Neuroprotective Effects of Theobromine in permanent bilateral common carotid artery occlusion rat model of cerebral hypoperfusion

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Abstract
Cerebral hypoperfusion (CH) is a common underlying mechanism of dementia disorders linked to aberrations in the neurovascular unit. Hemodynamic disturbances adversely affect cellular energy homeostasis that triggers a sequence of events leading to irrevocable damage to the brain and neurobehavioral discrepancies. Theobromine is a common ingredient of many natural foods consumed by a large population worldwide. Theobromine has shown health benefits in several studies, attributed to regulation of calcium homeostasis, phosphodiesterase, neurotransmission, and neurotrophins. The current study evaluated the neuroprotective potential of theobromine against CH in the permanent bilateral common carotid artery occlusion (BCCAO) prototype. Wistar rats were distributed in Sham-operated (S), S + T100, CH, CH + T50, and CH + T100 groups. Animals received permanent BCCAO or Sham treatment on day 1. Theobromine (50, 100 mg/kg) was given orally in animals subjected to BCCAO for 14 days daily. CH caused neurological deficits (12-point scale), motor dysfunction, and memory impairment in rats. Treatment with theobromine significantly attenuated neurological deficits and improved sensorimotor functions and memory in rats with CH. In biochemistry investigation of the entire brain, findings disclosed reduction in brain oxidative stress, inflammatory intermediaries (tumor necrosis factor-α, interleukin-1β and -6, nuclear factor-κB), markers of cell demise (lactate dehydrogenase, caspase-3), acetylcholinesterase activity, and improvement in γ-aminobutyric acid quantity in rats that were given theobromine for 14 days daily after CH. Histopathological analysis substantiated attenuation of neurodegenerative changes by theobromine. The findings of this study indicated that theobromine could improve neurological scores, sensorimotor abilities, and memory in CH prototype.

Keywords Theobromine · Cerebral hypoperfusion · GABA · Inflammation · Memory · Oxidative stress

Introduction
Pre-existing cardiovascular (e.g., heart failure, cardiac arrest, high blood pressure) and metabolic disorders (e.g., atherosclerosis and diabetes) cause hemodynamic changes in the brain that forms the basis of several progressive neurocognitive disorders (Zhao and Gong 2015). Abnormality in autoregulatory mechanisms of cerebral blood flow (CBF) causes a hypoperfusion state, resulting in inefficient functional hyperemia and neuro-necroptosis (Ciacciarelli et al. 2020; Park et al. 2019). Cerebral hypoperfusion (CH) instigates extensive brain injury with increased severity in highly vulnerable regions, resulting in a comprehensive range of neurological and neuropsychiatric deficits (Duncombe et al. 2017). Mitochondrial dysfunction, adenosine triphosphate (ATP) depletion, excitotoxicity, and intracellular calcium excess trigger catabolic pathways in CH (Daulatzai 2017). Sequentially, oxidative stress followed by resident immune cells, leucocytes, and pro-inflammatory cytokines finally integrate to initiate widespread neurological deficits and irreparable brain and vascular injury (Bell and Zlokovic 2009).

Traditionally methylxanthines, including theobromine and its derivatives, are used as anti-anginal, diuretics, cardiac, respiratory, and brain stimulants, vasodilators, bronchodilators, anti-cancer, hepatoprotective, and hypo-cholesterolaemia agents Wei et al. 2021; Jang et al. 2020;...
Theobromine antagonizes phosphodiesterase activity (nonselective inhibitor) (Monteiro et al. 2016) and adenosine receptors (affinity in decreasing order adenosine $A_1$, $A_{2B}$, $A_{3A}$, and $A_{2A}$ receptors) (Jacobson et al. 2020) that enhances levels of the second messenger, cyclic adenosine monophosphate (cAMP). The inhibitory effects of cAMP on contractile proteins and intracellular calcium levels (Levy and Bailey 2000) cause vasodilation that may revive CBF against CH. In contrast to adenosine $A_1$ receptors (inhibitory role), adenosine $A_{2A}$ (Gomes et al. 2011) and $A_{2B}$ receptors (Coppi et al. 2020) depict a crucial part in the progression of ischemic injury by enhancing neuroinflammation (Pedata et al. 2014), and inhibition of these receptors attenuate ischemic brain damage (Chen et al. 1999). Furthermore, theobromine inhibits intracellular calcium influx, poly(ADP-ribose)polymerase-1, and nitric oxide toxicity (Martinez-Pinilla et al., 2015), which can alleviate oxidative brain damage in hypoperfusion states. Recent findings indicate that theobromine inhibits amyloid-$\beta_{40-42}$ deposition (Sugimoto et al. 2019; Cova et al. 2019), mTOR (mammalian target of rapamycin) and nuclear factor-kappa B (NF-$\kappa$B) effects, low- and very-low-density lipoproteins, pro-inflammatory cytokines (Martinez-Pinilla et al., 2015), and augments neurotrophic factors and high-density lipoproteins (Islam et al. 2019). These findings indicate that theobromine might be able to attenuate CH-triggered pathogenic mechanisms.

In contrast to caffeine, the psychostimulant effect of theobromine lacks dependence and withdrawal (Jacobson et al. 2020; Monteiro et al. 2016; Meredith et al. 2013). Theobromine has good lipophilicity, penetrates biological barriers, and has a greater plasma half-life relative to caffeine that accounts for better therapeutic outcomes in many disorders (Martinez-Pinilla et al., 2015). Theobromine has mild adverse effects and a high therapeutic index relative to other methylxanthines. In animal studies, high doses of theobromine (Lethal dose 50 in rats 950–1356 mg/kg) exhibited thymic and testicular withering and loss of fetal weight. In human studies, high doses (1–1.5 g/day orally) of theobromine (Lethal dose 50 in humans 1000 mg/kg) showed minor side effects such as nausea, anorexia, sweating, and headache (Monteiro et al. 2016; Salihovic et al. 2014). In the current investigations, we intended to evaluate the neuroprotective properties of theobromine in permanent 2-vessel occlusion (2-VO) rat prototype.

