group has the minor ceramide overproduction that has been repeatedly associated to programmed cell death [19-22] and at the same time the ratio Cho/PL was unaffected in penumbra producing the lowest values for the GSSG/GSH ratio in mitochondrial fraction isolated from this critical zone. We and other authors have previously demonstrated that there is an inverse relationship between these two parameters [78-80] because an increased Cho/PL ratio impairs the entry of GSH into the mitochondrial matrix due to the effects on membrane structure and physiology that finally inhibits various GSH carriers [81]. The failure in restoring mitochondrial GSH leads to accumulation of free radicals, mitochondrial membrane damage with loss of integrity (via peroxidation of cardiolipin), and release of citochrome c to the cytosol initiating the caspase-3 activation cascade [10,13,78-81].

Prostaglandin production was also lower in the olive oil supplemented group. This was directly reflected in a lesser inflammation as demonstrated previously by other researches [15-17]. The activity of PLA2 and the production of PGE2 and F2α are key factors linked to the stroke outcome [16,17]. In addition, PLA2 is responsible for initiating not only the pro-inflammatory cascade, but also for the activation of ceramide production via arachidonate [18]. Once activated, inflammatory cells can release a variety of cytotoxic agents including

more cytokines, matrix metalloproteinases, nitric oxide, superoxide, and other oxygenated and nitrogenated free radicals [15-17]. In fact, cyclooxygenase pathway is one of the main targets for the palliative treatment of stroke in humans [7,16]; and interleukin determination in peripheral blood is used for the prognosis of the stroke patients [15,17]. The cytokines (IL-1, -6 and -10) play a crucial role in the outcome of ischemic stroke and they are strongly influenced by the type of dietary polyunsaturated fatty acids [27]. In the presence of inflammation of glia, even low alterations of the redox status can induce neuronal death under hypoxic conditions [11]. We observed that dietary lipid manipulation significantly modify the levels of cytokines. The best relationship between the anti- (IL-10) and the pro-inflammatory interleukins (IL-1 and IL-6) was achieved under olive oil dietary supplementation. It was previously reported that levels of IL-6 > 13 pg/mL could predict intrahospital mortality with a sensitivity of 85 % and specificity of 93 % [15]. Moreover, both Il-1 and IL-6 positively correlated with the volume of infracted region in humans [11,15,16]. Previous research from other labs have demonstrated lower levels of IL-10 in the stroke patients with negative onset after stroke [16,17]. Thus, we think that increased IL-1 and -6 and reduced IL-10 in those animals fed on diets other than olive oil-supplementation was associated to clinical deterioration. Thus, olive oil seems to stabilize the inflammatory response. These findings reinforce the idea that peripheral measurement of cytokines should be useful for the prognosis of ischemic stroke [11,15-17] and -more important- that these mediators can be modify by dietary lipid manipulation [27].

All the changes induced by the dietary fatty acyl composition here demonstrated converge finally in the main and most important fact which is the activation of the proteases involved in cell survival. In acute ischemic stroke it was suggested that necrosis and apoptosis are both present in the core, while apoptosis predominate in penumbral zone [12]. Inhibition of caspase-3 has become (together with inflammation control) one of the main palliative tools during the stroke outcome in humans [1]. Thus, it is relevant to discuss the role of caspase-3

and calpain in this context. There is a consensus that free radicals, ceramide overproduction, and GSSG level are key factors in the activation of protease systems [9,10,12,13,22,32]. However, the question of how the programmed cell death is implemented remains as continuous controversy. Previous experimental data have demonstrated a complex interrelationship between the two systems [82,83]. In fact, activation of one of these proteases may lead to inactivation of the other; in other words, they function as inter-dependent and exclusive effectors able to replace one another during the programmed cell death cascade [84].

Taken together, our results justify the lower velocity of recruitment of penumbral neurons into core observed for the olive oil-fed rats, demonstrating the crucial influence that the quality of dietary lipids has on the evolution of ischemic stroke. There are many possible speculations concerning the complex relationship between the variables studied here investigated that could explain our findings. Fig. 8 presents a graphical summary of these interdependencies. However, certain limitations to the conclusions of our study are acknowledged since we do not know if the experimental conditions simulated in our rats can be extrapolated to those associated with real human situations. Modern diets are enriched in saturated fats and in oils with polyunsaturated fatty acids from the n-6 series (for example, soybean oil is the most consumed oil in many countries of Latin America). Notwithstanding, the experimental evidence presented here supports the recommendation to increase not only the n-3 derived fatty acids [77,85] but also the ingestion of virgin olive oil as a preventive resource for ischemic brain damage. Another important derivation is that further investigation should be focused on the isolation and development of bioactive compounds (nutraceuticals) likely present in the unsaponifiable fraction of olive oils that could have important connotations from the pharmaceutical point of view. This is in line with the promising epidemiological, experimental, and clinical evidence emerging from the use of resveratrol [41] and tyrosol [36,38].

Conclusion

Dietary fats profoundly influence the oxidative status of the brain and the inflammatory response to ischemic injury by modulating the activities of NOS and PLA2 activities, interleukin and prostaglandin production, ceramide concentration, and glutathione import into the mitochondrial matrix. These effects finally determine the activation of the two main protease systems involved in programmed cell death (caspase-3 and calpains). Olive oil seems to be a biological source for the isolation of highly protective agents that block expansion of brain core at the expense of penumbral neurons after ischemic stroke.

Acknowledgements

This study was supported by a grant from Consejo Nacional de Investigaciones Científicas y Técnicas (CCT-CONICET) PIP #0697 and #0203, UNLP 11M-175.

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Figure legends

Fig. 1. (A) Representative TTZ-stained slide from an ischemic lesion produce surgically by MACAO as described in the Material and methods section and its digitalization using the Erdas v8.0 Image Software (core, penumbral zone, and undamaged contralateral hemisphere were indicated and visualized with different intensities of grey (original images were processed in colors). (B) Representative section of ischemic brains from the different experimental diets after TTZ stained method. All slides correspond to the same coronal section. The infarct or ischemic cores are visualized as clear zones.

Fig. 2. (A) Core volumes (Cv, mm^3) measured after 24 (with bars), 48 (grey bars) and 72 (black bars) hours post stroke in brains after MACAO procedure and TTZ staining, digitalization and data processing with Erdas v8.0. Each group of bars represents one type of dietary treatment with commercial oils (S, soybean; O, olive; C, coconut; G, grape seed) and they are the mean of four independent experiments ± SD. Differences ($p < 0.01$) among the same time post stroke measurements are indicated with distinct superscript letters. Asterisks indicate statistical differences ($p< 0.01$) between 48 or 72 hours post stroke compared to 24 hours within the same experimental group. (B) Penumbral volume (Pv, mm^3) represented in the same way as described in part (A) of this figure.

Fig. 3. Representation of the ratio between penumbral volume (Pv) and core volume (Cv) as a function of the time post stroke (24, 48, 72 h). Different symbols represent one type of the four dietary treatments with commercial oils (S, soybean; O, olive; C, coconut; G, grape

seed). Each result is the mean of four independent experiment ± SD. Data are processed to obtain the regression curves with linear adjustment ($0.97 < r^2 < 0.99$) and the slopes of each independent regression were calculated electronically. Asterisks indicate statistical differences between results of 48 or 72 h compared to 24 h of the same experimental group ($p < 0.01$).

Fig. 4. Representative electrophoretic profile of whole DNA extracted from the core of ischemic lesions after 48 h post stroke. The analysis was performed on agarose gels (1 %) using DAPI to visualize the results. The slabs were photographed and scanned using a commercial equip as described in Material and methods section. The numbers indicated are relative intensity units along the two scanning positions (indicated with solid or dashed lines). Lanes were seed with different samples as indicated (S, soybean; O, olive; C, coconut; G, grape seed).

Fig. 5. Phospholipase A2 (PLA2) activities measured as calcium-dependent (c) and – independent (i) isoforms determined as described in the experimental part by regulating the amount of calcium ions in the incubation mixture. Results correspond to different brain zones analyzed after 48-h post stroke. Each group of bars represents one type of dietary treatment with commercial oils (S, soybean; O, olive; C, coconut; G, grape seed) and they are the means of four independent experiments ± SD. Differences ($p < 0.01$) among the same time of measurements (Sh, sham-operated rats; Cl, contralateral hemisphere; Pe, penumbral zone; Co, ischemic core) are indicated with distinct superscript letters.

