Journal of Functional Foods 64 (2020) 103693

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Grape seed proanthocyanidin extract and swimming training enhances neuronal number in dorso-medial prefrontal cortex in middle-aged male rats by alleviating oxidative stress

S. Abhijit[a], Sunil Jamuna Tripathi[b], B.S. Shankaranarayana Rao[b], S. Asha Devi[a,*]

[a] Laboratory of Gerontology, Department of Zoology, Bangalore University, Bengaluru 560 056, Karnataka, India
[b] Department of Neurophysiology, National Institute of Mental Health and Neurosciences, Bengaluru 560 029, Karnataka, India

ARTICLE INFO

Keywords:
Age
Grape seed proanthocyanidin extract
Prefrontal cortex
Neuroprotection

ABSTRACT

The dorsomedial prefrontal cortex (dmPFC) is critical for working memory, which declines with age. The mechanisms for aging dmPFC are oxidative stress, antioxidant defense and anatomical alterations. This study was carried out to test whether swimming training and grape seed proanthocyanidin extract (GSPE) are suitable interventions for curtailing free radical generation in the dmPFC in middle-aged male rats. The results indicated that superoxide and hydrogen peroxide increased in the middle-aged, as did lipid and protein oxidations, while superoxide dismutase and catalase activity decreased. There were positive correlations between age and lipofuscin. Importantly, the rats experienced reductions in neuronal number, volume and density in the dmPFC. Rats under combined interventions displayed more alleviation of the age changes than rats with single interventions suggesting the possibilities of utilizing GSPE as a functional supplement in conjunction with swimming exercise towards improving neuronal survivability, and preventing cognitive decline beyond middle-age.

1. Introduction

The stride of modern medicine has led to an increase in longevity but is not devoid of age-associated impairments in cognition and declined motor co-ordination. Working memory (WM) is associated with the temporary storage and control of information required for learning, reasoning and language comprehension (Baddeley, 2010; Granon & Poucet, 2000), and is dependent on the processing capacity of the medial prefrontal cortex (mPFC). The anterior cingulate cortex (ACC) of the dorsomedial PFC (dmPFC) connects the nucleus accumbens (ACB), ventral tegmental area (VTA), amygdala (AMY) and mediodorsal nucleus of the thalamus (Schweimer & Hauber, 2005). In addition to be connected to the thalamus, striatum, AMY, and ACB the prelimbic cortex (Prl) have direct connection with the CA1 subfield of the ventral hippocampus. The ACC connects the motor, somatosensory, visual, and retrosplenial cortices. The dmPFC has an important role in the processing of WM in a delayed alternation task (DAT). Imaging studies on non-human primates have revealed a tonic activity in the ACC during the delay period of a WM task (Procyk & Joseph, 2001) with the ACC being associated with reward expectancy (Amiez, Joseph, & Procyk, 2006). A study on Prl has shown its involvement in monitoring and processing of WM task in rats (Gisquet-Verrier and Delatourm, 2006). The ACC and Prl sub-serve the various aspects of WM wherein the ACC is more associated with reward and the Prl with acquired memory (Walton et al., 2003).

Brain aging is associated with a progressive increase in mitochondrial free radical (FR) generation. The increase in lipid peroxidation (LPO) and protein oxidation (Anand, Asha Devi, & Ravikiran, 2014) exemplify the changes in the redox state of the aging brain with impaired endogenous antioxidant mechanisms (Asha Devi & Ravi Kiran, 2004).

Normal brain aging is also associated with the substantial shrinkage of the brain's volume in humans (Peters, 2006) and a previous study on rats aged 4-(adult) and 18-months (middle-age) showed reduced volume and neuronal number in the aging hippocampal CA1 region have shown that grape seed proanthocyanidin extract (GSPE) at a dosage of 400 mg/kg body weight for a period of 16 weeks combined with swimming exercise prevents morphological changes in the hippocampus (HC) (Abhijit et al., 2018) and alleviates the spatial WM decline through the elevated expression of m1AChR in the mPFC in middle-aged rats (Abhijit, Subramanyam, & Asha Devi, 2017).

Proanthocyanidins are oligomers or polymers of polyhydroxy flavan-3-ol units, (+)-catechin and (−)-epicatechin (Porter et al., 1986), and are present in large amounts in the polyphenols of grape

* Corresponding author.
E-mail address: sambeashadevi@bub.ernet.in (S. Asha Devi).

https://doi.org/10.1016/j.jff.2019.103693
Received 15 June 2019; Received in revised form 6 November 2019; Accepted 19 November 2019
Available online 11 December 2019

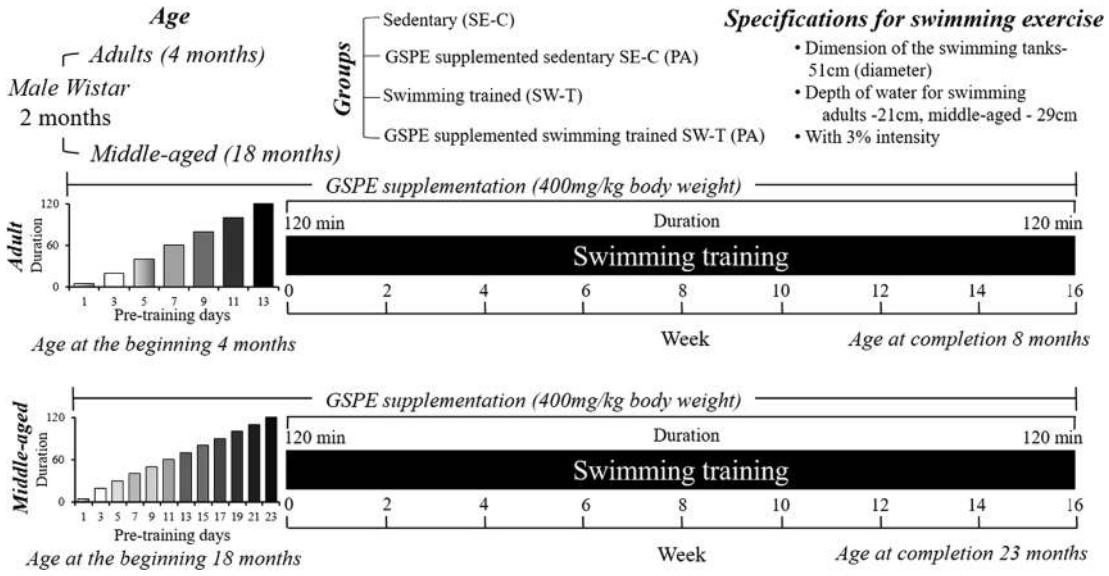

Fig. 1. Timeline of swimming training and GSPE supplementation in adult and middle-aged rats.

seeds. Further studies by Xianchu et al.(2018) on mouse model subjected to forced swimming for 28 days have shown that GSPE could be used as an effective functional food to delay exhaustive swimming-induced fatigue and improve exercise capacity. Findings on the synergistic interaction between the HC and PFC and spatial memory (Wang et al., 2006) with coordinated cellular activities during the WM (Hyman, Zilli, Paley, & Hasselmo, 2005) have been used to further investigate the PFC in the present study.

Studies have revealed the neuroprotective effects of swimming-induced adaptive response and tolerance to oxidative stress (OS) (Radak et al., 2017). Further, although studies related to GSPE and exercise in improving cognitive ability have been carried out; the effects of these factors on the mPFC have not yet been studied extensively.

In the present study, we evaluated the possible synergistic effect of GSPE administration and swimming training on the mPFC with the following hypotheses: (1) GSPE and swimming training, may protect the mPFC by ameliorating the antioxidant defense system and curtailing the FR generation associated with normal aging. (2) The anatomical alterations in the ACC and Prl are related to elevate mitochondrial FRs (3). The bioavailability of constituents of GSPE is responsible for its synergistic effect. To test these hypotheses, we examined the mPFC in terms of (1) endogenous antioxidants and mitochondrial FR generation along with oxidative damage by analyzing the lipid peroxidation (LPO) and protein oxidation, (2) the bioavailability of GSPE's monomeric constituents, and (3) the neuronal number and volume of the ACC and Prl.

2. Material and methods

2.1. Experimental procedures

Animal care and maintenance - Seventy-two healthy male *Wistar* rats between the ages of 2 to 2.5 months were obtained from the Central Animal Facility of the Indian Institute of Science (Bengaluru) and were maintained in the departmental animal facility until the age of 4 months, the rats were fed *ad libitum* standard laboratory chow (Amrut Lab Animal Feed, Bengaluru, India) and tap water. They were maintained at 22 ± 1 °C with a 12-h/12-h light/dark cycle. Upon reaching the age of 4 months rats were randomly segregated equally into two groups: adults (4 months) and middle-aged (18 months) and were maintained as described above.

All experiments involving the animals were carried out in conformity with the Institutional Animal Ethics Committee (IAEC) after obtaining permission (IAEC/402/19/9/2014/CPCSEA) in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Experimental grouping and supplementation - The animals from each age were randomly divided into four sub-groups (n = 9 per sub-group): as SE-C, (sedentary controls); SE-C(PA),(sedentary supplemented with GSPE); SW-T, (swimming trained); and SW-T(PA), (swimming trained supplemented with GSPE). The supplemented rats received an oral dose of 400 mg/kg body weight/day of GSPE over a period of 16 weeks. Gravinol (Kikkoman Co. Ltd, Noda, Japan) is a natural substance extracted from grape seeds with ethanol and water as eluents and then purified and condensed, avoiding use of any toxic solvents. It is composed of 96% proanthocyanidin and other minor components such as protein, fat, ash and moisture and is referred as grape seed proanthocyanidin extract (GSPE). In the present study, we have supplemented rats with a similar dose of GSPE as of our earlier studies (Abhijit et al., 2017) in order to further understand the possibilities, if any, of GSPE's effect in increasing the neuronal density in the adult and middle-aged rats.

Swimming Training - Each day the rats were transferred to the exercise training room and allowed to swim in a circular tank of 51 cm diameter, with water at a depth of 21 cm for the adults and 29 cm for the middle-aged. Swimming training was initiated with pre-training the animals to swim with a load of 3% of their body weight tied to their tails. During pre-training, the duration was increased progressively from 5 mins till they swam for 2 hrs and thereafter continued for 16 weeks as the training period. The pre-training period, however, lasted for 21 days for the middle-aged animals against 14 days for the adults as shown in Fig. 1.

2.2. Estimation of redox status in the mPFC

2.2.1. Isolation of mitochondria

10 k mitochondrial pellets was obtained from mPFC by the method of Geller and Winge (1982). Homogenizing buffer was prepared with 0.17 M KCl, 0.01 M EDTA, 0.01 M tris-HCl (cat# T-3253-250G, Sigma-Aldrich, USA) and 1% bovine serum albumin (BSA, cat# A-2153, Sigma-Aldrich, USA). mPFC was homogenized in homogenizing buffer in 1:1.5 ratio and further diluted in 6 times in the same buffer and centrifuged for 15 min at 300g. Resultant supernatant was collected and the precipitate was dissolved in the buffer (6X) and again centrifuged for 15 min at 300g. Supernatant was collected and were centrifuged for 15 min at 10000g. Precipitate was collected and suspended in the 5X

homogenizing buffer and again centrifuged for 15 min at 10,000g. 10 K mitochondrial pellets were obtained from the precipitate following separation of supernatant. 10 K pellets were finally suspended in 3x 0.1 M phosphate buffer (pH 7) and stored at −80 °C till analysis.

2.2.2. Free radicals

2.2.2.1. Superoxide. Superoxide was spectrophotometrically estimated by the method used by Das, Padma, Sagar, Ramesh, and Koratkar (1990). To 200 μL of mitochondrial 10 K pellet, 80 μL of 0.1% nitro blue tetrazolium (cat# N5514-10TAB, Sigma-Aldrich, USA) dissolved in PBS (pH 7.4) was added and the mixture was incubated in a hot water bath for 45 min followed by centrifugation at 1000g for 10 min at 4 °C. The difarmazan formed was then dissolved in 1 mL of glacial acetic acid and absorbance was read at 560 nm.

2.2.2.2. Hydrogen peroxide. H_2O_2 generation was spectro photometrically determined in the 10 K pellet by the method used by Josephy, Elingg, and Mason (1982). Briefly, 100 μL of 100 nmol 3,5,3′,5′-tetramethylbenzidine (cat# 860336-5G, Sigma-Aldrich, USA) in 0.2 N HCl, 50 μL of horseradish peroxide (cat# RM664-5000U, Himedia, India) and 100 μL of 10 K pellet were dissolved in 0.2 M of an acetate buffer (pH 5.0), and absorbance of the blue product was measured at 700 nm against blank and H_2O_2 were determined by calibration from standard and expressed as nmol H_2O_2 generated / mg mitochondrial protein.