**Materials and methods**

**Experimental subjects**

The IAEC permitted the investigation procedure (Ref. no. SSP/IAEC/2019/009 on date 17-11-2019), and animal experimentations were performed as per the guiding ethics of CPCSEA, GOI (New Delhi). Wistar rats (9–10-month adults) of male sex (body weight range 230 ± 10 g) were acquired from AIIMS, New Delhi (India), acclimatized for 14 days, and later subjected to experiments between 0900 and 1600 h period. CPCSEA registered institutional establishment (Regd. no. 1616/PO/Re/S/12/CPCSEA) was utilized to house rats in separate cages (polyacrylic) under an archetypal laboratory environment. The animal caretakers were kept blinded to the various drug regimens.

**Cerebral hypoperfusion**

The standard technique of bilateral common carotid artery occlusion (BCCAO) was applied to initiate CH in rats (Bhuvanendran et al. 2019; Yanpallewar et al. 2005). Initially, atropine sulfate (0.5 mg/kg, i.p.) was given in rats before ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) injections, and subsequently, reflexes were gauged to ascertain anesthesia. A ventral midline cut was given in the neck (in the middle of the neck and sternum) between sternocleidomastoid and sternohyoid muscles parallel to the trachea. Common carotid arteries (CCA) were identified adjacent to the sternocleidomastoid muscle, and both right and left CCA was bisected carefully from the adventitial sheath and vago-sympathetic nerve. A 3–0 silk suture (TRUSILK®) was sterilized, and both CCAs were double-ligated. Ligation of 1st carotid, either left or right, was swapped throughout this procedure (Soria et al. 2013). The skin was sutured and was applied with Neosporin®. Body temperature (37 ± 0.5 °C) was maintained throughout the surgery, including the recuperative period where free access to pulverized pellet diet and water was permitted. Rats displaying post-surgery-induced hesitancy in water intake were administered buprenorphine (0.05%, i.p) once (Saxena et al. 2015).

**Experimental methods**

Theobromine (Mol. weight 180.16, purity >98%, T4500-Merck) was suspended in 0.2% carboxymethylcellulose (CMC) to prepare a fresh homogenous suspension. Theobromine suspension was administered orally (50 and 100 mg/kg) in rats (Shojaii-Zarghani et al. 2021; Jang et al. 2020; Mendiola-Precena et al. 2017). The selection of theobromine doses is based on human consumption. The chosen doses are substantially lower relative to theobromine intake...
in dietary cocoa or chocolate products by humans. Data from earlier reports indicate that a 25–225 times increase in plasma theobromine concentrations relative to extreme theobromine intake by humans is devoid of any significant toxic repercussions in rats (Shively et al. 1984). The distribution of rats in 5 diverse groups ($n = 6$) was accomplished in a single-blind mode using a random distribution method to minimize selection bias. An experimenter blinded to the treatment to be allocated to each animal dispersed the animals into the following groups: (i) Sham-operated (S), (ii) S + T100, (iii) CH, (iv) CH + T50, (v) CH + T100. CH was initiated in rodents on 1st day by performing BCCAO surgery. After 90 min of CCA, ligation rats were subjected to vehicle (0.2% CMC, 5 ml/kg, $p.o.$) or theobromine (50 and 100 mg/kg, $p.o.$) treatments that continued for 14 successive days. In sham-operated (S) rats, similar surgery was performed without occluding CCAs and was administered vehicle or theobromine (100 mg/kg) for 14 consecutive days.

**Behavioral analysis**

Neurological abilities of rats were measured on days 2, 5, 7, and 13 (Fig. 1) by utilizing a standard 12-point modified neurological scale (NSS) (Liu et al. 2021; Umukoro et al. 2019). This scale is based on abnormal gait and hemiplegia. In NSS, each rat was tested for twisting thorax, forelimb flexion, beam-walk, and hanging on a wire, and a cumulative score was calculated individually to present a neurological deficit score (NDS). The sensorimotor performance of rats was assessed on day 1 (before surgery) and days 2, 4, 8, and 12 after occlusion. An accelerating rotarod technique was used to assess the sensorimotor abilities in rats, and fall-off latency was noted (Oyamada et al. 2008). Spatial memory was appraised using the elevated plus-maze test (EPM) (Parle and Dhingra 2003; Itoh et al. 1990). Animals were given acquisition trials on day 12 and retrieval trials on day 13 in EPM, and transfer latency (TL) was recorded. The data of TL were used calculate inflexion ratio (IR): $IR = (L_0 - L_1)/L_0$ ($L_0$ = Initial TL, $L_1$ = Retention TL). A decrease in IR indicates memory loss and an increase in IR indicates improvement in memory (Rajesh et al. 2017). On day 14, the animals were given trials in a novel object recognition test (NORT) to estimate discrimination index (DI) for evaluation of working memory abilities (Ennaceur 1998; Ennaceur and Delacour 1988).

**Biochemical parameters**

Animals were sacrificed using the cervical dislocation manner with sodium pentobarbitone (150 mg/kg) anesthesia. The entire brain was dissected out and rinsed in freezing isotonic...
0.9% w/v sodium chloride solution (sterile). After-homogenerate formation (10% w/v phosphate-buffered saline), the samples were centrifugated (CPR-30 Remi Compufuge, Vasai, India) at a 12,000 × g force for 15 min at 4°C. The extra liquid (supernatant) was secluded to estimate biochemical parameters following standard procedures. Thiobarbituric acid reactive substances (TBARS) were assessed by reaction of malondialdehyde in samples with thiobarbituric acid (Ohkawa et al. 1979) that forms a chromophore whose variability in optical density (O.D.) was recorded at 532 nm using twin-beam UV-spectrophotometer. Molar extinction coefficient (ε) = 1.56 × 10^5 /M/cm was applied to compute TBARS stated as nmol per mg protein. In estimating glutathione, Ellman’s reagent converts glutathione in the sample to yellow colored 2-nitro-5-thiobenzoic acid whose O.D. variability is noted at 412 nm. Glutathione (µmol GSH per mg protein) was quantified by using ε = 1.36 × 10^4 /M/cm (Ellman, 1959). Superoxide dismutase (SOD) activity was evaluated by adopting the method of Winterbourn et al. (1975). The SOD in the sample hinders the reduction of nitro blue tetrazolium (NBT) by O₄⁻ and formazan production. The rate of SOD (µmol NBT reduced per min per mg protein) was enumerated using ε (formazan) = 15,000 /M/cm. Catalase activity (µmol H₂O₂ decomposed per min per mg protein of brain) was computed using ε = 43.6 /M/cm at λₚ₅₀ = 240 nm (Claiborne 1985). The process elaborated by Sastry et al. (2002) was implemented to assess total nitrites. A typical curve of sodium nitrite (concentration range 0.01–0.1 mM) was designed, nitrite content in samples was equated and then specified as µmol per mg protein. The lactate dehydrogenase activity (µmol NADH oxidized/min/mg protein) was scrutinized by the procedure of Horecker and Kornberg (1948) using ε = 6220 M⁻¹ cm⁻¹ at λₚ₅₀ = 340 nm. The acetylcholinesterase (AChE) rate was recorded by the method of Ellman et al. (1961). Rate of AChE (µmol acetylthiocholine (AcTh) iodide hydrolyzed per min per mg protein) was computed utilizing ε = 1.36 × 10^4 /M/cm at λₚ₅₀ = 412 nm. The paper chromatography method was used to assess levels of GABA by using a mobile phase consisting of n-butanol, glacial acetic acid, and water in a ratio of 1:5:10. GABA concentration (pmol/ml) in eluted solution was appraised at 509 nm and by the standard curve of GABA (Swamy et al. 2013). Protein was estimated by the scheme elaborated by Lowry et al. (1951).