Fig. 6. (A) Arachidonic acid content in homogenates from different brain zones after 48-h post stroke. The fatty acid was quantified by c-GLC as described in the Experimental methods and procedures section using eicosamonoenoic (20:1) as internal standard. Results (nmoles 20:4) mg total lipids) are expressed as the means of 4 independent experiments ± SD. Each

group of bars represents one type of dietary treatment with commercial oils (S, soybean; O, olive; C, coconut; G, grape seed). Differences ($p < 0.01$) among the same time of measurements (Sh, sham-operated rats; Cl, contralateral hemisphere; Pe, penumbral zone; Co, ischemic core) are indicated with distinct superscript letters. Prostaglandin (PG) levels are represented in panels (B) for PGE2 and (C) for PGF2α. Results (ng/mg protein) were the means of four independent experiments ± SD. The way of representation and the statistical differences are as indicated in part (A) of this figure.

Fig. 7. (A) Caspase-3 activity from different brain zones after 48 h post stroke. The protease was assayed using a commercial kit as described in the Material and methods section. Results (U/mg protein) are expressed as the means of four independent experiments ± SD. Each group of bars represents one type of dietary treatment with commercial oils (S, soybean; O, olive; C, coconut; G, grape seed). Differences ($p < 0.01$) among the same time of measurements (Sh, sham-operated rats; Cl, contralateral hemisphere; Pe, penumbral zone; Co, ischemic core) are indicated with distinct superscript letters. Calpain isoform activities were measured as described in Material and methods section. They are represented in panels (B) (milli-calpain or m-calpain) and (C) micro-calpain or μ-calpain. Results (U/mg protein) were the means of four independent experiments ± SD. The way of representation and the statistical differences are as indicated in part (A) of this figure.

Fig. 8. Schematic representation of the main effects produced by dietary lipids on various parameters that trigger the outcome of the ischemic stroke. The quality of the fatty acyl chains can modify the production of radical species (an also the type of them) being one of the factors that modulate antioxidant enzyme activities, water- and lipid-soluble scavenger levels, and ultimately the damage to DNA, lipids and proteins that interact with redox-dependent signaling cascades. Lipid metabolism is also altered through enzyme changes and

modifications in both the availability of fatty acyl chains as substrates –via phospholipase A2 (PLA2)- for prostaglandin biosynthesis or ceramide production. Modification in the ratio cholesterol (Cho)/phospholipd (PL) influences the entry of reduced glutathione into the mitochondrial matrix. The levels of cytokines together with those of prostaglandins determine the inflammation scenario that characterizes each type of experimental group. The balance between Ca-dependent and -independent nitric oxide synthethase (NOS) contribute to the modulation of redox signaling pathways, the formation of nitrogenated radical species, perfusion rate, and other events linked to cell survival. All these changes finally determine the activities of the two main protease systems involved in programmed cell death (caspase-3 and milli/micro-calpain activities) and lead to differences in the recruitment of penumbral neurons into ischemic core.

Table 1. Non-enzymatic antioxidant defense system and biomarkers of pro-oxidative damage in whole brain homogenates from rats fed on the experimental diets.

Parameters	S	O	C	G
Ascorbate (pmoles/mg prot.)	6.7 ± 0.3^{a}	5.8 ± 0.2^{b}	5.9 ± 0.8^{b}	6.8 ± 0.4^{a}
α-Tocopherol (pmoles/mg lipids)	1121 ± 86^{a}	1356 ± 78^{b}	1303 ± 91^{b}	855 ± 66^{c}
Retinol (nmoles/mg prot.)	566 ± 25^{a}	689 ± 32^{b}	560 ± 49^{c}	499 ± 51^{d}
GSSG (nmoles/mg prot.)	5.3 ± 0.2^{a}	2.1 ± 0.1^{b}	3.2 ± 0.2^{c}	7.8 ± 0.3^{d}
GSH (nmoles/mg prot.)	44.1 ± 3.2^{a}	40.0 ± 4.1^{a}	38.7 ± 3.1^{a}	55.6 ± 3.0^{b}
GSSG/GSH	0.12 ± 0.02^{a}	0.05 ± 0.01^{b}	0.08 ± 0.02^{c}	0.14 ± 0.02^{a}
FRAP assay (µmoles/mg prot.)	645 ± 22^{a}	897 ± 31^{b}	887 ± 44^{b}	605 ± 24^{c}
TBARS (nmoles MDA/mg prot.)	2.22 ± 0.10^{a}	1.15 ± 0.05^{b}	0.61 ± 0.03^{c}	2.35 ± 0.12^{a}
Conjugated dienes (ODU/mg lipids)	0.11 ± 0.01^{a}	0.05 ± 0.01^{b}	0.03 ± 0.006^{c}	0.12 ± 0.01^{a}
Protein carbonyls (mmol/mg prot.)	1.87 ± 0.11^{a}	1.66 ± 0.07^{b}	1.60 ± 0.11^{b}	2.53 ± 0.13^{c}
NOx (pmoles/mg de prot.)	1897 ± 65^{a}	994 ± 47^{b}	755 ± 41^{c}	2012 ± 54^{d}

Antioxidants and biomarkers of oxidative stress were determined in brain homogenates from rats fed on the experimental diets supplemented with the commercial oils (S, soybean; O, olive; C, coconut; G, grape seed). Methods used for measurements were mentioned in detail in Research methods and procedures section. Each result (units are indicated in the table) is the mean ± SD of 4 independent experiments assayed in duplicate. NOx, nitrate plus nitrite, ODU, optical density units, TBARS, thiobarbituric acid reactive susbtances. Statistical differences among results are indicated with distinct superscript letters ($p < 0.01$).

Table 2. Antioxidant enzyme activities in brain homogenates from rats feeding the experimental diets.

Treatment	S	O	C	G
Total-SOD (U/mg. prot.)	31.2 ± 1.5^{a}	19.5 ± 1.3^{b}	24.6 ± 1.3^{c}	28.9 ± 1.6^{a}
Mn-SOD (U/mg. prot.	4.6 ± 0.3^{a}	2.3 ± 0.2^{b}	4.8 ± 0.4^{a}	3.9 ± 0.5^{a}
Cu,Zn-SOD (U/mg. prot.)	26.6 ± 1.4^{a}	17.2 ± 1.1^{b}	19.8 ± 0.6^{c}	25.0 ± 1.2^{a}
G-Re (U/mg. prot.)	62.3 ± 1.8^{a}	43.6 ± 1.0^{b}	58.1 ± 2.2^{a}	80.9 ± 2.1^{c}
G-Px (U/mg. prot.)	16.5 ± 1.4^{a}	11.4 ± 0.9^{b}	17.3 ± 1.5^{a}	22.3 ± 1.1^{c}
G-Tr (U/mg. prot.)	43.7 ± 1.2^{a}	45.6 ± 1.9^{b}	42.8 ± 1.3^{c}	44.4 ± 1.3^{a}
Catalase *k*/mg. prot.	0.90 ± 0.05^{a}	0.43 ± 0.11^{b}	1.12 ± 0.10^{a}	2.15 ± 0.22^{c}

Enzyme activities were determined in aliquots of total brain homogenates from control rats fed under the four experimental diets supplemented with commercial oils (S, soybean; O, olive; C, coconut; G, grape seed). Methodology used was described in the Research methods and procedures section (units of activities were consigned in the table). Each result is the mean ± SD of four independent experiments assayed in duplicate. G-Re, gluthatione reductase; G-Px, glutathione peroxidase; G-Tr, glutathione transferase. Statistical differences among the results (ANOVA + Tukey test) are indicated with different superscript letters ($p < 0.01$).

Table 3. Ca dependent- and independent-NOS activities in homogenates from sham operated rats and from core, penumbra, and contralateral hemispheres after different times post-stroke.