Oxidative stress - Brains were harvested, and mPFCs were excised approximately (Spijker, 2011) between bregma −4.68 mm to bregma −0.60 mm. The isolated mPFCs were weighed, snap-frozen in liquid nitrogen and stored at −80 °C until the time of the analysis. For biochemical estimations of stress markers, the motor cortices were removed from the isolated mPFCs.

2.2.2.3. Lipid peroxidation. A 10% tissue homogenate of mPFC was prepared in 1.15% KCl and assayed by the method used by Ohkawa, Ohishi, and Yagi (1979). 200 μL of the tissue homogenate was vortexed with 8% sodium dodecyl sulphate (cat# 161-0301, Bio-rad, USA). 1.5 mL of 0.8% TBA (cat# T5500-25G, Sigma-Aldrich, USA) and 1.5 mL of 20% acetic acid (pH 3.5) were added and kept for 50 min in a boiling water bath. After 1 hr, the reaction mixture was removed from the water bath, cooled and butanol: pyridine mixture (15:1) was added to the reaction tube, mixed well and centrifuged at 300*g* for 10 min. Absorbance of the clear layer was measured at 532 nm against blank butanol:pyridine mixture. 1,1,3,3-Tetramethoxy propane (cat# T-1642, Sigma-Aldrich, USA) was used as standard and extent of lipid peroxidation was expressed as nmol of MDA/mg protein.

2.2.2.4. Protein oxidation. Protein carbonyl content was estimated using 3% tissue homogenate prepared in 0.25 M of a sucrose buffer and by the method of Levine, Williams, Stadtman, and Shacter (1994). In brief, tissue homogenate was centrifuged at 1700g for 10 min at 4 °C. The supernatant was collected and equally divided into 2, 4 dinitrophenol hydrazine (DNPH, cat# V800012-25G, Sigma-Aldrich, US), treated and DNPH untreated fractions and was incubated for 1 hr at room temperature. An equal volume of 10% TCA was added to both the fractions and centrifuged at 1700g for 10 min at 4 °C. The resultant precipitate was washed three times with ethyl acetate and ethanol mixture (1:1), dissolved in 600 μL of 6 M guanidine hydrochloride (cat# G4505-100G, Sigma-Aldrich, USA) and centrifuged at 1000g for 10 min at 4 °C. The supernatant was collected and absorbance was read at 365 nm against respective DNPH untreated fraction. A molar extinction coefficient $2.2 \times 10^4\ M^{-1}\ cm^{-1}$ was used to obtain the extent of protein carbonyl incorporation in mPFC and expressed as μmol DNPH incorporated / mg protein.

2.2.2.5. Lipofuscin (LF). For LF estimation in the ACC, the fixed section belonging to bregma 2.28 mm were cleared in xylene, air dried, coverslipped and examined under a fluorescence microscope (Olympus IX 71, Japan) at ex 560 nm (Brunk & Terman, 2002) using the 20x objective lens (LCPlanFI 20x/0.04/∞ and optical lens WH10x/22, Olympus, Japan). Fluorescent intensity was quantified as corrected total cell fluorescence (CTCF) using NIH-ImageJ-FIJI (Wayne Rasband, Version 1.52a, NIH, USA) where integrated fluorescence density was subtracted by area of ROI × mean adjacent background fluorescence (Ansari et al., 2013).

2.2.3. Antioxidant defense

After the separation of motor cortex from the isolated mPFC, GSH system and antioxidant enzyme activities were studied.

2.2.3.1. Superoxide dismutase (SOD) activity. SOD activity was determined in 100 μL cytosolic fraction obtained following 10 K mitochondria pellets by the method of Misra and Fridovich (1972). In brief, 20 μL of 30 mM epinephrine (cat# E-4250, Sigma-Aldrich, USA) in 0.05% acetic acid was added to 990 μL of cytosolic fraction and 880 μL of 0.05 M carbonate buffer (pH 10.2) containing 0.1 mM EDTA, vortexed and absorbance was recorded at 480 nm against blank for 4 min.. SOD activity was expressed as unit/mg protein. One unit is equivalent to the amount of enzyme capable of inhibiting oxidation of epinephrine by 50%.

2.2.3.2. Catalase (CAT) activity. CAT activity was determined spectrophotometrically by the method of Aebi (1984). In brief, 100 μL of cytosolic fraction of tissue and ethanol was incubated in an ice bath for 30 min and then at 25 °C for 10 min. 10 μL of Triton X-100 (cat# T9284, Sigma-Aldrich, USA), 200 μL of 50 mM phosphate buffer and 250 μL of 0.06 M hydrogen peroxide were added and absorbance was read at intervals of 30 sec for 2 min at 240 nm. Enzyme activity was determined by using a molar extinction coefficient 43.6 M/ min / cm was used to determine enzyme activity. One unit of CAT activity is equal to mM of H_2O_2 hydrolysed / min / mg protein.

2.2.3.3. Glutathione (GSH). GSH level was evaluated in 2.5% tissue homogenate prepared in 0.15% KCl as described by Kuo, Maita, Sleight, and Hook (1983). Briefly, 2.0 mL of the tissue homogenate was deproteinized with 3 mL 30% solution containing 1.67% metaphosphoric acid, NaCl and 0.2% EDTA. The sample was centrifuged at 10,000 g for 20 min at 4 °C. 0.5 mL of diluted supernatant fraction was mixed with 2 mL of 0.3 M Na_2HPO_4 buffer (pH 8) and 2 mL of 0.04% 5,5-dithiobis(2-nitrobenzoic acid) (cat# D8130-1G, Sigma-Aldrich, USA) in 10% sodium citrate. The absorbance of the yellow color developed was read immediately after mixing at 412 nm against a blank that contained metaphosphoric acid, NaCl and EDTA in place of the supernatant. GSH values were determined by calibration from standard GSH curve and expressed as μM GSH / mg protein.

2.2.3.4. Glutathione peroxidase (GPx) activity. GPx activity was assayed spectrophotometrically by the method of Flohé and Gunzler (1984). The reaction mixture consisted of 500 μL of phosphate buffer, 100 μL 0f reduced glutathione (cat# G4251-300MG, Sigma-Aldrich, USA) 100 μL of 1.5 mM nicotinamide adenine diucleotide phosphate tetrasodium salt hydrate (cat# 5755-100MG, Sigma-Aldrich, USA) and 100 μL of 0.24U GR (cat# G-3664-2500U, Sigma-Aldrich, USA). One hundred microliters of tissue extract was added to the reaction mixture and incubated at 37 °C for 10 min. Fifty microliters of t-butyl hydroperoxide (cat# B-2633, Sigma-Aldrich, USA) was added to 450 μL of tissue reaction mixture and measured at 340 nm for 180 s in a spectrophotometer. A molar absorption coefficient of 6.2×10^3 M/cm was used to determine enzyme activity. One unit of GPx activity is equal to μM NADPH oxidized/min/mg protein.

2.2.3.5. Glutathione reductase (GR) activity. GR activity was estimated

spectrophotometrically by the method of Carlberg and Mannervik (1981). The supernatant obtained from the 2% tissue homogenate in 0.1 M PBS (pH 7.4) followed by centrifugation at 300g for 10 min was taken for the enzyme assay. The reaction mixture was 2.0 mL of 0.1 M (pH 7.4) phosphate buffer, 0.1 mL of 1 mM oxidized glutathione (cat# G4376-250MG, Sigma-Aldrich, USA), 0.1 mL of 250 μM of flavin adenine dinucleotide, 0.05 mL of 80 mM EDTA and 0.1 mL of supernatant and incubated at 37 °C for 15 min followed by the addition of 0.1 mL of 4 mM β-nicotinamide adenine diucleotide phosphate-tetrasodium salt hydrate (cat# 5755-100MG, Sigma-Aldrich, USA). Decrease in absorbance was read at 340 nm for 2 min. A molar extinction coefficient of NADPH, 6.22 × 10^3/M/cm was used to determine enzyme activity. One unit of GR activity is equal to μM NADPH oxidized/min/mg protein.

2.2.3.6. Glutathione-S-transferase (GST) activity. GST activity was estimated spectrophotometrically by the method of Habig, Pabst, and Jakoby (1974). In brief, 2.2 mL of phosphate buffer (100 mM; pH 6.5), 0.1 mL glutathione reduced (cat# G4251-300MG, Sigma-Aldrich, USA) and 0.1 mL of 30 mM 1-Chloro-2,4-dinitrobenzene (cat# 138631-100G, Sigma-Aldrich, USA) were taken in the cuvette. Reaction was initiated by adding 0.6 mL of supernatant with the reference cuvette having 0.6 mL phosphate buffer. The increase in absorbance was recorded at 340 nm for a total period of 5 mins and for every 30 sec against blank. GST activity was expressed as nM conjugate formed/min/cm using molar extinction coefficient, 9.6 × 10^3 M^{-1}cm of GSH-CDNB conjugate.

2.3. Protein measurement

Biochemical estimations were normalized to protein content using Bovine serum albumin (cat# A-2153, Sigma-Aldrich, USA) as standard protein as per Lowry, Rosebrough, Farr, and Randall (1951).

2.4. Bioavailability of monomeric subunits of GSPE

The animals was fasted overnight after the final day of exercise and after two hours of GSPE supplementation. Rats were transcardially perfused with ice-cold 0.1 M phosphate buffered saline (pH 7.0). The isolated mPFCs were weighed, snap-frozen in liquid nitrogen and stored at −80 °C until time of the analyses.

2.4.1. Phenolic extraction of samples for the LC-MS/MS analyses

The active monomeric constituents of GSPE were extracted by incubating the mPFC homogenate with 1 mL enzyme solution (250 U β-glucuronidase with sulfatase activity) (cat# G7017-1ML, Sigma-Aldrich, USA) in 0.4 M sodium phosphate monobasic (pH = 4.5) for 45 min at 37 °C. Further, samples were extracted in a 0.01% butylated hydroxytoluene-ethyl acetate mixture. Ethyl acetate combined fractions were dried in a nitrogen evaporator and reconstituted in a 500 μL mobile phase, sonicated for one min and introduced into 2 mL autosampler HPLC vials (ThermoFisher Scientific, USA) through a Maxil syringe-filter (13 mm × 0.45 μm).

2.4.2. Measurement of gallic acid, catechin and epicatechin

Gallic acid (GA), catechin [(+)-CT] and epicatechin [(−)-EC] bioavailable concentrations in the mPFCs were quantified by the method described by Wu et al.(2003). A Synergi™ C18 4U Hydro RP 80 Å column (4 μm, 250 × 3 mm i.d.) (Phenomenex, USA) was used for separations on a Series 200 HPLC (Perkin Elmer, USA). The column effluents were introduced into the PE SCIEX API 3000 LCMS-MS (AB Sciex, USA) system. Multi-level calibration curves were constructed with the standards of GA (cat# G0625-500G, Sigma-Aldrich, USA) , (+)-CT (cat# C1251-10G, Sigma-Aldrich, USA) and (−)-EC (cat# E1753-1G, Sigma-Aldrich, USA) for mass data analyses using Analyst® 4.1 software (AB Sciex, USA).

2.5. Stereological analysis

Following completion of training, rats were anesthetized with halothane (Piramal Healthcare, India) and was transcardially perfused with 0.9% saline followed by 4% formaldehyde. Brains were removed carefully, post-fixed for 48 h in the same fixative and subsequently processed for vibratome (Leica, Wetzlar, Germany) sectioning. 40 μm thick coronal sections were obtained through the rostrocaudal axis of the mPFC (approximately between bregma 4.68 mm to 1.56 mm) (Paxinos & Watson, 2007). Every twelveth section was mounted on albumin-coated glass slides. Mounted sections were stained with 0.1% cresyl violet (cat# C5042-10G, Sigma-Aldrich, USA). Briefly, sections were remobilized in chloroform for 2 mins and were hydrated using decreasing ethanol grades, stained, differentiated and dehydrated in increasing grades of ethanol. Stained sections were cleaned in xylene, air dried, mounted with vectamount (cat# H5000, Vector Labs, USA) and coverslipped. These brain sections were used for stereological studies.