**Enzyme-linked immunosorbent assay (ELISA)**

Dual antibody sandwich ELISA procedure was adopted to compute the cytokines and cell death biomarkers as per instruction manual provided in ELISA kits procured from Krishgen Biosystems, Mumbai (TNF-α, KB3145, IL-1/β, KLR0119, IL-6, KLR0135) and KinesisDX, CA, USA (capase-3, K11-5114 and NF-κB, K11-0288). A typical curve of biomarkers (concentrations standard rat TNF-α 450, 225, 56.25, 28.13, 14.06, 7.03, and 3.51 pg/ml; IL-1β of concentrations 4.8, 2.4, 1.2, 0.6, and 0.3 pg/ml; IL-6 24, 12, 6, 3, and 1.5 pg/ml; NF-κB 12, 6, 3, 1.5, and 0.75 ng/ml; caspase-3 6.4, 3.2, 1.6, 0.8, and 0.4 ng/ml) was plotted to estimate TNF-α (pg/ml), IL-1β (pg/ml), IL-6 (pg/ml), caspase-3 (ng/ml), and NF-κB (ng/ml) in the samples at 450 nm by using ELISA reader (iMARK, BIORAD).

**Histopathological assessment**

Animals were given anesthesia, and reflexes were assessed. Subsequently, intracardiac perfusion through the left ventricle ensued using 10% neutral buffered formalin solution (10% NBF) by means of a gravity-fed perfusion device. Hippocampus and cortical (frontal lobe) regions were secluded and immersed in 10% NBF with 0.05% natriumazid (pH 7.0) (fixative:tissue = 10:1) for 6 days (4 °C). Fixed tissues are kept in 70% ethyl alcohol at 4 °C. Later thin Sect. (5.0 µm) were carved out using a rotary microtome and applied with hematoxylin and eosin (H&E) dye. Permanant slides were scrutinized using light microscopy at × 40 magnifications. In histomorphometric analysis, cortical (frontal lobe pyramidal neurons) and hippocampus (CA 1 and 3) neuron densities (per µm²) were determined by counting viable neurons using ImageJ software (NIH Image 1.61; National Institute of Health; Bethesda, MD).

**Statistical analysis**

A trained experimenter blinded to drug regimens specified to different cohorts examined and appraised the data. Outliers were absent (Grubb’s test) in the data, and the Kolmogorov-Smirnov test and Levene’s test established normal distribution of variables and homogeneity of variance (HOV p > 0.05, Levene’s test), respectively. Else, in case of unequal variance (HOV p < 0.05, Levene’s test), Welch’s ANOVA (p < 0.05, F’-statistic) and Games-Howell posthoc tests can be useful. Means of normally distributed variables were scrutinized and related by one-way ANOVA (data from NORT, EPM, and biochemical) or repeated measures two-way ANOVA (data of mNSS and rotarod test). In case ANOVA outcomes are significant (p < 0.05) in F-statistics, multiple comparison tests viz. Tukey’s HSD (Honest Significant Difference) or Bonferroni were applied. Data is stated as mean ± Standard Deviation (S.D.). Statistical significance was deemed at p < 0.05.
Results

Theobromine attenuated neurological and sensorimotor dysfunctions in CH rat prototype

CH on 1st day depreciated neurological performance (day 2, 5, 7, and 13 $p<0.001$) relative to sham-operation [$F_{(12,100)}=6.01$, $p<0.001$]. Oral administration of theobromine (50 and 100 mg/kg) decreased neurological deficits (day 2 $p<0.01$, $p<0.01$, day 5 $p<0.001$, $p<0.01$, day 7 $p<0.05$, $p<0.01$, day 13 $p<0.001$) in rats against CH in reference to rats that received CH and vehicle treatments alone (Fig. 2 A). Although sensorimotor performance of rats before permanent 2-VO on 1st day showed no substantial disparity in latencies to fall (s) from rotating rod, however, significant intergroup variation in latency to fall (s) emerged out from day 2 onwards. Rats rendered to vehicle administration and CH showed a significant (day 2, 4, 8, and 12 $p<0.001$) decrease in the latency to fall (s) relative to sham-operated rats [$F_{(16,125)}=3.93$, $p<0.001$] (Fig. 2B). These outcomes exhibited that CH adversely affected the sensory and motor functions in rats. Administration of theobromine (100 mg/kg) continuously after CH attenuated sensorimotor deficits in rats when measured on day 2 ($p<0.01$), day 4 ($p<0.001$), day 8 ($p<0.001$), and day 12 ($p<0.01$) in reference to rats that were given CH and vehicle treatments. Post-treatment with theobromine (50 mg/kg) also mitigated CH induced motor deficits (day 4 $p<0.05$ and day 8 $p<0.01$) in rats.