NOS activities	Diet	Hours	Sham	Stroke		
				Core	Penumbra	Contralateral
Ca-independent (pmoles/mg prot.min)	S	24	17.2 ± 0.5[a]	36.9 ± 1.1[b]	22.4 ± 0.5[c]	15.6 ± 0.7[a]
		48	16.8 ± 0.6[a]	40.1 ± 1.3[b]*	25.5 ± 0.4[c]*	15.1 ± 0.5[a]
		72	17.3 ± 0.7[a]	44.6 ± 1.0[b]*	30.2 ± 0.6[c]*	16.3 ± 0.4[a]
	O	24	10.1 ± 0.2[a]	12.3 ± 0.4[a]	14.0 ± 0.4[b]	11.3 ± 0.3[a]
		48	9.8 ± 0.3[a]	18.6 ± 0.3[b]*	9.0 ± 0.3[a]*	10.4 ± 0.4[a]
		72	9.9 ± 0.2[a]	14.1 ± 0.4[b]	7.1 ± 0.2[c]*	10.0 ± 0.3[a]
	C	24	13.5 ± 0.4[a]	15.0 ± 0.6[a]	14.2 ± 0.5[a]	14.9 ± 0.7[a]
		48	13.1 ± 0.4[a]	14.8 ± 0.4[a]	14.1 ± 0.3[a]	14.1 ± 0.5[a]
		72	14.0 ± 0.5[a]	14.2 ± 0.3[a]	15.0 ± 0.4[a]	13.6 ± 0.4[a]
	G	24	19.2 ± 0.5[a]	45.8 ± 2.2[b]	31.4 ± 1.7[c]	21.0 ± 0.8[a]
		48	20.2 ± 0.7[a]	52.0 ± 2.4[b]*	36.4 ± 1.8[c]*	19.8 ± 0.6[a]
		72	18.8 ± 0.5[a]	55.5 ± 3.1[b]*	41.1 ± 2.0[c]*	21.5 ± 0.5[a]
Ca-dependent (pmoles/mg prot.min)	S	24	25.1 ± 0.3[a]	8.1 ± 0.1[b]	13.3 ± 0.3[c]	23.5 ± 0.4[a]
		48	24.6 ± 0.4[a]	8.8 ± 0.3[b]	16.5 ± 0.4[c]*	23.0 ± 0.3[a]
		72	25.8 ± 0.5[a]	7.9 ± 0.4[b]	14.0 ± 0.5[c]	24.2 ± 0.5[a]
	O	24	20.2 ± 0.4[a]	11.3 ± 0.2[b]	17.5 ± 0.4[c]	19.2 ± 0.4[c]
		48	19.7 ± 0.5[a]	11.5 ± 0.3[b]	20.3 ± 0.5[c]*	20.3 ± 0.5[a]
		72	20.0 ± 0.3[a]	12.2 ± 0.4[b]	25.1 ± 0.6[c]*	18.9 ± 0.4[a]
	C	24	12.2 ± 0.3[a]	7.3 ± 0.2[b]	20.7 ± 0.6[c]	14.1 ± 0.3[a]
		48	13.1 ± 0.4[a]	7.5 ± 0.3[b]	19.9 ± 0.5[c]	12.7 ± 0.3[a]
		72	12.8 ± 0.4[a]	6.5 ± 0.5[b]	21.3 ± 0.4[c]	13.5 ± 0.5[a]
	G	24	36.8 ± 1.1[a]	4.4 ± 0.1[b]	14.0 ± 0.3[c]	41.2 ± 2.2[a]
		48	38.5 ± 1.6[a]	5.0 ± 0.2[b]	16.1 ± 0.4[c]	40.4 ± 2.3[a]
		72	37.2 ± 2.0[a]	5.1 ± 0.3[b]	15.7 ± 0.5[c]	38.8 ± 2.2[a]

Ca-independent (iNOS) and Ca-dependent (nNOS plus eNOS) activities were determined in homogenates from sham operated animals and in core, penumbra, and contralateral hemispheres (after different times post-stroke) using a commercial kit as described in the Research methods and procedures section. Homogenates from the experimental diets supplemented with commercial oils were indicated by capital letters (S, soybean; O, olive; C, coconut; G, grape seed). Statistical differences among results (brain zones analyzed) from the same diet and time were identified with distinct superscript letters. Differences among results from the same type of homogenate and diet treatment associated to time post-stroke (compared to 24 h) are indicated with asterisks (ANOVA plus Tukey test, $p < 0.01$).

Table 4. Non-enzymatic antioxidant defense system and biomarkers of pro-oxidative damage in homogenates from sham operated rats and from core, penumbra, and contralateral hemispheres 48 h post-stroke.

Parameters	Diet	Sham	Stroke		
			Core	Penumbra	Contralateral
Ascorbate (pmoles/mg prot.)	S	7.2 ± 0.2^a	4.4 ± 0.1^b	3.0 ± 0.1^c	7.5 ± 0.3^a
	O	5.9 ± 0.2^a	4.3 ± 0.2^b	4.2 ± 0.1^b	6.0 ± 0.2^a
	C	5.0 ± 0.3^a	2.2 ± 0.1^b	2.0 ± 0.1^b	5.1 ± 0.3^a
	G	7.0 ± 0.2^a	3.8 ± 0.2^b	2.9 ± 0.1^c	6.8 ± 0.1^a
α-Tocopherol (pmoles/mg lipids)	S	1109 ± 76^a	725 ± 44^b	561 ± 33^c	1210 ± 70^a
	O	1340 ± 63^a	887 ± 45^b	1012 ± 51^c	1414 ± 67^a
	C	1303 ± 49^a	666 ± 57^b	513 ± 42^c	1294 ± 55^a
	G	870 ± 52^a	505 ± 36^b	411 ± 44^c	889 ± 45^a
Retinol (nmoles/mg prot.)	S	551 ± 22^a	402 ± 25^b	353 ± 19^c	566 ± 28^a
	O	691 ± 29^a	603 ± 18^b	677 ± 22^c	701 ± 31^a
	C	572 ± 35^a	355 ± 30^b	317 ± 25^c	585 ± 34^a
	G	484 ± 30^a	412 ± 32^b	381 ± 23^c	480 ± 35^a
GSSG/GSH	S	0.12 ± 0.01^a	0.16 ± 0.01^b	0.16 ± 0.02^b	0.11 ± 0.02^a
	O	0.06 ± 0.01^a	0.08 ± 0.01^a	0.07 ± 0.01^a	0.06 ± 0.02^a
	C	0.08 ± 0.02^a	0.12 ± 0.01^b	0.14 ± 0.02^b	0.07 ± 0.01^a
	G	0.15 ± 0.02^a	0.19 ± 0.02^b	0.24 ± 0.02^c	0.16 ± 0.01^a
TBARS (nmoles MDA/mg prot.)	S	2.31 ± 0.08^a	2.59 ± 0.11^b	2.62 ± 0.10^b	2.28 ± 0.10^a
	O	1.18 ± 0.03^a	1.46 ± 0.05^b	1.33 ± 0.01^b	1.22 ± 0.02^a
	C	0.66 ± 0.02^a	1.11 ± 0.02^b	1.43 ± 0.03^c	0.70 ± 0.01^a
	G	2.44 ± 0.10^a	3.15 ± 0.12^b	3.58 ± 0.20^c	2.40 ± 0.05^a
NOx (pmoles/mg de prot.)	S	1.92 ± 0.10^a	2.46 ± 0.11^b	2.98 ± 0.10^c	1.87 ± 0.12^a
	O	1.55 ± 0.05^a	2.02 ± 0.10^b	1.67 ± 0.11^a	1.61 ± 0.20^a
	C	1.46 ± 0.06^a	2.10 ± 0.10^b	2.41 ± 0.06^c	1.51 ± 0.10^a
	G	2.61 ± 0.11^a	3.33 ± 0.22^b	3.56 ± 0.20^c	2.54 ± 0.10^a
Protein carbonyls mmol/mg prot.)	S	1901 ± 51^a	2311 ± 49^b	2458 ± 33^c	1888 ± 60^a
	O	901 ± 42^a	1202 ± 51^b	943 ± 39^a	922 ± 37^a
	C	737 ± 31^a	866 ± 29^b	915 ± 34^c	741 ± 42^a
	G	2101 ± 59^a	2541 ± 48^b	2757 ± 55^c	2155 ± 44^a

Antioxidants and biomarkers of oxidative stress were determined in brain homogenates from rats fed on the experimental diets supplemented with the commercial oils (S, soybean; O, olive; C, coconut; G, grape seed). Methods used for measurements were mentioned in Research methods and procedures section. Each result (units are indicated in the table) is the mean ± SD of 4 independent experiments assayed in duplicate. Statistical differences among data are indicated with distinct superscript letters ($p < 0.01$).