2.5.1. Estimation of volume, neuronal number and density of anterior cingulate cortex by optical fractionator method

The optical fractionator technique is random systematic sampling method that allows uniform sampling of a known fraction of section thickness under a known area and is independent of the size, shape, and orientation of the cells. The optical fractionator combines the optical dissector and fractionator-sampling scheme. Therefore, optical fractionator technique was employed to obtain the number of pyramidal neurons in the ACC. The pyramidal cells were visualized and live images were captured using a high-resolution camera (Optronics, Microfire, USA) attached to a microscope (Olympus BX51, Japan) with a motorized LEP ×, y stage and motorizedz-axis (LEP MAC 5000). Contours were drawn at the anatomical boundary of ACC at low magnification (4x; Olympus Japan UPlanFL 4x/0.13 ∞/- and optical lens WH10x-H/22 Olympus, Japan) on every twelfth section between bregma 4.20 mm to 1.56 mm (Fig. 6E) and cells were counted using a high magnification lens (100X, Olympus Japan UPlanFL N 100x/1.03 oil Iris ∞/0.17/FN26.5 andoptical lens WH10x-H/22 Olympus, Japan) only if the cell body with the nucleus came into focus within the dissector height (Table S1). The cell body in touch with the red line (exclusion line) or its extensions were not considered for neuronal counting. Further, while counting the cells the section thickness was recorded in each optical fractionator frame to avoid the possible bias due to tissue shrinkage during processing (West, 2013). Total number of pyramidal neurons in ACC and Prl was obtained by multiplying the total number of cells counted (ΣQ^-) with the reciprocal sampling fractions

$$N = \Sigma\bar{Q} \times \left[\left(\frac{t\bar{Q}}{h}\right) \times \left(\frac{1}{asf}\right) \times \left(\frac{1}{ssf}\right)\right]$$

where t_{Q^-} is the number-weighted mean section thickness, h is the height of the disector, *asf* is the area sampling fraction, *ssf* is the section sampling fraction (Fabricius, Wörtwein, & Pakkenberg, 2008; West et al., 1991). The "asf" consisted of area of X, Y steps, i.e., 300 μm × 300 μm (ACC), 375 μm × 375 μm (Prl); divided by area of counting frame, i.e., 45 μm × 45 μm (ACC), 35 μm × 35 μm (Prl); "tQ^-" ∼ 22.512 ± 0.14 μm (mean ± SEM); and h is 16 μm, "ssf" is 12 as every 12th section was considered for the neuronal counting. Both the left and the right hemispheres were estimated for the total volume, neuronal number, and density of ACC pyramidal cells between layer II and VIb (Tripathi et al., 2019; 2018). Sampling was optimized to produce a coefficient of error $(CE)^3$ (Gundersen, Jensen, Kieu, & Nielsen, 1999). Values of less than 0.05 were calculated automatically by Stereo Investigator 8.1 software.

2.6. Statistical analyses

The statistical analysis was conducted using Prism® 6.01 (GraphPad Inc., USA) and R 3.4.4 (IIT-Madras, CRAN). All data are given as mean ± SEM. To determine a suitable analysis, all data were initially tested for homogeneity by Levene's test and normaility by Shapiro-Wilk's test using R statistical package, and a two-way ANOVA analysis was considered with Tukey's HSD test to assess the differences between the age groups and interventions by Prism® 6.01. The statistical significance threshold was set at $P < 0.05$. A two-tailed Pearson's correlation test was employed to obtain the relationship between the various parameters.

3. Results

3.1. Redox status in the medial prefrontal cortex

3.1.1. Free radicals

3.1.1.1. Superoxide. Age-dependent [$F_{(1,24)} = 1804$, $P < 0.0001$] increase in $O_2^{\bullet-}$ were significantly lowered by the interventions [$F_{(3,24)} = 24.54$, $P < 0.0001$], and the two-way ANOVA suggests a modulator role of the age × intervention interaction [$F_{(3,24)} = 15.36$, $P < 0.0001$]. Although both interventions reduced $O_2^{\bullet-}$ in the middle-aged rats, GSPE inhibited $O_2^{\bullet-}$ more efficiently than swimming exercise. Furthermore, GSPE was effective in reducing $O_2^{\bullet-}$ in the middle-aged SE-C(PA) group compared to the SE-C ($P < 0.0001$), SW-T ($P < 0.01$) and SW-T (PA) ($P < 0.05$) groups. A similar trend was seen with swimming training in the middle-aged groups (Fig. 2A).

3.1.1.2. Hydrogen peroxide. Significant increases in H_2O_2 generation with age [$F_{(1,24)} = 42.21$, $P < 0.0001$] were attenuated in response to the intervention as revealed by two-way ANOVA [$F_{(3,24)} = 111.1$, $P < 0.0001$]. Further, the age × intervention interaction [$F_{(3,24)} = 10.71$, $P < 0.001$] significantly affected H_2O_2 compared to adults. When the adult SE-C group was compared either with combined or single interventions a significant reduction ($P < 0.0001$) in H_2O_2 was evident (Fig. 2B). In the middle-aged rats, H_2O_2 was lowered by single interventions compared to the SE-C animals.

3.1.1.3. Lipid peroxidation. A Two-way ANOVA with single pooled variance revealed an increase in MDA with age [$F_{(1,24)} = 136.6$, $P < 0.0001$], and interventions [$F_{(3,24)} = 28.50$, $P < 0.0001$] resulted in a significant reduction in MDA. Also a significant effect of age × intervention interaction [$F_{(3,24)} = 40.44$, $P < 0.0001$] was evident. A depletion ($P < 0.05$) in MDA in the adult SW-T rats in relation to the SE-C rats confirms alleviated OS. In the middle-aged animals, combined interventions reduced MDA ($P < 0.0001$). However, single interventions were also beneficial in reducing MDA levels (Fig. 2C).

3.1.1.4. Protein oxidation. A two-way ANOVA revealed an age-dependent increase [$F_{(1,24)} = 93.28$, $P < 0.0001$] in PrC levels with an intervention-dependent decline [$F_{(3,24)} = 7.592$, $P < 0.001$]. An age × intervention interaction [$F_{(3,24)} = 14.65$, $P < 0.0001$] indicated the modulatory potential of interventions and age on PrC level. Combined intervention was more effective ($P < 0.0001$) than swimming training ($P < 0.001$) in the adult SE-C rats. In the middle-aged rats, a combined intervention, as opposed to single interventions was more effective in alleviating the PrC ($P < 0.05$) (Fig. 2D).

Fig. 2E shows a positive correlation between the free radicals and extent of lipid and protein damage with $O_2^{\bullet-}$and MDA ($r = +0.85$, $+0.85$), and PrC ($r = +0.72$, $+0.63$). A similar trend was seen between H_2O_2 and MDA ($r = +0.64$, $+0.70$), and PrC ($r = +0.87$, $+0.86$) with age.

3.1.1.5. Fluorescence microscopy of age pigments in the ACC and Prl. A two-way ANOVA confirmed that age-related LF accumulation was significantly depressed by[$F_{(1,24)} = 96.32$, $P < 0.0001$] by the interventions [$F_{(3,24)} = 141.9$, $P < 0.0001$] with a significant modulatory role of the age × intervention interaction [$F_{(3,24)} = 92.35$, $P < 0.0001$]. Fig. 3A represents LF autofluorescence in the ACC (layer II). A combined intervention in the adult rats resulted in the maximum reduction ($P < 0.0001$) of LF. Interventions either administered singly or combined revealed an equal effect in reducing LF autofluorescence in the middle-aged groups over the SE-C ($P < 0.0001$).

LF in the Prl significantly increased with age [$F_{(1,24)} = 198.7$, $P < 0.0001$] and was alleviated by the interventions [$F_{(3,24)} = 123.3$, $P < 0.0001$]. Also a significant effect of age × intervention interaction [$F_{(3,24)} = 27.49$, $P < 0.0001$] was noticed through a two-way ANOVA. When SE-C of both the age groups were compared, an increase in LF ($P < 0.0001$) was seen in the middle-aged Prl. Swimming training either with GSPE or without GSPE supplementation reduced the extent of LF in the adult Prl ($P < 0.0001$) over the SE-C. GSPE supplementation in the adult sedentary also lead to reduction in LF ($P < 0.001$) over the SE-C. Interventions significantly reduced ($P < 0.0001$) LF accumulation in the middle-aged groups. Further, combined intervention revealed reduction ($P < 0.05$) in LF over the SE-C (PA), irrespective of the age groups (Fig. 3B).

Pearson's correlation test revealed a positive correlation between MDA and LF ($r = +0.75$, $+0.80$); ($r = +0.88$, $+0.80$), between PrC and LF ($r = +0.86$, $+0.85$); ($r = +0.73$, $+0.88$), and between MDA and PrC ($r = +0.70$); ($r = +0.60$) in the ACC and Prl of adults and middle-aged groups respectively (Fig. 3C).

3.1.2. Antioxidant defense

3.1.2.1. Superoxide dismutase activity. A two-way ANOVA confirmed restoration of age [$F_{(1,24)} = 45.97$, $P < 0.0001$] associated with a decline in SOD activity through the interventions [$F_{(3,24)} = 127.2$, $P < 0.0001$]. Further, asignificant effect of the age × intervention interaction [$F_{(3,24)} = 17.25$, $P < 0.0001$] was also seen for enzyme activity. SOD activity was reduced in the middle-aged SE-C group ($P < 0.0001$) with respect to the adult SE-C group. A synergistic effect of GSPE and swimming training became evident, as a significant increase in SOD activity was evident in adult ($P < 0.001$) and middle-aged ($P < 0.0001$) rats as well. Similar increases but of lesser magnitudes in SOD activity in the SW-T group was seen in middle-aged animals. Interestingly, middle-aged SE-C (PA) group exhibited an increase in SOD activity ($P < 0.01$), unlike the adults, in comparison to their controls (Fig. 4A).

3.1.2.2. Catalase activity. A two-way ANOVA revealed age-related lowered CAT activity [$F_{(1,24)} = 27.2$, $P < 0.0001$] in the mPFC while interventions [$F_{(3,24)} = 58.87$, $P < 0.0001$] resulted in higher CAT activity with the age × intervention interaction [$F_{(3,24)} = 5.168$, $P < 0.01$]. Enhanced CAT activity was observed in combined intervened groups irrespective of age. Interestingly, unlike the adults, middle-aged animals exhibited an increase in CAT activity following GSPE supplementation ($P < 0.01$) and swimming exercise ($P < 0.0001$) when compared with their respective controls (Fig. 4B).

3.1.2.3. Glutathione levels. A two-way ANOVA analysis revealed significant effects of age [$F_{(1,24)} = 766.8$, $P < 0.0001$], intervention [$F_{(3,24)} = 74.00$, $P < 0.0001$] and age × intervention interaction [$F_{(3,24)} = 102.8$, $P < 0.0001$] on modulating GSH levels in the mPFC. GSH was significantly ($P < 0.05$) higher in middle-aged rats when compared to adult rats. Intervention-mediated increases in GSH were found irrespective of age, although the increase was more pronounced in the middle-aged animals. In the adults, combined intervention ($P < 0.0001$) or swimming training by itself ($P < 0.001$) resulted in higher GSH levels. Unlike the adults, interventions in the middle-