Theobromine attenuated memory loss against CH in rats

In the EPM test, TL and IR were estimated to gauge the outcomes of theobromine on spatial memory function of rats against CH. In EPM test acquisition trials, there was no substantial intergroup disparity in day 12 TL [$F_{(4,25)}=0.3212$, $p<0.001$] (Fig. 3 A). In the retention trials (day 13), a substantial upsurge ($p<0.001$) in the TL (Fig. 3B) was pragmatic in response to CH when juxtaposed with vehicle-treated sham-operated rats. Theobromine (50 and 100 mg/kg) administration considerably diminished the CH prompted upsurge in the TL ($p<0.01$, $p<0.001$) when juxtaposed with CH and vehicle alone treatments [$F_{(4,25)}=120.7$, $p<0.001$]. In harmony with the results of TL, the IR was significantly reduced ($p<0.001$) by CH and vehicle treatments in reference to sham-operation accompanied by vehicle administration (Fig. 3 C). Theobromine (50 and 100 mg/kg) enhanced ($p<0.05$, $p<0.001$) the IR against CH in rats when compared to CH and vehicles administered rats [$F_{(4,25)}=113.7$, $p<0.001$]. Furthermore, appraisal of recognition type memory in NORT by measuring DI on day 14 disclosed waning of working memory owing to permanent 2-VO on the 1st day. CH prompted considerable decline ($p<0.001$) in reference to sham-operation [$F_{(4,25)}=26.97$, $p<0.001$]. Oral post-treatment with theobromine (50 and 100 mg/kg) for 14 days abrogated memory deficits ($p<0.05$, $p<0.001$) against CH when related with CH and vehicle administrations alone (Fig. 3D). Experimental data showed that theobromine

Fig. 2 Theobromine (50 and 100 mg/kg) post-treatment for 14 days attenuated neurological and sensorimotor deficits in rats subjected to cerebral hypoperfusion (CH) on day 1. Neurological deficit scores (12-point scale) (A) and latency to fall (s) in rotarod test (B) evaluated on different days. Data expressed as mean ± S.D. ($n=6$). # # # ($p<0.001$) vs. Sham-operated (S) group; * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) vs. CH group.
(100 mg/kg) significantly reduced the TL \((p<0.001)\) and, improved the IR \((p<0.01)\) and DI \((p<0.05)\) in comparison to theobromine (50 mg/kg) contrary to the outcomes of CH. Hence, it can be inferred that theobromine showed dose-dependent improvement in memory in rats against CH.

Theobromine decreased the brain oxidative and nitrosative stress against CH

CH caused a substantial \((p<0.001)\) increase in the lipid peroxidation (TBARS content) and total nitrites, and diminution of endogenous antioxidants (GSH, SOD, and catalase activities) when related to vehicle and sham treatments (Fig. 4). Post-treatment with theobromine (50 and 100 mg/kg) abrogated the lipid peroxidation \((p<0.05, p<0.001)\) \([F_{(4,20)}=24.57, p<0.001]\) and nitrites \((p<0.05, p<0.01)\) \([F_{(4,20)}=14.33, p<0.001]\), and significantly boosted the GSH \((p<0.05, p<0.001)\) \([F_{(4,20)}=27.92, p<0.001]\), SOD \((p<0.05, p<0.001)\) \([F_{(4,20)}=25.18, p<0.001]\), and catalase \((p<0.05, p<0.001)\) \([F_{(4,20)}=17.95, p<0.001]\) activities in CH rats when related to rats that undergone CH and vehicle administration. Biochemical outcomes disclosed that theobromine at 100 mg/kg dose prompted a dose-dependent waning of TBARS \((p<0.05)\) and upsurge in endogenous...
Theobromine decreased brain inflammatory cytokines against CH

CH significantly elevated appearance of brain inflammatory cytokines (TNF-α, IL-1β, IL-6) \((p<0.001)\) in correlation to sham-operation. Theobromine (50 and 100 mg/kg) repressed CH-induced escalation in TNF-α \((p<0.05,\ p<0.001)\) [F(4,20)=28.32, \(p<0.001\)] (Fig. 6 A), IL-1β \((p<0.01, \ p<0.001)\) [F(4,20)=23.42, \(p<0.001\)] (Fig. 6B), and IL-6 \((p<0.05, \ p<0.001)\) [F(4,20)=19.41, \(p<0.001\)] (Fig. 6C) in reference to vehicle alone treatment in CH rats. Theobromine 100 mg/kg triggered considerable decrease \((p<0.05)\) in the appearance of inflammatory cytokines measured in this study relative to theobromine 50 mg/kg when administered for equivalent length in rats receiving permanent 2-VO.

Theobromine lowered brain acetylcholinesterase activity and upregulated GABA levels against CH

CH initiated considerably \((p<0.001)\) escalated the rate of brain AChE action and abrogated GABA concentration when juxtaposed to vehicle and sham treatments. A noteworthy decline in rate of AChE \((p<0.05, \ p<0.05)\) [F(4,20)=15.09, \(p<0.001\)] (Fig. 5 A) and improvement in GABA levels \((p<0.05, \ p<0.01)\) [F(4,20)=17.01, \(p<0.001\)] (Fig. 5B) against CH was pragmatic in response to theobromine (50 and 100 mg/kg) administration when related to CH and vehicle treatments only. These repercussions specified that theobromine post-conditioning considerably attenuated the CH-induced loss of cholinergic and GABAergic neurotransmission in rats.
Theobromine post-treatment (50 and 100 mg/kg) for 14 days ameliorated neurotransmitters level against cerebral hypoperfusion (CH). Theobromine significantly lowered (A) acetylcholinesterase (AChE) activity and enhanced (B) γ-aminobutyric acid (GABA) in the brain of rats exposed to CH on day 1. Data expressed as mean ± S.D. (n = 5). ### (p < 0.001) vs. Sham-operated (S) group; * (p < 0.05), ** (p < 0.01) vs. CH group.