Table 5. Antioxidant enzyme activities in brain homogenates from sham operated rats and from core, penumbra, and contralateral hemispheres 48 h post-stroke.

Parameters	Diet	Sham	Stroke		
			Core	Penumbra	Contralateral
Total-SOD (U/mg. prot.)	S	30.0 ± 1.1[a]	37.9 ± 1.0[b]	36.7 ± 1.3[b]	29.2 ± 0.8[a]
	O	17.1 ± 0.8[a]	21.8 ± 1.1[b]	22.4 ± 0.9[b]	17.5 ± 0.7[a]
	C	27.0 ± 0.9[a]	27.5 ± 0.8[a]	28.2 ± 1.0[a]	27.8 ± 0.6[a]
	G	29.9 ± 1.1[a]	42.5 ± 2.0[b]	39.5 ± 1.7[b]	31.1 ± 1.0[a]
Mn-SOD (U/mg. prot.	S	5.0 ± 0.2[a]	8.1 ± 0.2[b]	8.8 ± 0.1[b]	4.9 ± 0.3[a]
	O	2.2 ± 0.1[b]	2.3 ± 0.1[a]	2.2 ± 0.2[a]	2.3 ± 0.2[a]
	C	4.9 ± 0.3[a]	5.0 ± 0.1[a]	4.8 ± 0.2[a]	5.1 ± 0.3[a]
	G	4.8 ± 0.2[a]	9.2 ± 0.3[b]	9.0 ± 0.2[b]	5.1 ± 0.2[a]
Cu,Zn-SOD (U/mg. prot.)	S	25.0 ± 1.0[a]	29.8 ± 0.6[b]	27.9 ± 0.7[b]	24.3 ± 0.9[a]
	O	14.9 ± 0.6[a]	19.5 ± 0.5[b]	20.2 ± 0.8[b]	15.0 ± 0.7[a]
	C	22.1 ± 0.7[a]	22.5 ± 0.8[a]	23.4 ± 1.0[a]	22.7 ± 0.9[a]
	G	25.1 ± 1.1[a]	33.3 ± 1.0[b]	30.5 ± 0.8[b]	26.0 ± 0.7[a]
G-Re (U/mg. prot.)	S	68.2 ± 2.0[a]	75.6 ± 2.1[b]	78.5 ± 2.0[b]	66.7 ± 1.9[a]
	O	41.5 ± 1.5[a]	45.4 ± 1.1[b]	50.3 ± 1.2[c]	40.7 ± 1.3[a]
	C	56.2 ± 1.3[a]	57.5 ± 1.5[a]	54.2 ± 1.3[a]	57.1 ± 1.4[a]
	G	78.9 ± 2.0[a]	86.6 ± 2.3[b]	88.8 ± 3.0[b]	79.1 ± 1.9[a]
G-Px (U/mg. prot.)	S	18.7 ± 1.0[a]	25.4 ± 0.9[b]	26.1 ± 1.1[b]	18.0 ± 0.8[a]
	O	10.3 ± 0.5[a]	11.5 ± 0.6[a]	12.2 ± 0.7[a]	11.1 ± 0.6[a]
	C	16.5 ± 1.0[a]	17.3 ± 0.9[a]	16.8 ± 0.8[a]	16.1 ± 1.0[a]
	G	23.0 ± 1.1[a]	30.3 ± 1.0[b]	28.7 ± 0.7[b]	23.5 ± 1.0[a]
G-Tr (U/mg. prot.)	S	45.5 ± 1.5[a]	46.5 ± 1.3[a]	44.8 ± 1.1[a]	46.2 ± 1.5[a]
	O	54.1 ± 1.2[a]	51.8 ± 1.5[a]	53.7 ± 1.2[a]	55.2 ± 1.5[a]
	C	40.1 ± 2.0[a]	41.6 ± 1.8[a]	40.0 ± 2.2[a]	43.1 ± 2.1[a]
	G	50.4 ± 2.1[a]	52.2 ± 1.9[a]	48.5 ± 2.3[a]	47.3 ± 2.3[a]
Catalase (*k*/mg. prot.)	S	1.12 ± 0.03[a]	2.25 ± 0.05[b]	2.31 ± 0.03[b]	1.10 ± 0.02[a]
	O	0.40 ± 0.01[a]	0.44 ± 0.02[a]	0.65 ± 0.03[b]	0.39 ± 0.02[a]
	C	1.15 ± 0.05[a]	1.17 ± 0.02[a]	1.12 ± 0.03[a]	1.12 ± 0.04[a]
	G	2.17 ± 0.11[a]	3.33 ± 0.12[b]	3.01 ± 0.14[b]	2.15 ± 0.10[a]

Enzyme activities were determined in homogenates from sham operated animals and in core, penumbra, and contralateral hemispheres (48 h post-stroke) using the methodologies mentioned in the Research methods and procedures section. Homogenates from the experimental diets supplemented with commercial oils were indicated by capital letters (S, soybean; O, olive; C, coconut; G, grape seed). Statistical differences among results (brain zones analyzed) from the same diet were identified with distinct superscript letters (ANOVA plus Tukey test, $p < 0.01$).

Table 6. Cho/PL and GSSG/GSH ratios in sonicated mitochondrial suspensions from sham operated rats and from core, penumbra, and contralateral hemispheres 48 h post-stroke.

Parameters	Diet	Sham	Stroke		
			Core	Penumbra	Contralateral
Cho/PL	S	0.32 ± 0.02a	0.41 ± 0.02b	0.55 ± 0.03c	0.33 ± 0.03a
	O	0.21 ± 0.01a	0.32 ± 0.03b	0.25 ± 0.02a	0.22 ± 0.01a
	C	0.58 ± 0.03a	0.66 ± 0.03b	0.74 ± 0.02c	0.55 ± 0.02a
	G	0.36 ± 0.02a	0.41 ± 0.01b	0.50 ± 0.02c	0.37 ± 0.01a
GSSG/GSH	S	0.11 ± 0.01a	0.16 ± 0.01b	0.23 ± 0.02c	0.12 ± 0.02a
	O	0.06 ± 0.01a	0.11 ± 0.01b	0.09 ± 0.01b	0.05 ± 0.02a
	C	0.08 ± 0.01a	0.12 ± 0.01b	0.21 ± 0.02c	0.07 ± 0.02a
	G	0.13 ± 0.02a	0.20 ± 0.02b	0.25 ± 0.02c	0.14 ± 0.01a

Oxidized and reduced forms of glutathione were calculated as nmoles/mg mitochondrial protein and Cho and PL content were calculated as µmoles/mg total lipids considering an average of 670 for the MW of PL. They were determined in sonicated mitochondrial suspensions isolated from the brain of sham operated animals and in core, penumbra, and contralateral hemispheres (48 h post-stroke) using the methodologies mentioned in the Research methods and procedures section. Homogenates from the experimental diets supplemented with commercial oils were indicated by capital letters (S, soybean; O, olive; C, coconut; G, grape seed). Statistical differences among results (brain zones analyzed) from the same diet were identified with distinct superscript letters (ANOVA plus Tukey test, $p < 0.01$).

Table 7. Ceramide content in brain homogenates from sham operated rats and from core, penumbra, and contralateral hemispheres 48 h post-stroke.

Ceramide	Diet	Sham	Stroke		
			Core	Penumbra	Contralateral
µmoles/mg total lipids	S	1.6 ± 0.1a	1.7 ± 0.2a	3.2 ± 0.2b	1.5 ± 0.2a
	O	1.2 ± 0.2a	1.3 ± 0.1a	1.7 ± 0.1b	1.3 ± 0.1a
	C	1.4 ± 0.2a	1.6 ± 0.1a	4.4 ± 0.3b	1.5 ± 0.2a
	G	1.5 ± 0.1a	1.8 ± 0.2a	4.8 ± 0.1b	1.4 ± 0.2a

Ceramide content (expressed as µmoles palmitoyl ceramide, MW: 537.9/mg total lipids) was determined in homogenates from sham operated animals and in core, penumbra, and contralateral hemispheres (48 h post-stroke) using the methodologies mentioned in the Research methods and procedures section. Homogenates from the experimental diets supplemented with commercial oils were indicated by capital letters (S, soybean; O, olive; C, coconut; G, grape seed). Statistical differences among results (brain zones analyzed) from the same diet were identified with distinct superscript letters (ANOVA plus Tukey test, $p < 0.01$).