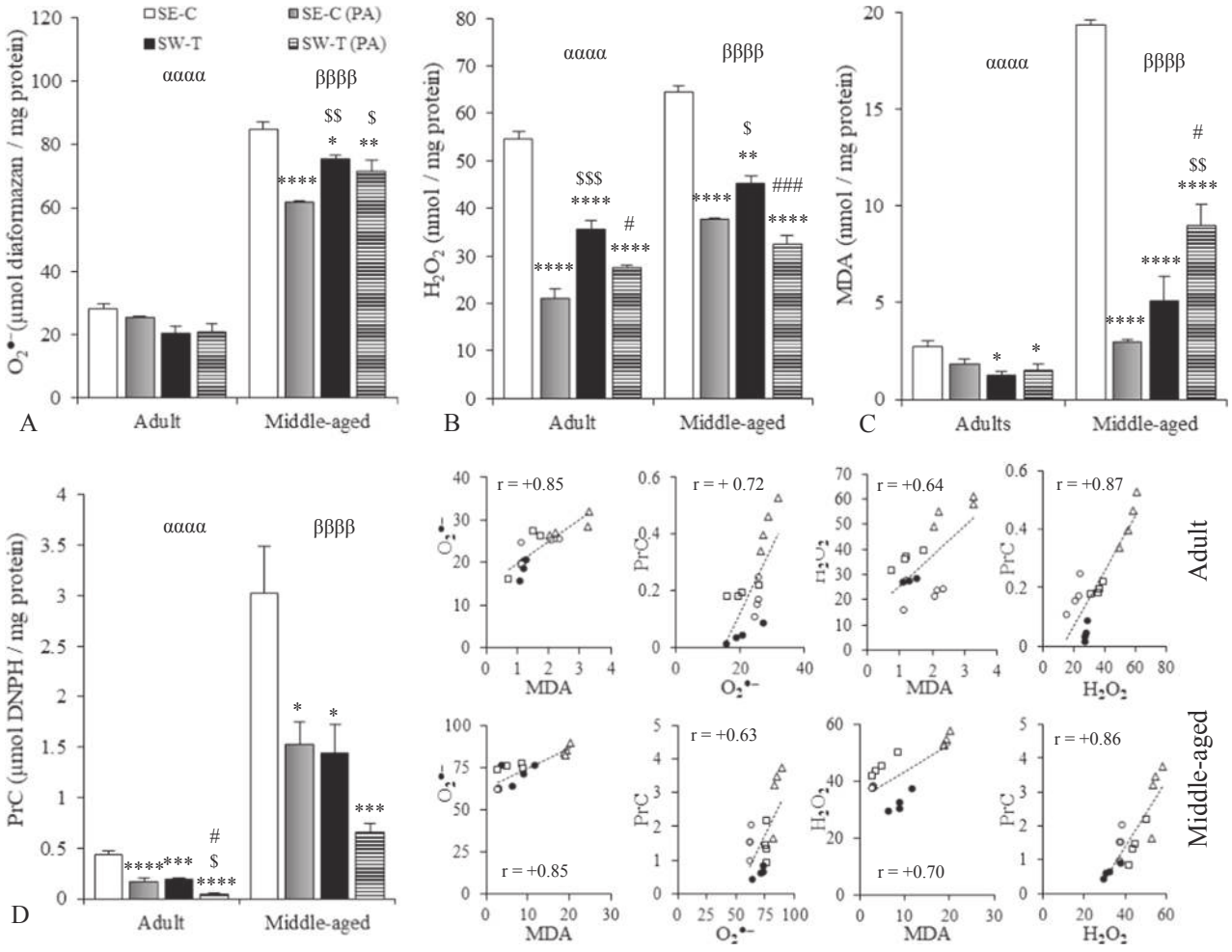

Fig. 2. Oxidative stress markers in medial pre-frontal cortex superoxide (A), hydrogen peroxide (B), malondialdehyde (C), protein carbonyl levels (D), Pearson's correlation between OS markers (E) in adult and middle-aged rats. The shape triangle in scattered plot represents SE-C; hollow circle, SE-C (PA); square, SW-T and solid circle, SW-T (PA). Values are mean ± SEM (n = 4). Two-way ANOVA with Tukey's HSD test revealed significance in mean difference as **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$, * $P < 0.05$ v/s SE-C, $^{\$\$\$}$ $P < 0.001$, $^{\$\$}$ $P < 0.01$; $^{\$}$ $P < 0.05$ v/s SE-C (PA), $^{\#\#\#}$ $P < 0.001$; $^{\#}$ $P < 0.05$ v/s SW-T, $^{\alpha\alpha\alpha\alpha}$ $^{\beta\beta\beta\beta}$ $P < 0.0001$ between the age groups. SE-C, sedentary control; SE-C (PA) and SW-T (PA), sedentary and swimming trained rats supplemented with GSPE, SW-T, swimming trained.

aged resulted in significantly high levels of GSH ($P < 0.0001$) with the SW-T(PA) group exhibiting the highest level followed by SE-C (PA) and SW-T (Fig. 4C).

3.1.2.4. Glutathione peroxidase activity. The influence of age [$F_{(1,24)} = 121.7$, $P < 0.0001$], interventions [$F_{(3,24)} = 51.06$, $P < 0.0001$] and the age × intervention interaction [$F_{(3,24)} = 25.70$, $P < 0.0001$] on GPx activity was seen in a two-way ANOVA. The middle-aged SE-C(PA) group displayed the highest GPx activity ($P < 0.0001$) compared to other groups. In the adults, combined intervention exhibited highest GPx activity ($P < 0.0001$) compared to the SE-C group, followed by SW-T ($P < 0.001$) and SE-C (PA) ($P < 0.05$). Also, the adult SW-T(PA) group showed a similar trend as middle-aged rats, indicating that swimming training can potentially elevate GPx activity, unlike GSPE alone (Fig. 4D).

3.1.2.5. Glutathione reductase activity. A two-way ANOVA analysis revealed that age [$F_{(1,24)} = 46.79$, $P < 0.0001$], intervention [$F_{(3,24)} = 254.7$, $P < 0.0001$] and the age × intervention interaction [$F_{(3,24)} = 30.01$, $P < 0.0001$] significantly modulated GR activity. The combined intervention resulted in increased enzyme activity ($P < 0.0001$) irrespective of age (Fig. 4E).

3.1.2.6. Glutathione S-transferase activity. A two-way ANOVA revealed that age [$F_{(1,24)} = 87.76$, $P < 0.0001$] and, interventions [$F_{(3,24)} = 4.539$, $P < 0.05$] had a significant effect on GST activity. Middle-aged SW-T(PA) rats exhibited increased GST activity (Fig. 4F).

3.2. Bioavailability of GSPE monomeric constituents in the mPFC

GA, (+)-CT and (−)-EC are the most abundant monomeric constituents of GSPE. These subunits of GSPE were confirmed based on co-chromatography with authentic standards and from collected MS line spectra having fragments mass/charge ratio of 125.1/79.8 for GA (Fig. S1.A), 203.5/125.4 for (+)-CT (Fig. S1.B), and 203.7/125.4 for (−)-EC (Fig. S1.C). Fig. 5 shows chromatograms of GSPE constituents from Gravinol, Gallic acid (Fig. 5A) and catechin and epicatechin (Fig. 5B). The concentration of these constituents is indicated in Fig. 5C.

3.2.1. Gallic acid

A two-way ANOVA revealed a significant influence of age, swimming training and their interaction effect [$F_{(1,8)} = 30670$, $P < 0.0001$], swimming training [$F_{(1,8)} = 30113$, $P < 0.0001$], age × swimming training [$F_{(1,8)} = 21967$, $P < 0.0001$] on the GA bioavailability in the mPFC. Chromatographs of GA(6A to D), (+)-CT and (−)-EC (Fig. 6E to H) in the supplemented SE-C and SW-T groups

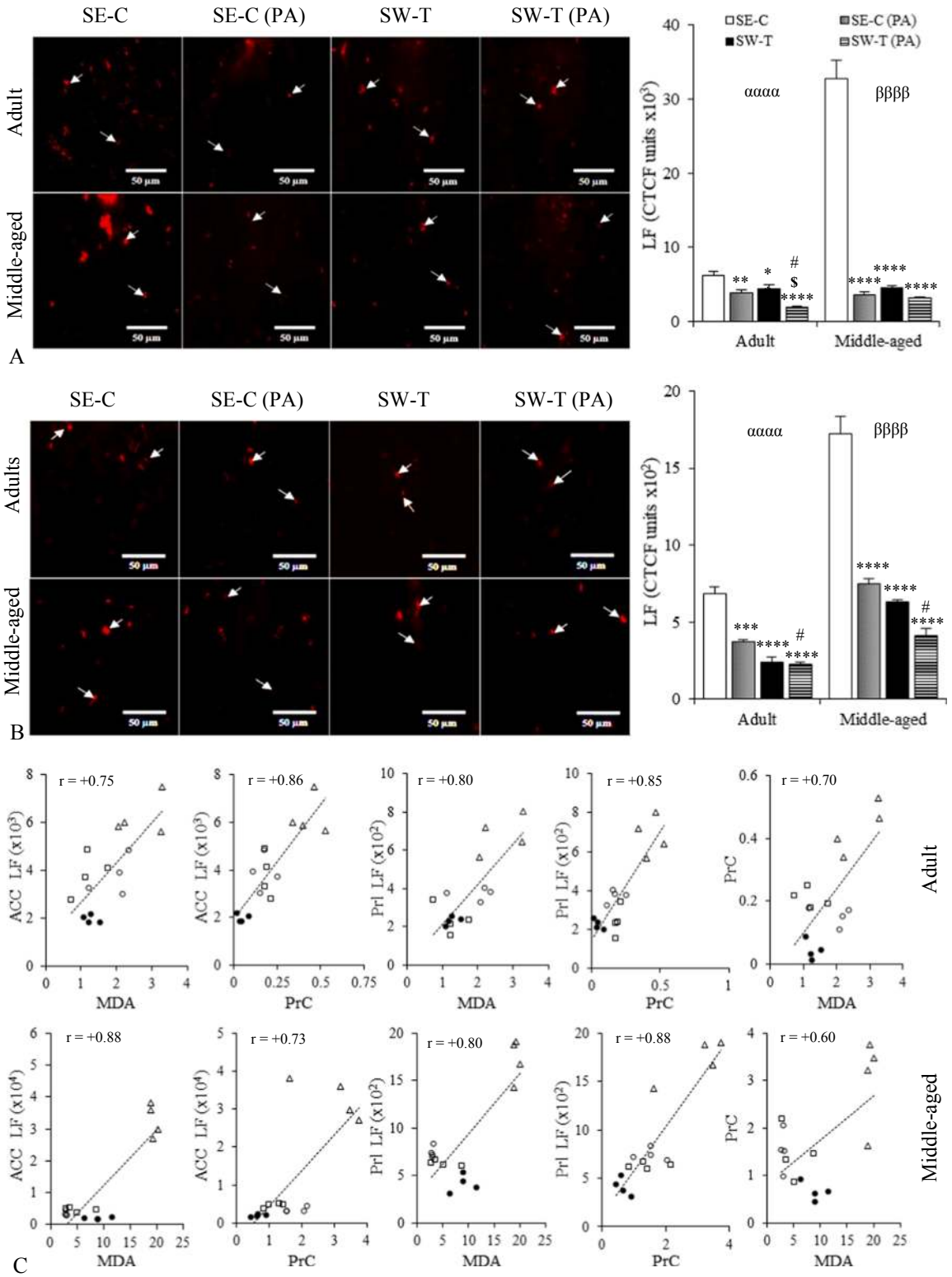

Fig. 3. Microphotographs of lipofuscin autofluorescence (ex 560 nm) in anterior cingulate cortex (A), pre-limbic cortex (B), of dorso-medial prefrontal cortex, scale 50 µm. Pearson's correlation between region LF accumulation and MDA, PrC (E) in adult and middle-aged rats. Values are mean ± SEM (n = 4). Two-way ANOVA with Tukey's HSD test revealed significance in mean difference as **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$, * $P < 0.05$ v/s SE-C, $^{\$}$ $P < 0.05$ v/s SE-C (PA), $^{\#}$ $P < 0.05$ v/s SW-T, $^{\alpha\alpha\alpha\alpha}$ $^{\beta\beta\beta\beta}$ $P < 0.0001$ between the age groups. All the abbreviations and shapes in scattered plot are similar to that described in Fig. 2.