Fig. 5 Theobromine post-treatment (50 and 100 mg/kg) for 14 days ameliorated neurotransmitters level against cerebral hypoperfusion (CH). Theobromine significantly lowered (A) acetylcholinesterase (AChE) activity and enhanced (B) γ-aminobutyric acid (GABA) in the brain of rats exposed to CH on day 1. Data expressed as mean ± S.D. (n = 5). ### (p < 0.001) vs. Sham-operated (S) group; * (p < 0.05), ** (p < 0.01) vs. CH group.

days reduced levels of pro-inflammatory cytokines in the brain of rats that received CH on day 1.
Theobromine negated neurodegenerative changes in CH rat prototype

In the histopathological investigation, rats subjected to permanent 2-VO showed neurodegenerative changes marked by blebbing of the plasma membrane (b), swelling (s), and pyknosis (p) in the cortex (frontal pyramidal neurons) and Cornu Ammonis 1 and 3 (CA1 and CA3) areas of the hippocampus of the brain. Sham-operation displayed none of the neurodegenerative signs. Theobromine (50 and 100 mg/kg) abolished neuropathological variations in these brain sub-regions neuronal membrane and chromosomal matter (Fig. 7). Histomorphometric examination disclosed that 2-VO-induced decrease in neuron density was attenuated by theobromine post-treatments with theobromine 100 mg/kg showing a more pronounced neuroprotective effect when compared to theobromine 50 mg/kg dose (Table 1). Hence, it can be inferred that oral administration of theobromine attenuated CH triggered neurodegeneration in the brain.

Theobromine attenuated biomarkers of neurodegenerative cascade against CH

The rate of LDH and caspase-3 appearance indicate the level of neurodegeneration and damage to the brain parenchyma. In harmony with previous outcomes, CH prompted an upsurge ($p < 0.001$) in the rate of LDH and brain caspase-3 appearance when juxtaposed to sham treatment. Theobromine (50 and 100 mg/kg) attenuated ($p < 0.05$, $p < 0.001$) the LDH activity $[F_{(4,20)}=23.53, p < 0.001]$ (Fig. 6E) and caspase-3 level $[F_{(4,20)}=19.94, p < 0.001]$ (Fig. 6F) when related to vehicle alone treatment against CH. Theobromine 100 mg/kg oral administration triggered a considerable decrease ($p < 0.05$) in LDH activity and caspase-3 level comparative to theobromine 50 mg/kg against CH. These results showed that post-treatment with theobromine protected against hypoperfusion-induced cell demise in the brain of rats.
low-density lipoproteins) of physiological and structural importance (Negre-Salvayre et al. 2008). Furthermore, CH triggers nitrosative stress through neutrophils, macrophages, and microglia-associated inducible nitric oxide synthase (iNOS). Peroxynitrites modify cell proteins (tyrosine nitrosylation, carbonylation) and DNA causing irreversible cell injury, necrosis, and microvascular abnormalities (Daulatzai 2017). Nitric oxide-dependent attenuation of respiratory complex I and II and instigation of poly(ADP-ribose) polymerase accentuate ROS-associated neurodegeneration. In the CH state, depletion of endogenous antioxidants augments the rate of free-radical yield and reactive, secondary intermediaries of oxidative insult. Studies on transgenic animals suggested that SOD and catalase actively confer neuroprotection against the decrease in CBF (Warner et al. 2004). In existing experiments, CH significantly augmented the TBARS in the brain homogenate samples and total nitrites, and both of these reactive by-products were abrogated by theobromine. TBARS directly specifies malondialdehyde (MDA), which happens to be notorious lipid peroxidation aldehyde, accountable for several biochemical aberrations (Ayala et al. 2014). Along with a decrease in lipid peroxidation, theobromine augmented endogenous brain antioxidants (GSH, SOD, and catalase) in the CH rat prototype.

CH is correlated with neurobehavioral deficits that involve hypoxia and hypo-nutrition-induced pro-oxidants and inflammatory cytokines (Saxena et al. 2015). Reactive oxygen species (ROS) are notorious for promoting endothelial dysfunction and local inflammatory responses via adhesion proteins, phospholipase A2, hypoxia-inducible factor-1α, TNF-α, and IL-1β (Chen et al. 2011). ROS modify cellular lipids and proteins to breach cell membrane integrity and cause cell death via genotoxicity (Liu and Zhang 2012). Oxidative decomposition of polyunsaturated fatty acids leads to the formation of a variety of overactive hydroperoxides, radicals, and aldehydes such as hexanal, acrolein, malondialdehyde, propanal, and 4-hydroxy 2-nonenal. These toxic mediators can alter protein structure and lipoproteins (e.g., Table 1 indicating cortical (frontal lobe) and hippocampus (CA 1 and CA 3) viable neuron density per µm²:

| Group     | Cortex (neuron density per µm²) | Hippocampus (neuron density per µm²) |
|-----------|----------------------------------|--------------------------------------|
| (i) S     | 35.833                           | 37.937                               |
| (ii) S + T100 | 36.722                           | 38.874                               |
| (iii) CH  | 15.733                           | 14.221                               |
| (iv) CH + T50 | 27.634                           | 29.363                               |
| (v) CH + T100 | 33.728                           | 36.263                               |

Discussion

CH is correlated with neurobehavioral deficits that involve hypoxia and hypo-nutrition-induced pro-oxidants and inflammatory cytokines (Saxena et al. 2015). Reactive oxygen species (ROS) are notorious for promoting endothelial dysfunction and local inflammatory responses via adhesion proteins, phospholipase A2, hypoxia-inducible factor-1α, TNF-α, and IL-1β (Chen et al. 2011). ROS modify cellular lipids and proteins to breach cell membrane integrity and cause cell death via genotoxicity (Liu and Zhang 2012). Oxidative decomposition of polyunsaturated fatty acids leads to the formation of a variety of overactive hydroperoxides, radicals, and aldehydes such as hexanal, acrolein, malondialdehyde, propanal, and 4-hydroxy 2-nonenal. These toxic mediators can alter protein structure and lipoproteins (e.g., low-density lipoproteins) of physiological and structural importance (Negre-Salvayre et al. 2008). Furthermore, CH triggers nitrosative stress through neutrophils, macrophages, and microglia-associated inducible nitric oxide synthase (iNOS). Peroxynitrites modify cell proteins (tyrosine nitrosylation, carbonylation) and DNA causing irreversible cell injury, necrosis, and microvascular abnormalities (Daulatzai 2017). Nitric oxide-dependent attenuation of respiratory complex I and II and instigation of poly(ADP-ribose) polymerase accentuate ROS-associated neurodegeneration. In the CH state, depletion of endogenous antioxidants augments the rate of free-radical yield and reactive, secondary intermediaries of oxidative insult. Studies on transgenic animals suggested that SOD and catalase actively confer neuroprotection against the decrease in CBF (Warner et al. 2004). In existing experiments, CH significantly augmented the TBARS in the brain homogenate samples and total nitrites, and both of these reactive by-products were abrogated by theobromine. TBARS directly specifies malondialdehyde (MDA), which happens to be notorious lipid peroxidation aldehyde, accountable for several biochemical aberrations (Ayala et al. 2014). Along with a decrease in lipid peroxidation, theobromine augmented endogenous brain antioxidants (GSH, SOD, and catalase) in the CH rat prototype.