Table 8. Cytokine contents in peripheral plasma from sham operated and 48 h post-stroke rats.

Cytokines (pg/mL)	Diet	Sham	Stroke
IL-1	S	2.5 ± 0.1a	8.8 ± 0.3b
	O	1.8 ± 0.05a	4.2 ± 0.03c
	C	2.2 ± 0.1a	8.3 ± 0.2b
	G	2.3 ± 0.1a	9.0 ± 0.3b
IL-6	S	3.0 ± 0.1a	14.7 ± 0.3b
	O	2.7 ± 0.1a	8.2 ± 0.2c
	C	2.6 ± 0.05a	13.9 ± 0.4b
	G	3.1 ± 0.1a	16.1 ± 0.3b
IL-10	S	20.3 ± 0.9a	14.4 ± 0.4b
	O	22.5 ± 0.7a	18.1 ± 0.5c
	C	18.9 ± 0.3a	13.7 ± 0.4b
	G	20.1 ± 0.8a	12.8 ± 0.3b
Ratio IL-10 /(IL-1 + IL-6)	S	3.7 ± 0.1a	0.6 ± 0.03c
	O	5.0 ± 0.05b	1.6 ± 0.1d
	C	3.9 ± 0.1a	0.6 ± 0.1c
	G	3.7 ± 0.1a	0.5 ± 0.05c

Cytokine contents (expressed as pg/mL) were determined in peripheral plasma from sham operated and 48-h post-ischemic animals using the methodologies mentioned in the Research Methods and Procedures section. Samples from the experimental diets supplemented with commercial oils were indicated by capital letters (S, soybean; O, olive; C, coconut; G, grape seed). Statistical differences among results for a determined type of cytokine were identified with distinct superscript letters (ANOVA plus Tukey test, $p < 0.01$).