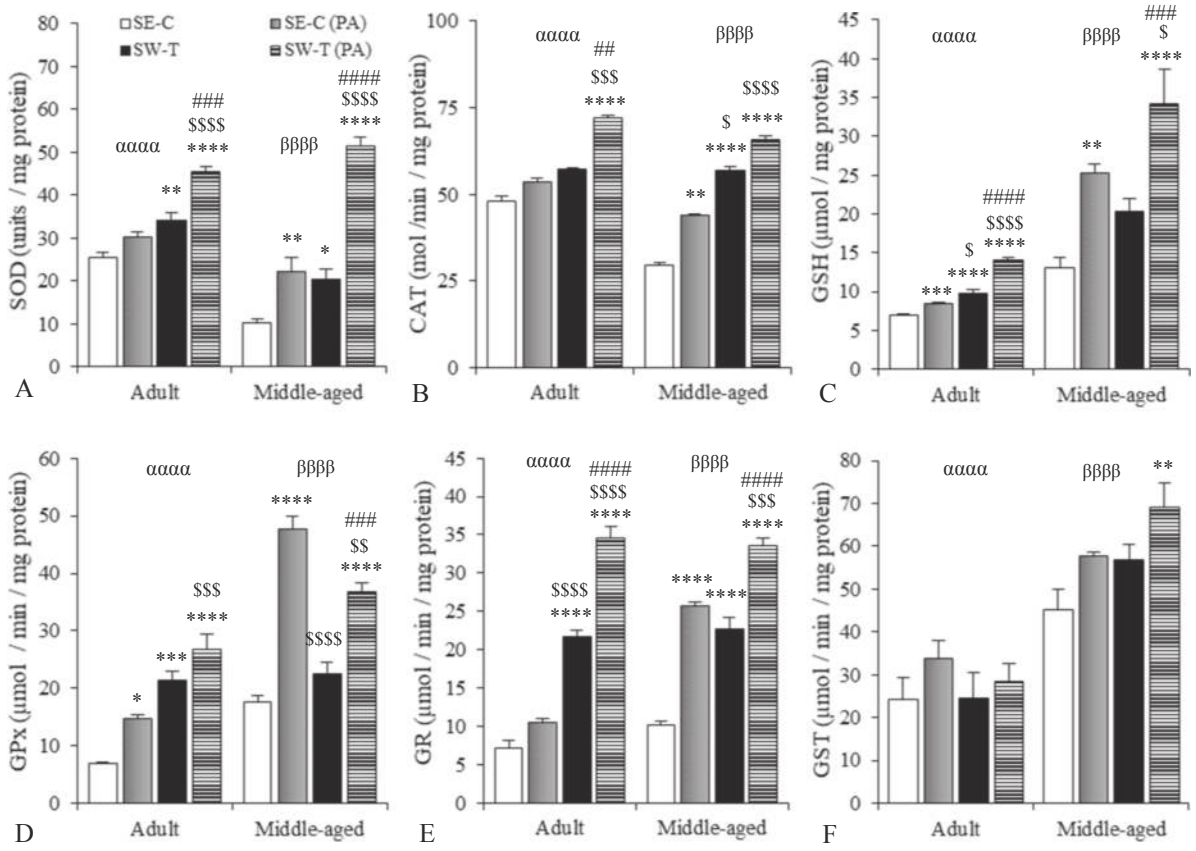

Fig. 4. Antioxidants in medial prefrontal cortex: activities of superoxide dismutase (A), catalase (B), glutathione concentration (C), activities of glutathione peroxidase (D), glutathione reductase (E), glutathione-s-transferase (F) in adult and middle-aged rats. Values are mean ± SEM (n = 4). Two-way ANOVA with Tukey's HSD test revealed significance in mean difference as**** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05 v/s SE-C, $^{\$\$\$\$}$ P < 0.0001; $^{\$\$\$}$ P < 0.001; $^{\$\$}$ P < 0.01; $^{\$}$ P < 0.05, v/s SE-C (PA), $^{\#\#\#\#}$ P < 0.0001; $^{\#\#\#}$ P < 0.001, $^{\#\#}$ P < 0.01 v/s SW-T, $^{\alpha\alpha\alpha\alpha}$ $^{\beta\beta\beta\beta}$ P < 0.0001 between the age groups. All the abbreviations are similar to that described in Fig. 2.

are shown for mPFC in the adult and middle-aged rats. Swimming training resulted in higher bioavailability of GA in both groups (P < 0.0001), although the extent of the increase was lesser in the middle-aged rats (Fig. 6I).

3.2.2. Catechin

Age and swimming training impacted (+)-CT bioavailability in mPFCs and a two-way ANOVA affirmed the impact of age [$F_{(1,8)}$ = 12400, P < 0.0001], swimming training [$F_{(1,8)}$ = 10195, P < 0.0001] and the age × swimming training interaction [$F_{(1,8)}$ = 8828, P < 0.0001]. Swimming training resulted in higher bioavailability of (+)-CT (P < 0.0001) in both age groups compared with their SE-C (PA) (Fig. 6J).

3.2.3. Epicatechin

The effect of age [$F_{(1,8)}$ = 497.9, P < 0.0001], swimming training [$F_{(1,8)}$ = 949.8, P < 0.0001], the age × swimming training interaction [$F_{(1,8)}$ = 271.2, P < 0.0001] with regard to the bioavailability of (−)-EC in the mPFC was revealed by a two-way ANOVA. The SW-T(PA) groups displayed higher (−)-EC bioavailability (P < 0.0001) than of SE-C(PA), SE-C (PA) and SW-T (PA) rats (Fig. 6K).

3.3. Stereological analysis

3.3.1. Anterior cingulate cortex

In Fig. 7A, the two panels represent microphotographs of cresyl violet stained sections of the ACC. A significant decline (P < 0.0001)

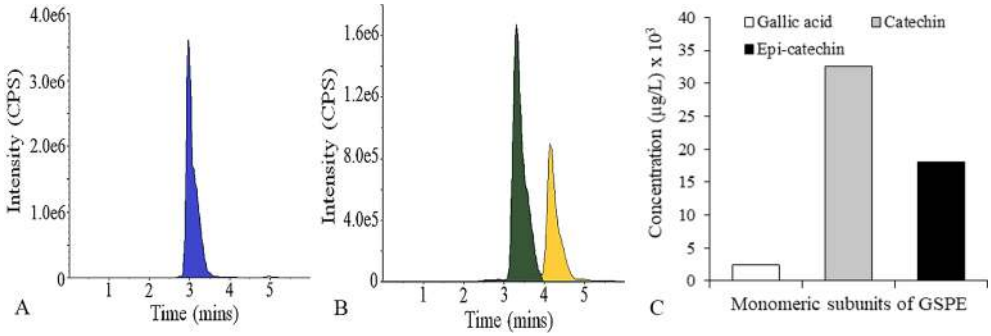

Fig. 5. Chromatograms of GSPE (Gravinol) constituents. GA (A), (+)-CT and (−)-EC (B) and concentration of the constituents in gravinol (µg/L) (C). CPS, counts per second; GSPE grape seed proanthocyanidin extract, GA, gallic acid; (+)-CT, catechin; (−)-EC, epicatechin.

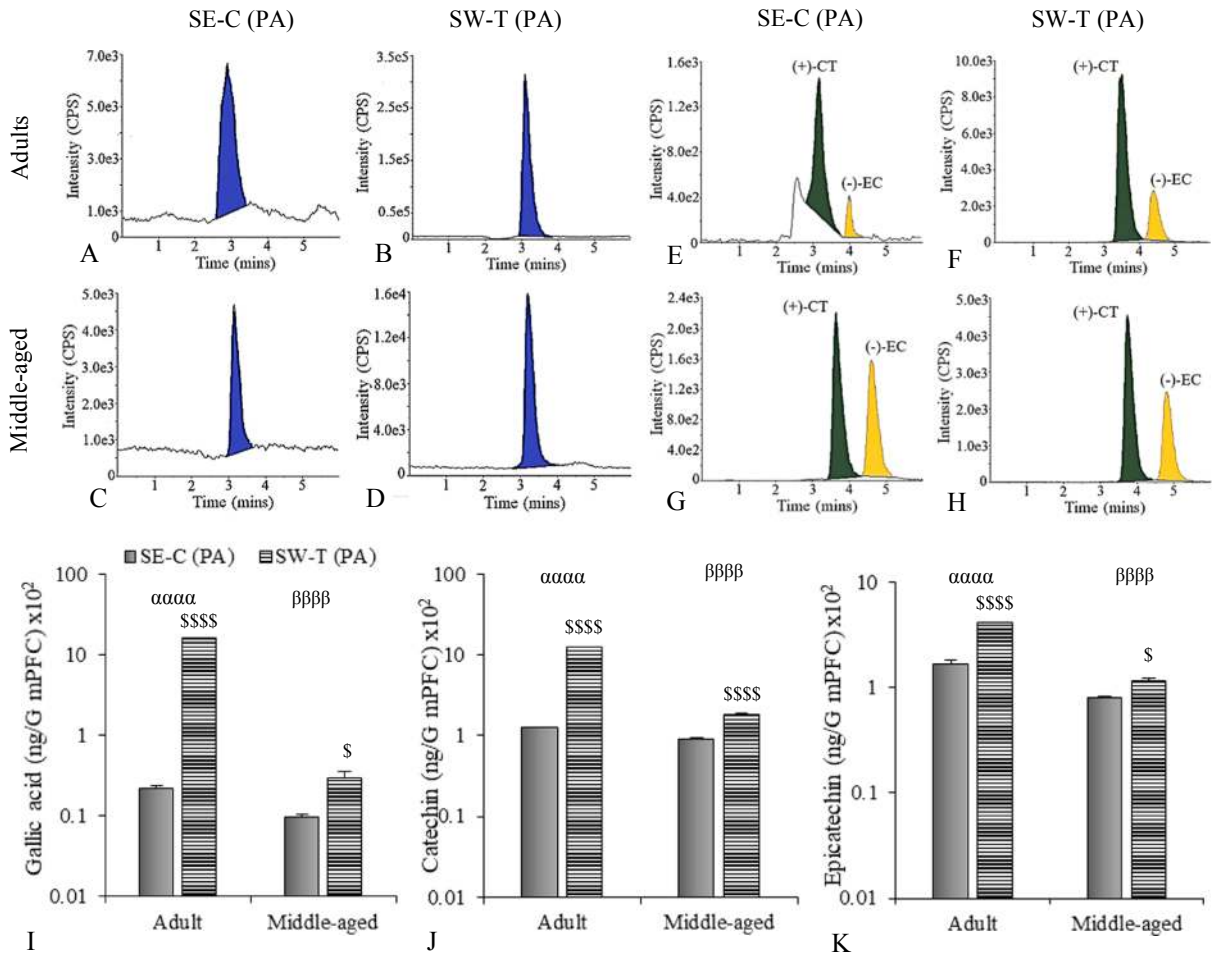

Fig. 6. Representative chromatograms of GA (A to D), (+)-CT and (−)-EC (E to H) in the medial prefrontal cortex of SE-C(PA) and SW-T(PA) adult and middle-aged rats and bioavailable concentration of GA(I), (+)-CT(J) and (−)-EC (K) are plotted as bar graphs. Values are mean ± SEM (n = 5). Two-way ANOVA followed by Tukey's HSD test revealed sifnificance in mean difference in bioavailable concentration as $^{\$\$\$\$}$ P < 0.0001; $^{\$}$ P < 0.05 v/s SE-C (PA), $^{\alpha\alpha\alpha\alpha\ \beta\beta\beta\beta}$ P < 0.0001 between age-groups. CPS, counts per second; GA, gallic acid; (+)-CT, catechin; (−)-EC, epicatechin. All other abbreviations are similar to that described in Fig. 2.

in neuronal number was revealed in the SE-C of adult and middle-aged rats. A two-way ANOVA revealed age [$F_{(1,32)}$ = 192.1, P < 0.0001] and interventions [$F_{(3,32)}$ = 54.68, P < 0.0001] significantly altering the neuronal number in the ACC. Among the adults, swimming training was effective in increasing the neuronal numbers (P < 0.0001) over the SE-C while in the middle-aged interventions either singly or combined were effective in increasing the neuronal number (P < 0.0001) over the SE-C groups (Fig. 7C).

An age-related decline (P < 0.0001) was evident in the ACC volume. A two-way ANOVA showed age as a significant modulatory factor for volume [$F_{(1,32)}$ = 348.1, P < 0.0001] with significant age × interventions interaction [$F_{(3,32)}$ = 4.413, P < 0.05]. Only adult SW-T (PA) exhibited a higher volume (P < 0.05) over the SE-C (Fig. 7D).

Interventions resulted in higher neuronal density with an insignificant (P = 0.9929) decrease in the middle-aged SE-C over the adult SE-C. A two-way ANOVA revealed age [$F_{(1,32)}$ = 29.95, P < 0.0001], interventions [$F_{(3,32)}$ = 25.69, P < 0.0001] and age × interventions interaction can significantly modulate neuronal density in the ACC. A synergistic effect of GSPE supplementation and swimming training was seen with the SW-T (PA) group showing highest neuronal density (P < 0.0001) over the SE-C in the adult and middle-aged as well. Single interventions were effective in increasing the higher neuronal density (P < 0.0001) over the middle-aged SE-C group. However, no significant change was seen in the adult over their SE-C group (Fig. 7E).

3.3.2. Prelimbic cortex

A significant decrease in the neuronal number was seen in the middle-aged SE-C group (P < 0.0001). A two-way ANOVA revealed age-related [$F_{(1,32)}$ = 127.1, P < 0.0001] and interventions-related [$F_{(3,32)}$ = 25.38, P < 0.0001] changes in the neuronal number. In Fig. 8A, the two panels represent microphotographs of cresyl violet stained sections of the Prl. In the adult, combined or single interventions were effective in increasing the neuronal number over the SE-C group. Further, in the middle-aged interventions increased the neuronal number although the response to interventions differed from the adult groups with SW-T revealing highest increase in the SW-T(PA) (P < 0.0001) followed by SW-T (P < 0.001) and SE-C (PA) (P < 0.05) over the SE-C group (Fig. 8C).