The circulatory deviations provoke uninvited proteins (e.g., matrix metalloproteinases) and immunity regulators (e.g., TNF-α, interleukins) that can damage the blood-brain
barrier and other cerebral capillary networks (Wang et al. 2020). Subsequently, migration of plasma leucocytes in the brain parenchyma, invasion of neurotoxins, and glial activation perpetuate inflammatory cascade. An upsurge in IL-1β levels early during hypoperfusion is implicated in activating inflammatory intermediaries viz. phospholipase A2, cyclooxygenase-2, and iNOS (Woodcock and Morganti-Kossmann 2013). An increase in IL-6 levels is also linked with brain infarct, thrombus formation, and an upsurge in TNF-α and ROS yield through microglia and astrocytes (Tang et al. 2015). TNF-α stimulates caspase-initiated apoptotic mechanisms through TNFR1 receptors, necrosis (via excitotoxic and nitric oxide pathways) and transcription activity of NF-κB substantially (Duncan et al. 2020; Ju Hwang et al., 2019). These proceedings deteriorate capillaries, tight junction proteins, and extracellular matrix, ensuing detrimental consequences such as white matter atrophy and loss of neurobehavioral functions (Wang et al. 2020; Fogal and Hewett 2008; Maher et al. 2003). In the existing investigation, CH substantially amplified the content of pro-inflammatory biomolecules (TNF-α, IL-1β, IL-6), which materialize their neurotoxic repercussions from the sub-acute period of CH and onwards. Furthermore, the activity of LDH and caspase-3 levels (cell death markers) was also enhanced by CH over 14 days. Oral administration of theobromine for 14 days abolished the TNF-α, IL-1β, IL-6, caspase-3 levels, and LDH activity in the brain of rats subjected to CH. Inflammatory cascade, the appearance of cytokines/chemokines, and apoptosis machinery are regulated by NF-κB, whose transcription activity is amplified when there is a drop in CBF (Saggu et al. 2016). Brain insult in the form of traumatic brain injury (TBI), CH, excitotoxic pathways, and several neurotoxins (free radicals) (Negre-Salvayre et al. 2008) can galvanize the NF-κB action resulting in an upsurge in cell degrading enzymes (e.g., matrix metalloproteinases), cytokines (e.g., IL-6, C-reactive protein), and inflammatory molecules (e.g., selectins, integrins, iNOS, cyclooxygenase-2) (Liu et al. 2017; Duncombe et al. 2017). In harmony with previous conclusions (Li et al. 2020), CH significantly enhanced the brain in the existing pre-clinical investigation. Treatment with theobromine (100 mg/kg) attenuated the CH-induced brain NF-κB levels that might have contributed to lowering inflammatory cytokines (TNF-α, IL-1β, IL-6). These findings disclosed that theobromine declined the CH triggered inflammation and secondary brain damage in rats.

Central cholinergic transmission regulates cognitive functions, several biological activities (e.g., anti-apoptotic factors, stress response, wakefulness, sleep), and the release of neuromodulators (e.g., dopamine, growth factors) (Resende and Adhikari 2009). Published data indicate that acetylcholine modulates several stages of cognitive processing (Ferreira-Vieira et al. 2016). Anti-inflammatory and vasodilatory effects of acetylcholine through nicotinic receptors located on microglia, astrocytes, and blood vessels prevent memory impairments (Maurer and Williams 2017; Jin et al. 2015). Evidence indicates a significant increase in the rate of brain AChE and decline in the rate of choline acetyltransferase (ChAT) action with a concomitant drop in acetylcholine generation lead to memory deficits after CH (Sun et al. 2020; Gatak et al. 2012). In the recent investigation, CH enhanced the central AChE activity and reduced the GABA content in rats. Reported data suggest that GABAergic depression can be a factor for overt glutamatergic excitatory transmission (Asomuha et al. 2010; Liu et al. 2015). GABA is well known for its protective effects (e.g., antioxidative, anti-apoptotic) and has been extensively investigated in pre-clinical studies, confirming its benefits against hyperglycemia, proliferative disorders, liver ailments, kidney disease injury, and neurodegeneration. CBF and energy demand/supply ratio are also improved by GABA (Chen et al. 2019; Ngo and Vo 2019). Neuronal hyperpolarization reduces metabolic activity, ROS, and inflammation, parallel to hypothermia (Lee et al. 2018; Neumann et al. 2013). Hence, in excitotoxicity origin brain dysfunction, GABAergic hyperpolarization can afford great relief. Theobromine was able to diminish the brain AChE activity and augment the GABA levels against CH. These findings corroborated that theobromine can increase cholinergic transmission and deter excitotoxic pathways by enhancing the GABA levels in CH states.

Pre-existing cardiovascular and metabolic disorders cause hemodynamic changes in the whole brain that forms the basis of CH (Traystman 2003), which can be closely simulated by the permanent BCCAO technique in rodents (Bacigalupi et al. 2010). 2-VO is a forebrain ischemia model that can be divided into acute (24 h), subacute (3 days), and chronic phases (>7 days) (Ma et al. 2020) in which hippocampal CA1 neurons are the most vulnerable and cortical (including neocortex) are late ischemic tolerant followed by subcortical structures such as caudate-putamen, striatum, and thalamus (Hossmann 2008). In the present study, CH produced substantial neurodegenerative deviations noticeable by pyknosis, swelling, and blebbing of membranes in the hippocampus (CA1 and CA3 regions) and cortical regions, and these changes were markedly attenuated by theobromine post-treatment in rats. Hence, the histopathological investigation data reinforced the present biochemical outcomes.