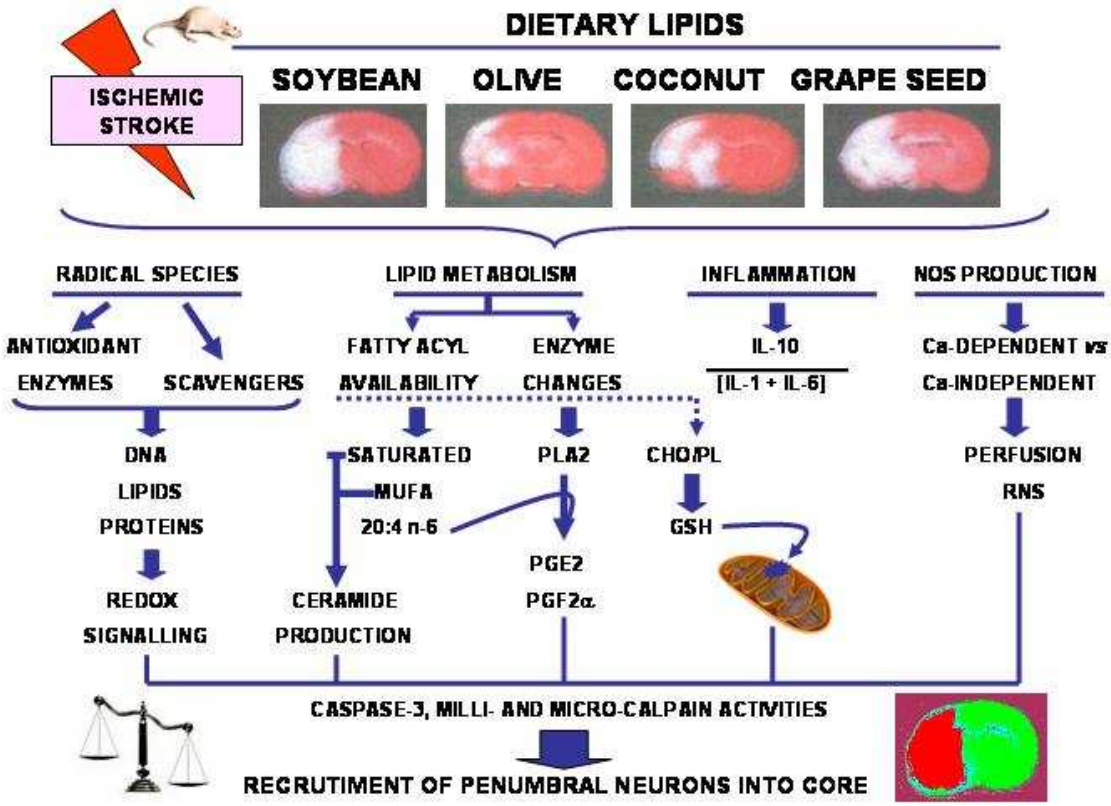


Figure 1

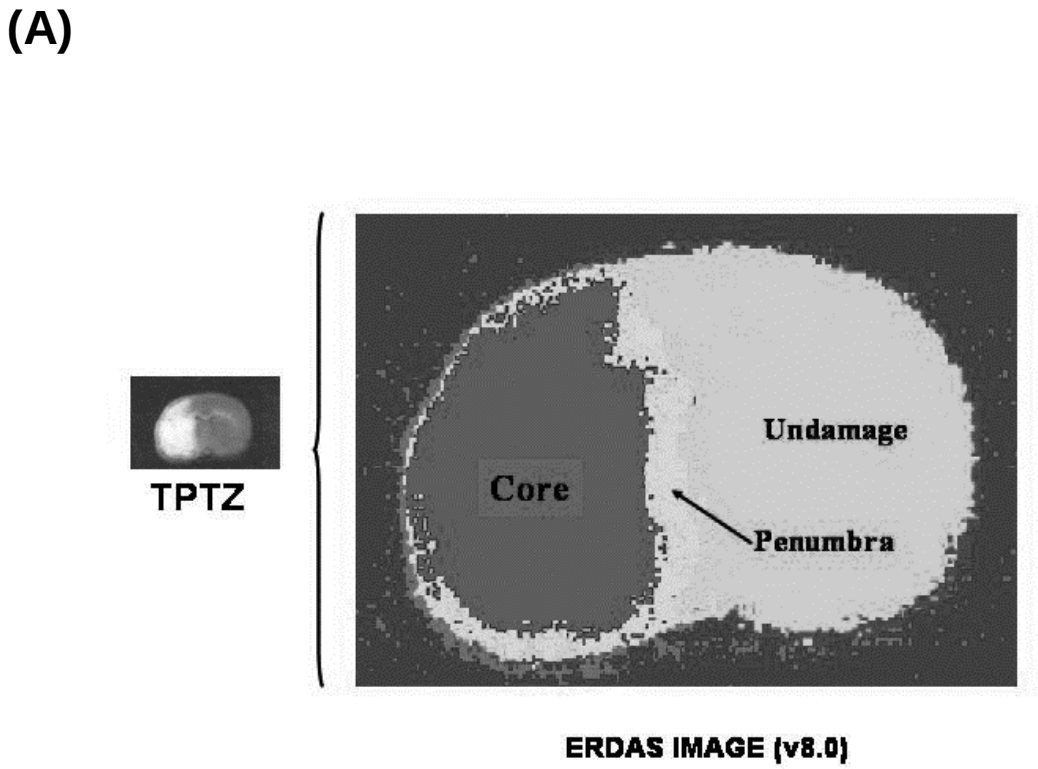
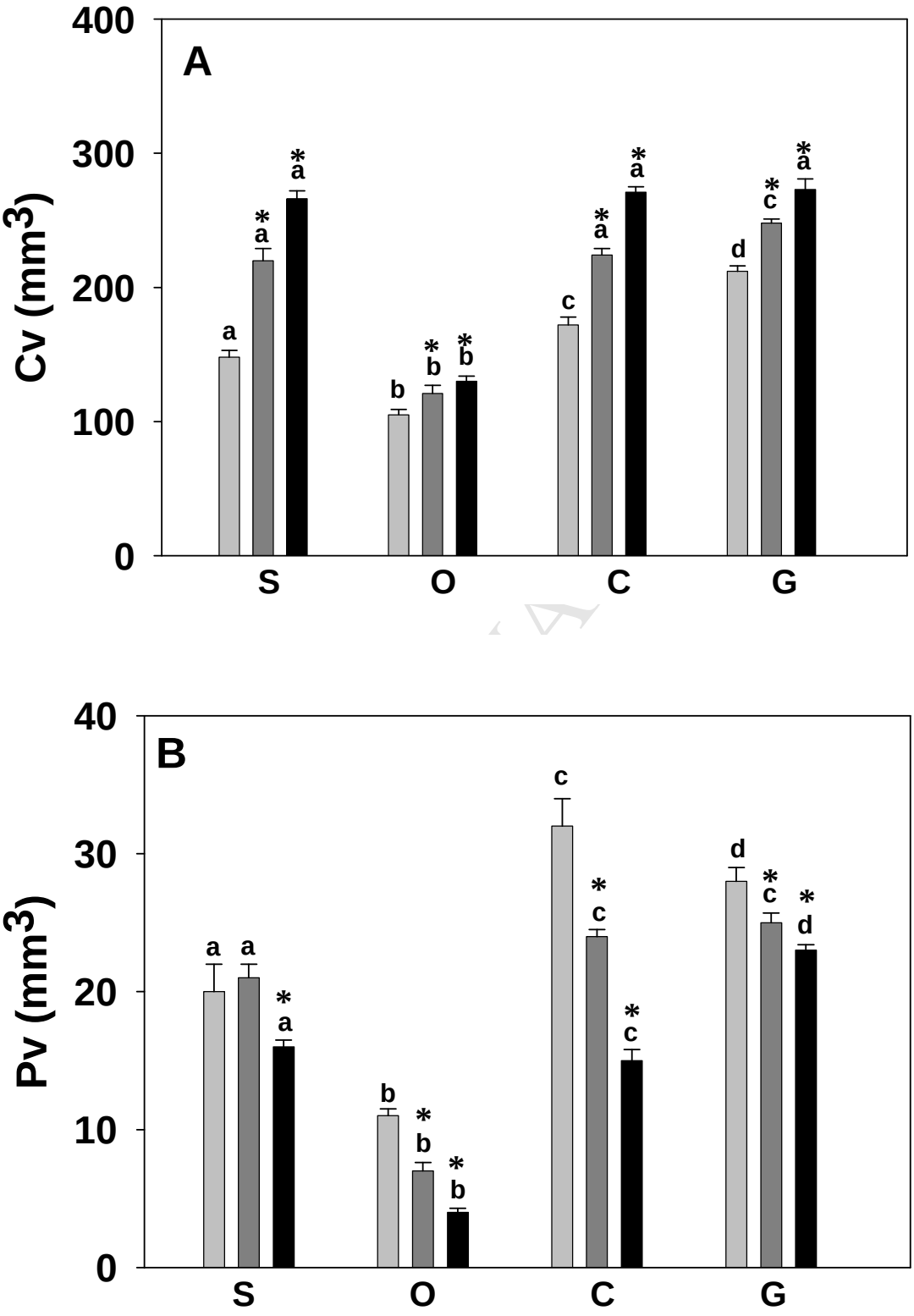


Figure 2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Cv (mm3)
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300
200
100
0
S
O
C
G
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a
B
Pv (mm3)
40
30
20
10
0
S
O
C
G
a
a
a
b
b
b
c
c
c
d
c
d

Figure 3

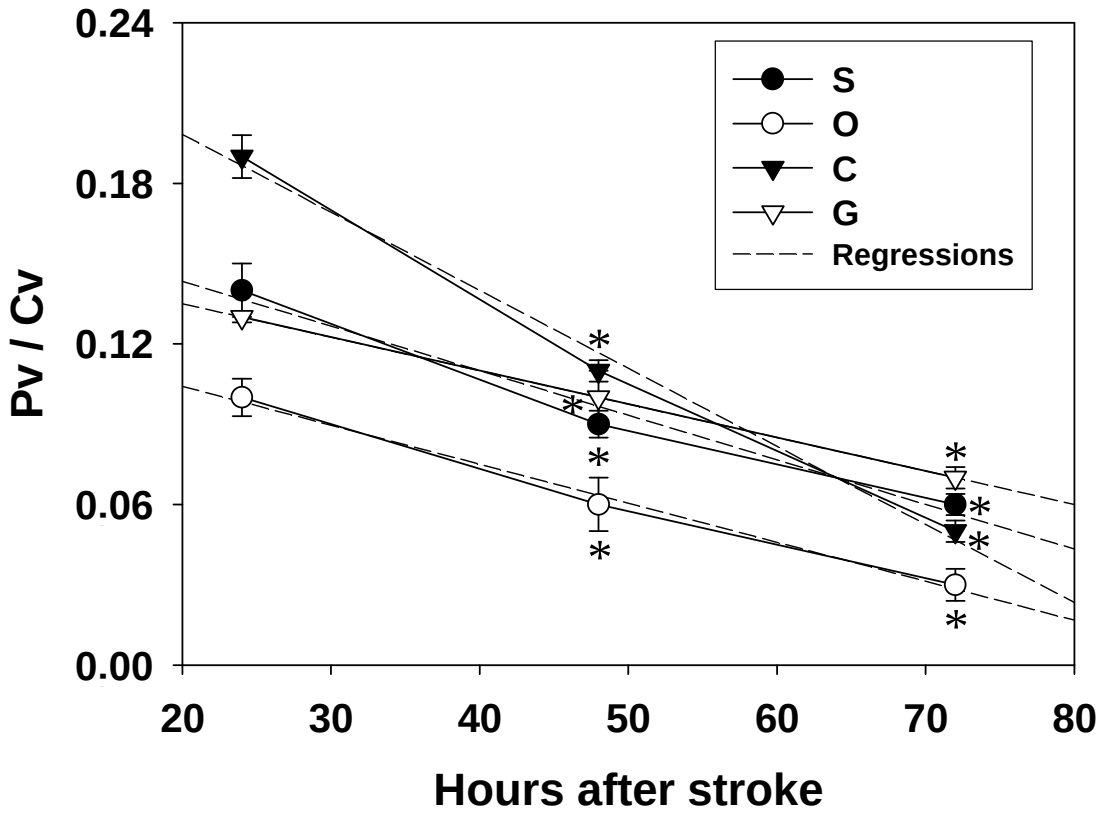
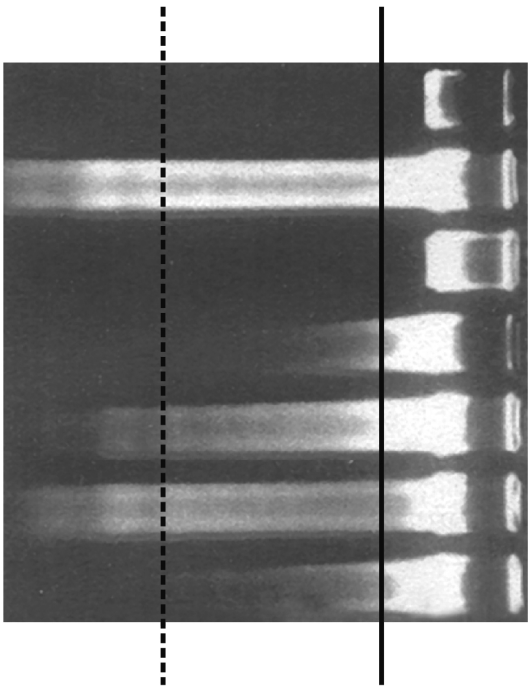


Figure 4



	Relative intensity	
	——	-----
SHAM	0	0
G	122	66
CONTRALATERAL	0	0
O	14	0
C	86	10
S	33	31
O	12	0