Fig. 8D shows an age-related decline (P < 0.0001) in the Prl volume. Age [$F_{(1,32)}$ = 187.4, P < 0.0001] interventions [$F_{(3,32)}$ = 18.51, P < 0.0001] and age × interventions interaction [$F_{(3,32)}$ = 5.364, P = 0.0042]. Single interventions (P < 0.001) resulted in increase in the volume with the combined intervention being more effective (P < 0.0001) over the SE-C in the adults. In the middle-aged, SW-T group alone showed higher neuronal density over their SE-C group.

Interestingly, our results confirm significant decline in Prl neuronal density (*P < 0.01*) as opposed to that of ACC although a two-way ANOVA revealed no significance between the age groups

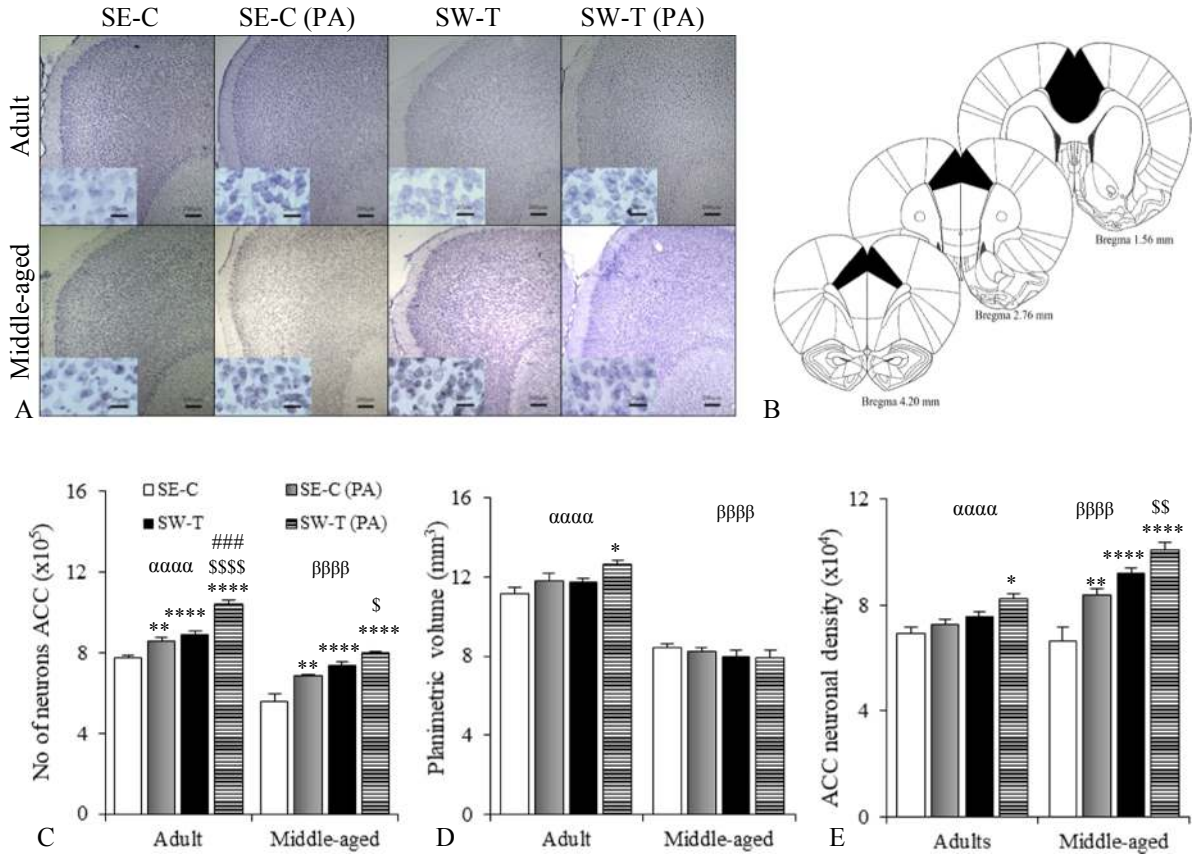

Fig. 7. Microphotograph represents ACC at 4x and ACC layer II at 100x with scale bar 200 μm and 20 μm respectively (A) between bregma 4.20 to 1.56 mm (B). Unbiased stereological estimates of ACC neuronal number (C), planimetric volume (D), neuronal density (E). Values are mean ± SEM (n = 5). Two-way ANOVA with Tukey's HSD test revealed significance in mean difference as **** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05 v/s SE-C, $^{\$\$\$\$}$ P < 0.0001; $^{\$\$}$ P < 0.01, $^{\$}$ P < 0.05 v/s SE-C (PA), $^{\#\#\#}$ P < 0.001 v/s SW-T, $^{\alpha\alpha\alpha\alpha\ \beta\beta\beta\beta}$ P < 0.0001 between the age groups. All other abbreviations are similar to that described in Fig. 2.

[$F_{(1,32)}$ = 0.006305, P = 0.9372]. Moreover interventions [$F_{(3,32)}$ = 5.559, P = 0.0035] and the age × interventions interaction [$F_{(3,32)}$ = 10.26, P < 0.0001] demonstrated significant modulatory effect on Prl neuronal density. In the adult, no difference was seen in the neuronal density unlike in the middle-aged wherein the combined intervention resulted in significantly increased neuronal density (P < 0.0001) over their SE-C group (Fig. 8E).

Table 1 represents degree of relatedness of neuronal density of the ACC and Prl with the antioxidant enzymes. The ACC density showed a positive correlation between density and antioxidant enzymes in the adult and middle-aged rats suggesting an efficient antioxidant defence in promoting neuronal density. A similar trend was seen in the Prl of middle-aged rats unlike the Prl in the adult wherein no positive correlation was seen between the enzymes in spite of increases in the neuronal number and volume.

4. Discussion

There is persistent search for natural products as alternatives of synthetic antioxidants to overcome OS-related lipid and protein oxidations (Lafka, Sinanoglou, & Lazos, 2007) and grape seeds with significant polyphenolic ingredients are much sought after antioxidant for the synthesis of nutraceutical functional foods (Davidov-Pardo, Moreno, Arozarena, Bleibaum, & Bruhn, 2012). In this study, the major GSPE phenolic compounds, catechin and epicatechin, including GA were measured by LC-MS-MS in the plasma and mPFC of adult and middle-aged rats.

Age-related cognitive decline in the spatial WM performance in normal aging despite the absence of MCI or dementia (Fabiani, 2012), parallels an increased redox imbalance and shrinkage in brain size. Parallel to the role of the HC in spatial memory, the mPFC has gained much attention for its organization and role in decision making. The current study emphasizes the neuroprotective role of GSPE and swimming training on the neuronal density in the ACC of middle-aged rats. An earlier study has demonstrated positive correlation between neuronal density in the hippocampus and remote working memory in rats subjected to a T-maze for aspontaneous attention task with 1 min spatial alteration delay (Abhijit et al., 2018) indicating neuronal density as a critical factor associated with cognitive decline in middle-age during normal aging. The observed loss of ACC volume and neuronal number in the middle-aged rats can be attributed to the unprecedented increase in FRs and associated lipid/protein damage with a five-fold increase in LF in the mPFC, which is supportive of the LF hypothesis on the accumulating risk of developing Alzheimer's disease during the later years of life (Giaccone, Orsi, Cupidi, & Tagliavini, 2011). An increase in neuronal density was noticeable in the intervened adult and middle-aged animals as well, although the latter age failed to exhibit a significant decline in neuronal density despitea significant loss of volume and increased neuronal number suggesting an age-related attrition in dendritic arborisation.

The present study showed higher levels of $O_2^{\bullet -}$ and H_2O_2 in the mPFC of middle-aged rats indicating an imbalance in redox state as a function of mitochondrial stress. The obtained results on the age-dependent increase in H_2O_2 concentration in the mPFC are akin to the reports of Kilbride, Telford, and Davey (2008) on 6- and 18-month-old rat brain synaptosomal fractions and are similar to earlier report from our laboratory on the HC in normal aging in rats (Abhijit et al., 2018). Our results on increased LPO and protein carbonylation in the mPFC are

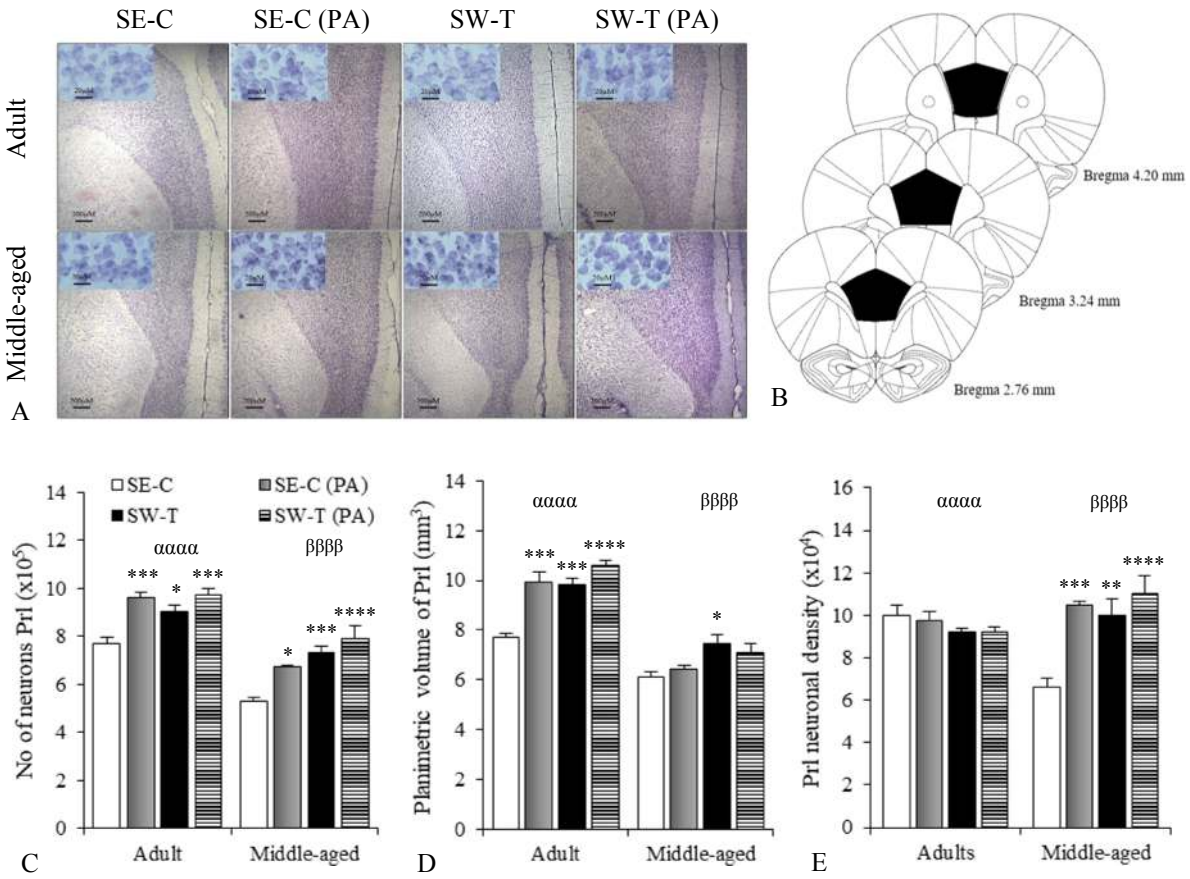

Fig. 8. Microphotograph represents Prl at 4x and Prl layer II at 100x with scale bar 200 μm and 20 μm respectively (A) between bregma 4.20 to 1.56 mm (B). Unbiased stereological estimates of ACC neuronal number (C), planimetric volume (D), neuronal density (E).Values are mean ± SEM (n = 5). Two-way ANOVA with Tukey's HSD test revealed significance in mean difference as **** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05 v/s SE-C, $^{\alpha\alpha\alpha\alpha}$ $^{\beta\beta\beta\beta}$ P < 0.0001 between the age groups. All other abbreviations are similar to that described in Fig. 2.

due to three- and one-fold increases in $O_2^{\bullet-}$ and H_2O_2 generation. Further, the reduction in SOD activity in the middle-aged mPFCs along with the increased mitochondrial $O_2^{\bullet-}$ supports the findings of Tsay et al.(2000) suggesting elevated OS. Interestingly, the attenuated levels of MDA and PrC levels in the GSPE-supplemented rats and swimming trained rats are concomitant with adaptive changes in the antioxidant enzyme activities as ascertained by the bioavailability of catechin, epicatechin and gallic acid. Further, a by-product of LPO and oxidized proteins is LF, an important a biomarker of aging in the post-mitotic cells, the accumulation of which is a result of the crossing of modified proteins and oxidized lipids (Kin, Palo, Haltia, & Wolfe, 1983). The prefrontal cortex is critically involved in higher cognitive functions including working memory. The age-associated cognitive deficits might be due to selective neuronal vulnerability among subregions of the prefrontal cortex. The estimation of an age-pigment like LF in the ACC and Prl may indicate the vulnerability of the subregions of prefrontal cortex to age-associated increase in oxidative stress. Our results on a significant age-dependent increase in the LF accumulation in the ACC than in the Prl cortex is suggestive of differential vulnerability to OS in the dmPFC. Further, the increased LF in the ACC corresponds to our earlier findings on decline in the reward based WM task in the T-maze (Abhijit et al., 2017). Interestingly, studies by Walton et al.(2003) have demonstrated that rats with an excitotoxic lesion of the ACC choose low-reward as opposed to climbing 30 cm barrier in a T-maze task for a high-reward prior to the lesion. Other studies using multiple single-unit recordings in ACC lesioned rats have shown the role of ACC in selection and monitoring of action output in a radial arm maze task (Lapish, Durstewitz, Chandler, & Seamans, 2008).