The present research assessed neurological, sensorimotor, and memory functions at different time intervals starting from 1st day. Results showed that permanent 2-VO produced CH that caused a significant increase in their neurological scores indicating deficits in balance, gait, sensory functions, and reflexes, and a decrease in motor activity measured over
14 days duration. Findings from previous studies suggest that methylxanthines such as pentoxifylline (Dong et al. 2018; Eun et al. 2000) and caffeine (Rehni et al. 2007; Bona et al. 1995) can defend against hypoxia-ischemia conditions (Cova et al. 2019; Kumral et al. 2010) via mechanisms linked to phosphodiesterase-4 and adenosine receptors inhibition. In healthy elderly humans, flavanol-rich cocoa improved CBF in the middle cerebral artery, which substantiates that cocoa and its constituents may benefit cerebral ischemia (Sorond et al. 2008). Parallel to these findings (Camandola et al. 2019; Onatibia-Astibia et al., 2017), current results indicated that theobromine, when given orally for two weeks, improved the neurological and sensorimotor abilities in rats marked by a decrease in NDS and upsurge in fall-off latency respectively. In the present study, theobromine significantly decreased the TL (day 13). It enhanced the IR (day 13) and DI (day 14), which indicated improved spatial and recognition-type working memory in rats against CH. Precise coordination between different brain structures such as the cortex, thalamus, hippocampus, amygdala, limbic system, medulla, and cerebellum regulates spatial orientation, awareness, recognition-type memory, balance, motor coordination, reflexes, sensory functions, and gait (Ackerman 1992). The biochemical analysis in the entire brain disclosed a decline in oxidative stress, inflammation, and cell death biomarkers and an increase in neurotransmitters, which aptly substantiated the behavioral findings in the present study. Commensurate with earlier findings, hippocampus and cortical tissues are the most vulnerable regions in BCCAO induced CH model supported by the H&E staining technique in this study. Besides, we observed a dose-dependent amelioration of biochemical outcomes and neurobehavioral functions in animals by theobromine.

**Conclusions**

In this study, the anti-oxidative and anti-inflammatory effects of theobromine abolished CH-associated cell death pathways with a concomitant upsurge in neurotransmitters (GABA) and decline in acetylcholinesterase activity, thereby resulting in improvement of neurological, sensorimotor, and memory functions in rats in the CH model. These are novel research findings indicating that theobromine can be used against hypoperfusion-associated cerebral disorders such as Alzheimer’s disease, vascular cognitive impairment, and dementia.

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**Authors’ contributions Javeed Ahmad Bhat:** Investigation, Data curation, Analysis. **Manish Kumar:** Conceptualization, design of methodology, supervision, validation, project administration, writing - original draft, writing - review & editing.

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**Availability of data and material** The data used to support the findings of this study can be made available upon a reasonable request to the corresponding author.

**Declarations**

**Conflict of interest** None declared.

**Ethics approval** The research protocol was approved by Institutional Animal Ethics Committee vide approval reference no. SSP/IAEC/2019/009 on date: 17-11-2019. Animals were housed within the institutional establishment (Animal House Facility) registered under CPCSEA (Regd. 1616/PO/Re/S/12/CPCSEA). All the animal tests were performed following the guiding principles of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment and Forests (Animal Welfare), Government of India (GOI), New Delhi.

**Consent to participate** Not applicable.

**Consent for publication** It is affirmed that all the authors have seen and agreed to the submission and publication of the research article and their inclusion of name(s) as co-author(s).

**References**

Ackerman S (1992) Discovering the brain. National Academies Press (US), Washington (DC)

Asomugha CO, Linn DM, Linn CL (2010) ACh receptors link two signaling pathways to neuroprotection against glutamate-induced excitotoxicity in isolated RGCs. J Neurochem 112:214–226. https://doi.org/10.1111/j.1471-4159.2009.06447.x

Ayala A, Muñoz MF, Argüelles S (2014) Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev 2014:360438. https://doi.org/10.1155/2014/360438

Bacigaluppi M, Comi G, Hermann DM (2010) Animal models of ischemic stroke. Part two: Modeling cerebral ischemia. Open Neurol J 4:34–38. https://doi.org/10.2174/1874205X01004020034

Bell RD, Zlokovic BV (2009) Neurovascular mechanisms and blood-brain barrier disorder in Alzheimer’s disease. Acta Neuropathol 118:103–113. https://doi.org/10.1007/s00401-009-0522-3

Bhuvanendran S, Bakar SNS, Kumari Y, Othman I, Shaikh MF, Hassan Z (2019) Embelin improves the spatial memory and hippocampal long-term potentiation in a rat model of chronic cerebral hypoperfusion. Sci Rep 9:1–11. https://doi.org/10.1038/s41598-019-50954-y

Bona E, Aden U, Fredholm BB, Hagberg H (1995) The effect of long term caffeine treatment on hypoxic-ischemic brain damage in the neonate. Pediat Res 38:312–318. https://doi.org/10.1203/00006450-199509000-00007

Camandola S, Plick N, Mattson MP (2019) Impact of coffee and cacao purine metabolites on neuroplasticity and neurodegenerative disease. Neurochem Res 44:214–227. https://doi.org/10.1007/s11064-018-2492-0
Camps-Bossacoma M, Pérez-Cano FJ, Franch A, Castell M (2018) Theobromine is responsible for the effects of cocoa on the anti-body immune status of rats. J Nutr 148:464–471. https://doi.org/10.1093/jn/nmx056

Chen C, Zhou X, He J, Xie Z, Xia S, Lu G (2019) The roles of GABA in ischemia-reperfusion injury in the central nervous system and peripheral organs. Oxid Med Cell Longev. https://doi.org/10.1155/2019/4028394

Chen H, Yoshioka H, Kim GS, Jung JE, Okami N, Sakata H, Maier CM, Narasimhan P, Goeders CE, Chan PH (2011) Oxidative stress in ischemic brain damage: Mechanisms of cell death and potential molecular targets for neuroprotection. Antioxid Redox Signal 14:1505–1517. https://doi.org/10.1089/ars.2010.3576

Chen JF, Huang Z, Ma J, Zhu J, Moratalla R, Standaert D, Moskowitz MA, Fink JS, Schwarzschild MA (1999) A(2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. J Neurosci 19:9192–9200. https://doi.org/10.1523/JNEUROSCI.19-21-09192.1999