Figure 5

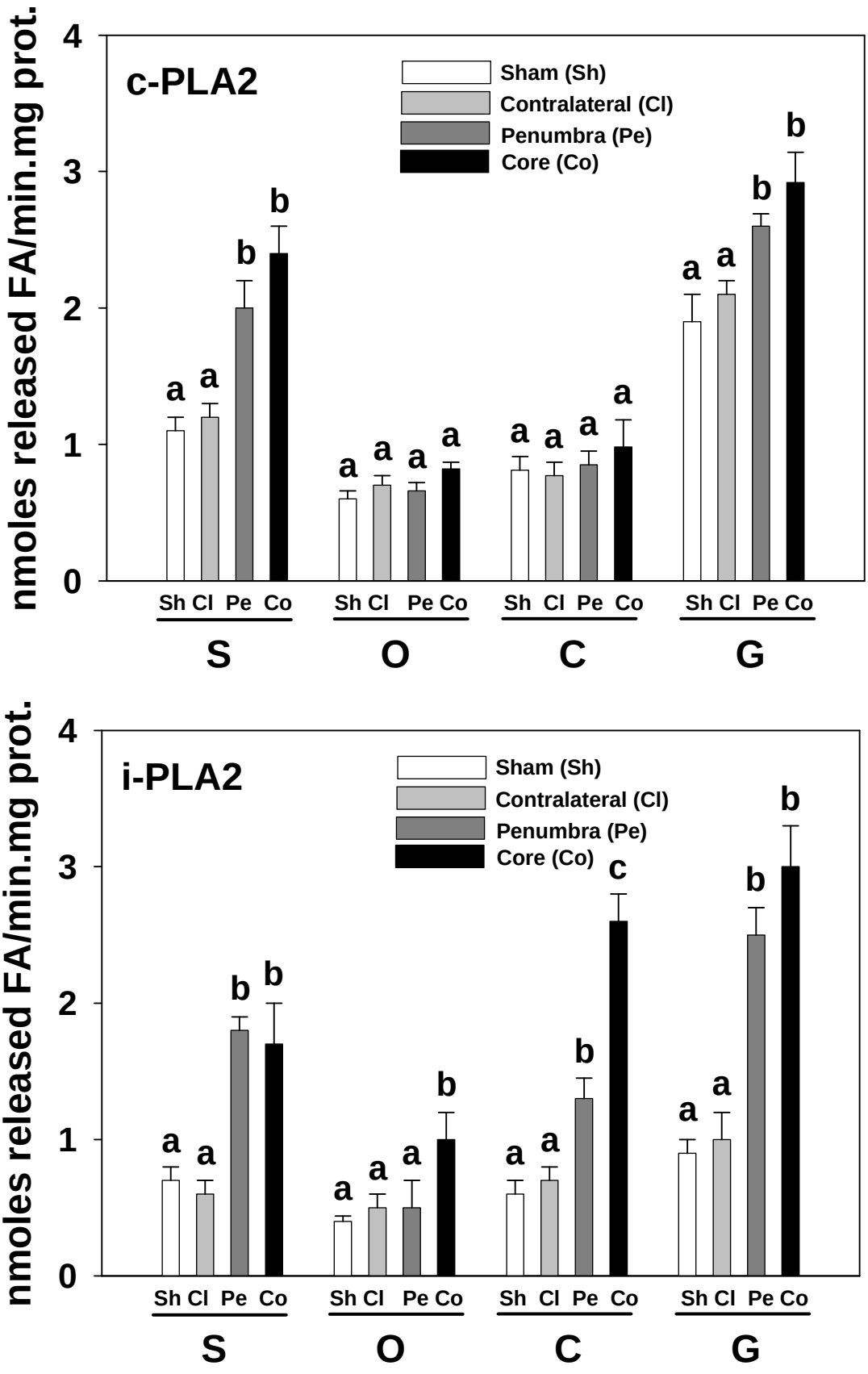

c-PLA2
Sham (Sh)
Contralateral (Cl)
Penumbra (Pe)
Core (Co)
nmoles released FA/min.mg prot.
0
1
2
3
4
a a b b
a a a a
a a a a
a a b b
Sh Cl Pe Co
S
O
C
G
i-PLA2
Sham (Sh)
Contralateral (Cl)
Penumbra (Pe)
Core (Co)
nmoles released FA/min.mg prot.
0
1
2
3
4
a a b b
a a a b
a a b c
a a b b
Sh Cl Pe Co
S
O
C
G

Figure 6

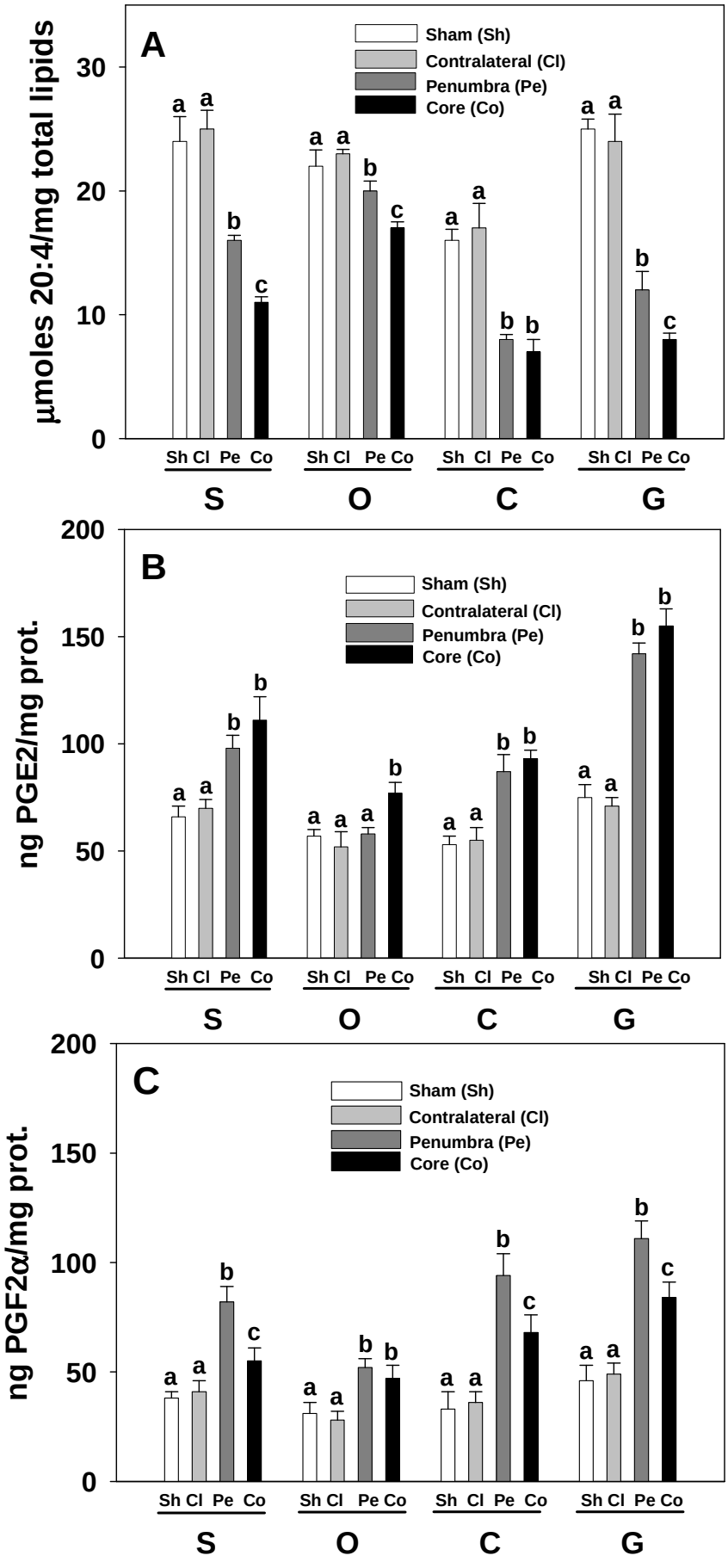

A
Sham (Sh)
Contralateral (Cl)
Penumbra (Pe)
Core (Co)
µmoles 20:4/mg total lipids
0
10
20
30
Sh Cl Pe Co
S
O
C
G
B
ng PGE2/mg prot.
0
50
100
150
200
C
ng PGF2α/mg prot.
0
50
100
150
200