The observed alleviation in LF accumulation in the dmPFC correlates with theinhibition of the oxidation of lipids and proteins following interventions. Earlier studies on swimming training (Asha Devi, Sagar Chandrasekar, Manjula, & Ishii, 2011) and GSPE supplementation (Abhijit et al., 2017) in improving the antioxidant defense in the aging brain supports our present findings. However, the findings on a lower extent of LF accumulation in the ACC and Prl among the swimming trainees and GSPE supplementees may be attributed to the Fe (III) chelating ability of (+)-CT (Cherrak et al., 2016). The present findings on neuronal loss in the ACC and Prl may be explained on the lipofuscin's ability to incorporate iron and thereby elevating the neuronal oxidative challenges with activation of caspase-3 and induction of PARP cleavage that initiate apoptosis (Höhn, Jung, Grimm, & Grune, 2010).

The present finding on reductions in SOD and CAT activities in the mPFC and the positive correlation with increased levels of $O_2^{\bullet-}$ and H_2O_2 with age are suggestive of an increased production of $OH^{\bullet}$ by means of the Fenton's reaction leading to an impaired GSH redox state (Crivello et al., 2007). Intracellular GSH concentration depends on GSH recycling as well as GSH biosynthesis. The results of the present study of unchanged GR activity with high GPx activity in the middle-aged controls indicate imbalanced GSH recycling. Further, age-related increases in the GST enzyme in the mPFC is consistent with the findings of Kim et al. (2003)

However, increases in the GR activity in response to the interventions in the middle-aged rats is suggestive of restored GSH cycling andthe ameliorative effect of swimming training and GSPE supplementation either singly or combined. The observed high levels of GSH in the middle-aged controls are suggestive of the *de novo* synthesis of GSH and in turn, the curtailing of the oxidative damage. Our results

Table 1
Correlation matrix between the glutathione and antioxidant enzymes with neuronal density of anterior cingulate and prelimbic cortex in adult and middle-aged rats.

Anterior cingulate cortex								
		Density	GSH	GPx	GR	GST	CAT	SOD
Adult	Density	–	***	**	**	ns	*	**
	GSH	+0.76	–	****	****	ns	**	****
	GPx	+0.72	+0.91	–	****	ns	**	****
	GR	+0.68	+0.92	+0.84	–	ns	**	****
	GST	−0.20	+0.03	+0.11	−0.01	–	ns	ns
	CAT	+0.58	+0.70	+0.72	+0.70	+0.36	–	***
	SOD	+0.72	+0.93	+0.87	+0.94	+0.15	+0.76	–
Middle-aged	Density	–	**	ns	****	**	****	**
	GSH	+0.66	–	*	****	**	**	****
	GPx	+0.44	+0.65	–	*	ns	ns	*
	GR	+0.77	+0.82	+0.70	–	****	****	****
	GST	+0.69	+0.66	+0.45	+0.74	–	****	***
	CAT	+0.85	+0.73	+0.35	+0.84	+0.76	–	****
	SOD	+0.72	+0.84	+0.56	+0.86	+0.73	+0.84	–
Prelimbic cortex								
		Density	GSH	GPx	GR	GST	CAT	SOD
Adult	Density	–	ns	ns	ns	ns	ns	ns
	GSH	−0.13	–	****	****	ns	**	****
	GPx	−0.09	+0.91	–	****	ns	**	****
	GR	−0.20	+0.92	+0.84	–	ns	**	****
	GST	+0.10	+0.03	+0.11	−0.01	–	ns	ns
	CAT	−0.26	+0.70	+0.72	+0.70	+0.36	–	***
	SOD	−0.29	+0.93	+0.87	+0.94	+0.15	+0.76	–
Middle-aged	Density	–	***	*	*	ns	ns	ns
	GSH	+0.80	–	*	****	**	***	****
	GPx	+0.57	+0.65	–	**	ns	ns	*
	GR	+0.48	+0.82	+0.70	–	***	****	****
	GST	+0.25	+0.66	+0.45	+0.74	–	***	****
	CAT	+0.43	+0.73	+0.35	+0.84	+0.76	–	****
	SOD	+0.49	+0.84	+0.56	+0.86	+0.73	+0.84	–

Values are Pearson's correlation coefficient (r) derived from two-tailed test. **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns, non-significant. GSH, glutathione; GPx, glutathione peroxidase; GST, glutathione-S-transferase; GRr, glutathione reductase; CAT, catalase; SOD, superoxide dismutase

emphasize that swimming training improves the primary defense against H_2O_2-mediated OS by elevating the CAT activity in view of the evidence on the increased expression of CAT proteins in exercised AD-Tg mice (Um et al., 2008). The observed increase in antioxidant enzyme activities with efficient GSH recycling may be explained on the basis of studies by Camiletti-Moirón et al. (2015), who have indicated that high intensity exercise protects the brain from induced OS by elevating the CAT activity in the brain via an up-regulation of Nrf-2 and of Harvey et al. (2009) reports on Nrf-2-dependent GSH recycling for mitigating oxidative damage. Thus, the increased CAT activity as well as the increase in GPx and GR activity among the intervened groups in adult and middle-aged rats indicates the possible upregulation in the Nrf-2 pathway in response to exercise. Interestingly, GSPE-supplemented rats exhibited increased CAT and GR activity on par with the swimming trained rats with further enhancement in the combined intervened groups suggesting a synergism between the two interventions.

Our results on detectable levels of GSPE monomeric constituents in the dmPFC indicate exercise mediated changes in the transportation of the constituents across the blood brain barrier. Exercise and grape polyphenols have been shown to improve the BDNF levels, which can facilitate synaptogenesis and, survival of neurons and improve cognition (Dani et al., 2017; Weinstein et al., 2012). The exact mechanism of transport needs to be elucidated further. Overall, the changes in GSH redox state or antioxidant defense in the middle-aged rats and their correlation with GSPE monomeric constituents in the mPFC of supplemented and swimming-trained rats demonstrate the protective role of GSPE constituents against age-associated OS. In case of human studies, dependency on GSPE-related increase in neuronal density and alleviation of oxidative stress in the mPFC may be attempted with varying dosages of GSPE combined with exercise training.

In summary, interventions singly were beneficial in protecting the dmPFC through alleviating mitochondrial FRs, lipid and protein oxidations and ameliorating the cytosolic antioxidant defences. However, a more pronounced effect was evident in animals that underwent combined interventions, thereby implying a possible synergism between swimming training and GSPE supplementation, more so, in the middle-aged rats that are vulnerable to OS-induced mitochondrial functions. Further, our data on gallic acid, (+)-catechin and (−)-epicatechin, through LC-MS/MS, demonstrates their depositions suggesting their passage through the blood brain barrier. Furthermore, the laboratory results from this study provide an important insight into beneficial effects of GSPE at 400 mg/kg b.w/day along with swimming exercise. Notably, the dose used in the current study on laboratory rats is equivalent to approximately 3.89 g/day for a 60 kg human, based on body surface area (Reagan-Shaw, Nihal, & Ahman, 2007). However, to derive similar effects in the middle-aged human subjects, clinical intervention trials are warranted.

Acknowledgements

This work was supported by financial assistance granted to Dr. S. Asha Devi by the Indian Council of Medical Research (Referral No. 54/9/CFP/GER/2011/NCD-II, dt.30.04.2012), New Delhi, INDIA and under the Promotion of University Research and Scientific Excellence (PURSE)-Department of Science and Technology (DST) programme (SR/59/Z-23/2010/38) dt.27.06.2011), New Delhi, INDIA.

Declaration of Competing Interest

The authors have no conflicts of interest related to the present study.

Ethics statement

All experiments involving the animals were carried out in conformity with the Institutional Animal Ethics Committee (IAEC) after obtaining permission (IAEC/402/19/9/2014/CPCSEA) in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103693.

References

Abhijit, S., Subramanyam, M. V. V., & Asha Devi, S. (2017). Grape seed proanthocyanidin and swimming exercise protects against cognitive decline: A study on M1 acetylcholine receptors in aging male rat brain. *Neurochemical Research, 42*(12), 3573–3586. https://doi.org/10.1007/s11064-017-2406-6.

Abhijit, S., Tripathi, S. J., Bhagya, V., Shankaranarayana Rao, B. S., Subramanyam, M. V., & Asha Devi, S. (2018). Antioxidant action of grape seed polyphenols and aerobic exercise in improving neuronal number in the hippocampus is associated with decrease in lipid peroxidation and hydrogen peroxide in adult and middle-aged rats. *Experimental Gerontology, 101*(1), 101–112. https://doi.org/10.1016/j.exger.2017.11.012.

Aebi, H. (1984). Catalase in vitro. *Methods in Enzymology. 105*, 121–126.

Amiez, C., Joseph, J. P., & Procyk, E. (2006). Reward encoding in the monkey anterior cingulate cortex. *Cerebral Cortex, 16*(7), 1040–1055. https://doi.org/10.1093/cercor/bhj046.

Anand, S., Asha Devi, S., & Ravikiran, T. (2014). Differential expression of the cerebral cortex proteome in physically trained adult rats. *Brain Research Bulletin, 104*(5), 88–91. https://doi.org/10.1016/j.brainresbull.2014.04.012.

Ansari, N., Muller, S., Steizer, E. H. K., & Pampaioni, F. (2013). Quantitative 3D cell-based assay performed with cellular spheroids and fluorescence microscopy. *Methods in Cell Biology, 113*, 295–309. https://doi.org/10.1016/B978-0-12-407239-8.00013-6.

Asha Devi, S., & Ravi Kiran, T. (2004). Regional responses in antioxidant system to exercise training and dietary vitamin E in aging rat brain. *Neurobiology of Aging, 25*(4), 501–508. https://doi.org/10.1016/S0197-4580(03)00112-X.

Asha Devi, S., Sagar Chandrasekar, B. K., Manjula, K. R., & Ishii, N. (2011). Grape seed proanthocyanidin lowers brain oxidative stress in adult and middle-aged rats. *Experimental Gerontology, 46*(11), 958–964. https://doi.org/10.1016/j.exger.2011.08.006.

Baddeley, A. (2010). Working memory. *Current Biology, 20*(4), R136–R140. https://doi.org/10.1016/j.cub.2009.12.014.

Brunk, U. T., & Terman, A. (2002). Lipofuscin: Mechanisms of age-related accumulation and influence on cell function. *Free Radical Biology and Medicine, 33*(5), 611–619. https://doi.org/10.1016/S0891-5849(02)00959-0.

Camiletti-Moirón, D., Aparicio, V. A., Nebot, E., Medina, G., Martínez, R., Kapravelou, G., ... Aranda, P. (2015). High-intensity exercise modifies the effects of stanozolol on brain oxidative stress in rats. *International Journal of Sports Medicine, 36*(12), 984–991. https://doi.org/10.1055/s-0035-1548941.

Carlberg, I., & Mannervik, B. (1981). Purification and characterization of glutathione reductase from calf liver. An improved procedure for affinity chromatography on 2,5, ADP sepharose 4B. *Analytical Biochemistry, 116*(2), 531–535.

Cherrak, S. A., Mokhtari-Soulimane, N., Berroukeche, F., Bensenane, B., Cherbonnel, A., Merzouk, H., & Elhabiri, M. (2016). In vitro antioxidant versus metal ion chelating properties of flavonoids: A structure-activity investigation. *PLoS One, 11*(10), 1–21. https://doi.org/10.1371/journal.pone.0165575.

Crivello, N. A., Rosenberg, I. H., Shukitt-Hale, B., Bielinski, D., Dallal, G. E., & Joseph, J. A. (2007). Aging modifies brain region-specific vulnerability to experimental oxidative stress induced by low dose hydrogen peroxide. *Age, 29*(4), 191–203. https://doi.org/10.1007/s11357-007-9039-7.

Dani, C., Andreazza, A. C., Gonçalves, C. A., Kapizinski, F., Henriques, J. A. P., & Salvador, M. (2017). Grape juice increases the BDNF levels but not alter the S100B levels in hippocampus and frontal cortex from male Wistar rats. *Anais da Academia Brasileira de Ciências, 89*(1), 155–161. https://doi.org/10.1590/0001-3765201720160448.

Das, U. N., Padma, M., Sagar, P. S., Ramesh, G., & Koratkar, R. (1990). Stimulation of free radical generation in human leukocytes by various agents including tumor necrosis factor is a calmodulin dependent process. *Biochemical and Biophysics Research Communication, 167*(3), 1030–1036.

Davidov-Pardo, G., Moreno, M., Arozarena, I., Bleibaum, R. N., & Bruhn, C. M. (2012). Sensory and consumer perception of the addition of grape seed extracts in cookies. *Journal of Food Science, 77*(12), S430–S438. https://doi.org/10.1111/j.1750-3841.2012.02991.x.

Fabricius, K., Wörtwein, G., & Pakkenberg, B. (2008). The impact of maternal separation on adult mouse behaviour and on the total neuron number in the mouse hippocampus. *Brain Structure and Function, 212*(5), 403–416. https://doi.org/10.1007/s00429-007-0169-6.

Fabiani, M. (2012). It was the best of times, it was the worst of times: A psychophysiologist's view of cognitive aging. *Psychophysiology, 49*(3), 283–304. https://doi.org/10.1111/j.1469-8986.2011.01331.x.

Flohé, L., & Gunzler, W. A. (1984). Assays of glutathione peroxidase. *Methods in Enzymology. 105*, 114–120.

Geller, B. L., & Winge, D. R. (1982). Rat liver Cu, Zn-superoxide dismutase. *Journal of Biological Chemistry, 257*(15), 8945–8952.

Giaccone, G., Orsi, L., Cupidi, C., & Tagliavini, F. (2011). Lipofuscin hypothesis of Alzheimer's disease. *Dementia and Geriatric Cognitive Disorders Extra, 1*(1), 292–296. https://doi.org/10.1159/000329544.

Gisquet-Verrierm, P., & Delatour, B. (2006). The role of the rat prelimbic/infralimbic cortex in working memory: Not involved in the short-term maintenance but in monitoring and processing functions. *Neuroscience, 141*(2), 585–596. https://doi.org/10.1016/j.neuroscience.2006.04.009.

Granon, S., & Poucet, B. (2000). Involvement of the rat prefrontal cortex in cognitive functions : A central role for the prelimbic area. *Psychobiology, 28*(2), 229–237.

Gundersen, H. J. G., Jensen, E. B., Kieu, K., & Nielsen, J. (1999). The efficiency of systematic sampling in stereology and its prediction. *Journal of Microscopy, 193*(3), 199–211. https://doi.org/10.1111/j.1365-2818.1987.tb02837.x.

Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-Transferase- the first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry. 249*(22), 7130–7139.

Harvey, C. J., Thimmulappa, R. K., Singh, A., Blake, D. J., Ling, G., Wakabayashi, N., ... Biswal, S. (2009). Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress. *Free Radicals in Biology and Medicine, 46*(4), 443–453. https://doi.org/10.1016/j.freeradbiomed.2008.10.040.

Höhn, A., Jung, T., Grimm, S., & Grune, T. (2010). Lipofuscin-bound iron is a major intracellular source of oxidants: Role in senescent cells. *Free Radical Biology and Medicine. 48*(8), 1100–1108. https://doi.org/10.1016/j.freeradbiomed.2010.01.030.

Hyman, J. M., Zilli, E. A., Paley, A. M., & Hasselmo, M. E. (2005). Medial prefrontal cortex cells show dynamic modulation with the hippocampal theta rhythm dependent on behavior. *Hippocampus, 15*(6), 739–749. https://doi.org/10.1002/hipo.20106.

Josephy, P. D., Elingg, T., & Mason, R. P. (1982). The horseradish peroxidase-catalyzed oxidation of 3,5,3',5'- tetramethylbenzidine. *Journal of Biological Chemistry, 257*(7), 3669–3675.

Kilbride, S. M., Telford, J. E., & Davey, G. P. (2008). Age-related changes in H_2O_2 production and bioenergetics in rat brain synaptosomes. *Biochimica et Biophysica Acta –Bioenergetics, 1777*(7–8), 783–788. https://doi.org/10.1016/j.bbabio.2008.05.445.

Kim, H.-G., Hong, S.-M., Kim, S.-J., Park, H.-J., Jung, H.-I., Lee, Y.-Y., ... Lim, C. J. (2003). Age-related changes in the activity of antioxidant and redox enzymes in rats. *Molecules and Cells, 16*(3), 278–284. http://www.ncbi.nlm.nih.gov/pubmed/14744015.

Kin, N. M. K. N. Y., Palo, J., Haltia, M., & Wolfe, L. S. (1983). High levels of brain dolichols in neuronal ceroid lipofuscinosis and senescence. *Journal of Neurochemistry, 40*(5), 1465–1473. https://doi.org/10.1111/j.1471-4159.1983.tb13592.x.

Kuo, C., Maita, K., Sleight, S. D., & Hook, J. B. (1983). Lipid peroxidation : A possible mechanism of cephaloridine-induced nephrotoxicity. *Toxicology and Applied Pharmacology, 67*(1), 78–88. https://doi.org/10.1016/0041-008x(83)90246-6.

Lafka, T. I., Sinanoglou, V., & Lazos, E. S. (2007). On the extraction and antioxidant activity of phenolic compounds from winery wastes. *Food Chemistry, 104*(3), 1206–1214.

Lapish, C. C., Durstewitz, D., Chandler, L. J., & Seamans, J. K. (2008). Successful choice behavior is associated with distinct and coherent network states in anterior cingulate cortex. *Proceedings of the National Academy of Sciences (USA), 105*(33), 11963–11968. https://doi.org/10.1073/pnas.0804045105.

Levine, B. R. L., Williams, J. A., Stadtman, E. R., & Shacter, E. (1994). Carbonyl assays for determination of oxidatively modified proteins. *Methods in Enzymology. 233*, 337–357.

Lowry, O. H., Rosebrough, N. J., Farr, L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry, 193*(1), 265–275.

Misra, H. P., & Fridovich, I. (1972). The role of superoxide anion in the epinephrine and a simple assay for superoxide dismutase. *Journal of Biological Chemistry, 247*(10), 3170–3175.

Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues thiobarbituric acid reaction. *Analytical Biochemistry, 95*(2), 351–358.

Paxinos, G., & Watson, C. (2007). *The Rat Brain in Stereotaxic Coordinates* (6th ed). London, UK: Academic Press.

Peters, R. (2006). Ageing and the brain. *Postgraduate Medical Journal. 82*(964), 84–88. https://doi.org/10.1136/pgmj.2005.036665.

Porter, L. J. (1986). Flavans and proanthocyanidins. In J. B. Harborne (Ed.). *The Flavonoids Advances in Research Since 1986* (pp. 23–55). London, UK: Chapman and Hall.

Procyk, E., & Joseph, J. P. (2001). Characterization of serial order encoding in the monkey anterior cingulate sulcus. *European Journal of Neuroscience. 14*(6), 1041–1046. https://doi.org/10.1046/j.0953-816X.2001.01738.x.

Radak, Z., Ishihara, K., Tekus, E., Varga, C., Posa, A., Balogh, L., ... Koltai, E. (2017). Exercise, oxidants, and antioxidants change the shape of the bell-shaped hormesis curve. *Redox Biology, 12*(8), 285–290. https://doi.org/10.1016/j.redox.2017.02.015.

Reagan-Shaw, S., Nihal, M., & Ahman, N. (2007). Dose translation from animal to human studies revisited. *Federation of American Societies for Experimental Biology Journal, 22*(3), 659–661. https://doi.org/10.1096/fj.07-9574LSF.

Schweimer, J., & Hauber, W. (2005). Involvement of the rat anterior cingulate cortex in control of instrumental responses guided by reward expectancy Involvement of the rat anterior cingulate cortex in control of instrumental responses guided by reward expectancy. *Learning and Memory. 12*, 334–342. https://doi.org/10.1101/lm.90605.

Spijker, S. (2011). Dissection of rodent brain regions. In K. W. Li (Vol. Ed.), *Neuroproteomics, Neuromethods: Vol. 57*, (pp. 13–27). Totowa, NJ: Humana Press. https://doi.org/10.1007/978-1-61779-111-6.

Tripathi, S. J., Chakraborty, S., Srikumar, B. N., Raju, T. R., & Shankaranarayana Rao, B. S. (2018a). Inactivation of basolateral amygdala prevents stress-induced astroglial loss in the prefrontal cortex. *Molecular Neurobiology,* 1–17. https://doi.org/10.1007/s12035-018-1057-1.

Tripathi, S. J., Chakraborty, S., Srikumar, B. N., Raju, T. R., & Shankaranarayana Rao, B. S. (2018b). Basolateral amygdalar inactivation blocks chronic stress-induced lamina-specific reduction in prefrontal cortex volume and associated anxiety-like behavior. *Progress in Neuro-psychopharmacology and Biological Psychiatry. 88*(7), 194–207.

Tsay, H. J., Wang, P., Wang, S. L., & Ku, H. H. (2000). Age-associated changes of superoxide dismutase and catalase activities in the rat brain. *J Biomedical Sciences, 7*(6), 466–474. https://doi.org/10.1007/bf02253362.

Um, H. S., Kang, E. B., Leem, Y. Y., Cho, I. H., Yang, C. H., Chae, K. R., Hwang, D. Y., & Cho, J. Y. (2008). Exercise training acts as a therapeutic strategy for reduction of the pathogenic phenotypes for Alzheimer's disease in an NSE/APPsw-transgenic model. *International Journal of Molecular Medicine, 22*(4), 529–539. https://doi.org/10.3892/ijmm_00000052.

Walton, M. E., Bannerman, D. M., Alterescu, K., & Rushworth, M. F. S. (2003). Functional specialization within medial frontal cortex of the anterior cingulate for evaluating effort-related decisions. *Journal of Neurosciences, 23*(16), 6475–6479. https://integrateddiabetes.com/choosing-a-cgm-3-heads-are-better-than-one/.

Wang, G., & Cai, J. (2006). Disconnection of the hippocampal – prefrontal cortical circuits impairs spatial working memory performance in rats. *Behavioural Brain Research, 175*(2), 329–336. https://doi.org/10.1016/j.bbr.2006.09.002.

West, M. J. (2013). Tissue shrinkage and stereological studies. *Cold Spring Harbor Protocols, 8*(3), 207–212. https://doi.org/10.1101/pdb.top071860.

West, M. J., Slomianka, L., & Gundersen, H. J. (1991). Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. *The Anatomical Record, 231*(4), 482–497. https://doi.org/10.1002/ar.1092310411.

Wu, Q., Wang, M., & Simon, J. E. (2003). Determination of proanthocyanidins in fresh grapes and grape products using liquid chromatography with mass spectrometric detection. *Analytical Chemistry, 75*(10), 2062–2068. https://doi.org/10.1002/rcm.2029.

Weinstein, A. M., Voss, M. W., Prakash, R. S., Chaddock, L., Szabo, A., White, S. M., ... Erickson, K. I. (2012). The association between aerobic fitness and executive function is mediated by prefrontal cortex volume. *Brain Behavior, and Immunity, 26*(5), 811–819. https://doi.org/10.1016/j.bbi.2011.11.008.

Xianchu, L., Ming, L., Xiangbin, L., & Zheng Lan, Z. (2018). Grape seed proanthocyanidin extract supplementation affects exhaustive exercise-induced fatigue in mice. *Food and Nutrition Research, 62*, 1421. https://doi.org/10.29219/fnr.v62.1421.

Further reading

Müller, S., Steizer, E. H. K., & Pampaioni, F. (2013). Quantitative 3D cell-based assay performed with cellular spheroids and fluorescence microscopy. *Methods in Cell, 113*, 295–309. https://doi.org/10.1016/B978-0-12-407239-8.00013-6.