Figure 7

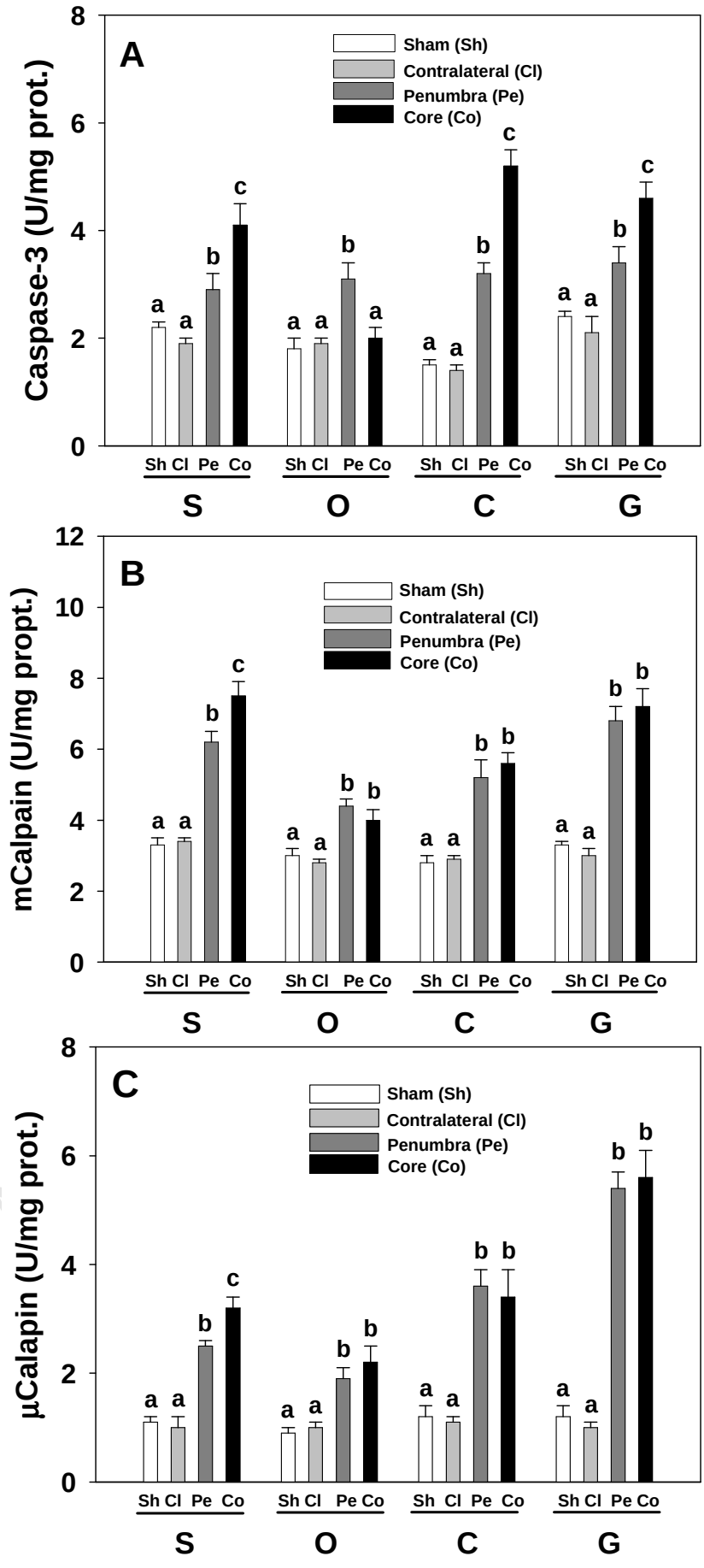
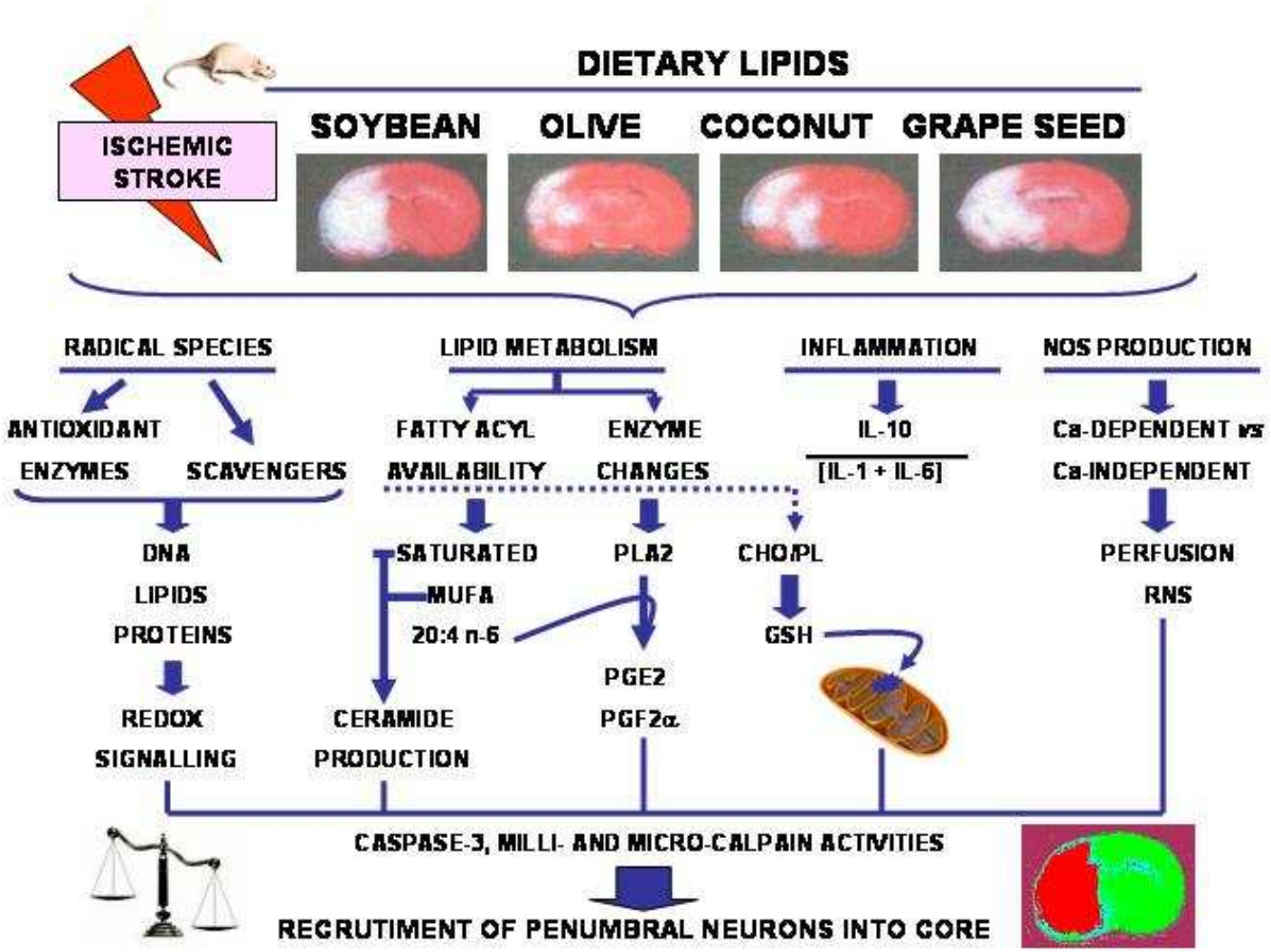

A
Caspase-3 (U/mg prot.)
Sham (Sh)
Contralateral (Cl)
Penumbra (Pe)
Core (Co)
Sh Cl Pe Co
S O C G
B
mCalpain (U/mg propt.)
C
µCalapin (U/mg prot.)

Figure 8 (please reproduce in gray scale)



Highlights

- ✓ Diets enriched with soybean and grape seed oils favor inflammation and oxidation.
- ✓ Olive oil maintains redox status and reduces damage to lipids, proteins and DNA.
- ✓ Ratio penumbra/core was reduced in olive-oil-fed rats.
- ✓ Phospholipase A2 activity and prostaglandin production were decreased by olive oil.
- ✓ Caspase-3 and calpains activities were less active in olive-oil-fed rats.