Ciacciarelli A, Sette G, Giubilei F, Orzi F (2020) Chronic cerebral hyperperfusion: An undefined, relevant entity. J Clin Neurosci 73:8–12. https://doi.org/10.1016/j.jocn.2020.01.026

Claiborne A (1985) Catalase activity. In: Greenland RA (ed) CRC Handbook of Methods for Oxygen Radical Research. CRC Press, Boca Raton, pp 283–284

Coppi E, Dettori I, Cherchi F, Bulli I, Venturini M, Lana D, Giovannini MG, Pedata F, Pugliese AM (2020) A2B adenosine receptors: When outsiders may become an attractive target to treat brain ischemia or demyelination. Int J Mol Sci 21:3697. https://doi.org/10.3390/ijms21249697

Cova I, Leta V, Mariani C, Panton L, Pomati S (2019) Exploring cocoa properties: is theobromine a cognitive modulator? Psychopharmacology 236:561–572. https://doi.org/10.1007/s00213-019-5172-0

Daulatzaei MA (2017) Cerebral hyperperfusion and glucose hypometabolism: Key pathophysiological modifiers promote neurodegeneration, cognitive impairment, and Alzheimer’s disease. J Neurosci Res 95:943–972. https://doi.org/10.1002/jnr.23777

Dong S, Maniar S, Manole MD, Sun D (2018) Cerebral Hyperperfusion and Other Shared Brain Pathologies in Ischemic Stroke and Alzheimer’s Disease. Transl Stroke Res 9:238–250. https://doi.org/10.1007/s12975-017-0570-2

Duncan JW, Younes ST, Hildebrandt E, Ryan MJ, Granger JP, Drummond HA (2020) Tumor necrosis factor-α impairs cerebral blood flow in pregnant rats: Role of vascular β-epithelial Na⁺ channel. Am J Physiol - Heart Circ Physiol 318:H1018–H1027. https://doi.org/10.1152/ajpheart.00748.2019

Duncombe J, Kitamura A, Hase Y, Ihara M, Kalaria RN, Horsburgh K (2017) Chronic cerebral hyperperfusion: A key mechanism leading to vascular cognitive impairment and dementia. Closing the translational gap between rodent models and human vascular cognitive impairment and dementia. Clin Sci 131:2451–2468. https://doi.org/10.1042/CS20160727

Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–77. https://doi.org/10.1016/0003-9861(59)90090-6

Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95. https://doi.org/10.1016/0006-2952(61)90145-9

Ennaceur A (1998) Effects of lesions of the Substantia Innominata/ Ventral Pallidum, Globus Pallidus and Medial Septum on rat’s performance in object-recognition and radial-maze tasks: Physostigmine and amphetamine treatments. Pharmacol Res 38:251–263. https://doi.org/10.1016/phrs.1998.0361

Ennaceur A, Delacour J (1988) A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav Brain Res 31:47–59. https://doi.org/10.1016/0166-4328(88)90157-X

Eun BL, Liu XH, Barks JD (2000) Pentoxifylline attenuates hypoxic-ischemic brain injury in immature rats. Pediatr Res 47:73–78. https://doi.org/10.1203/00006450-20001000-00014

Ferreira-Vieira TH, Guimaraes IM, Silva FR, Ribeiro FM (2016) Alzheimer’s disease: Targeting the cholinergic system. Curr Neuropharmacol 14:101–115. https://doi.org/10.2174/1570199X15666150716165726

Fogal B, Hewett SJ (2008) Interleukin-1β: A bridge between inflammation and excitotoxicity? J Neurochem 106:1–23. https://doi.org/10.1111/j.1471-4159.2008.05315.x

Franco R, Olatibia-Astibia A, Martinez-Pinilla E (2013) Health benefits of methylxanthines in cacao and chocolate. Nutrients 5:4159–4173. https://doi.org/10.3390/nu5104159

Gnatek Y, Zimmerman G, Goll Y, Najami N, Soreq H, Friedman A (2012) Acetylcholinesterase loosens the brain’s cholinergic anti-inflammatory response and promotes epileptogenesis. Front Mol Neurosci 5:66. https://doi.org/10.3389/fnmol.2012.00066

Gomes CV, Kaster MP, Tome AR, Agostinho PM, Cunha RA (2011) Adenosine receptors and brain diseases: Neuroprotection and neurodegeneration. Biochim Biophys Acta 1808:1380–1399. https://doi.org/10.1016/j.bbamer.2010.12.001

Horecker BL, Kornberg A (1948) The extinction coefficients of the reduced band of pyridine nucleotides. J Biol Chem 175:385–390. https://doi.org/10.1016/s0021-9258(18)57268-9

Hossmann KA (2008) Cerebral ischemia: Models, methods and outcomes. Neuropharmacology 55:257–270. https://doi.org/10.1016/j.neuropharm.2007.12.004

Islam R, Matsuaki K, Sumiyoshi E, Hossain ME, Hashimoto M, Katakura M, Sugimoto N, Shido O (2019) Theobromine improves working memory by activating the CaMKII/CREB/BDNF pathway in rats. Nutrients 11:888. https://doi.org/10.3390/nu11040888

Itoh J, Nabeshima T, Kamayama T (1990) Utility of an elevated plus-maze for the evaluation of memory in mice: effects of nootropics, scopolamine and electroconvulsive shock. Psychopharmacology 101:27–33. https://doi.org/10.1007/BF02253713

Jacobson KA, Gao ZG, Matrican P, Eddy MT,Carlsson J (2020) Adenosine A2A receptor antagonists: from caffeine to selective non-xanthines. Br J Pharmacol. https://doi.org/10.1111/bph.15103

Jang MH, Mukherjee S, Choi MJ, Kang NH, Pham HG, Yun JW (2020) Theobromine alleviates diet-induced obesity in mice via phosphodiesterase-4 inhibition. Eur J Nutr 59:3503–3516. https://doi.org/10.1007/s00394-020-02184-6

Jin X, Wang RH, Wang H, Long CL, Wang H (2015) Brain protection against ischemic stroke using choline as a new molecular bypass treatment. Acta Pharmacol Sin 36:1416–1425. https://doi.org/10.1038/aps.2015.104

Ju Hwang C, Choi D-Y, Park MH, Hong JT (2019) NF-κB as a Key Mediator of Brain Inflammation in Alzheimer’s Disease. CNS Neurosci - Drug Targets 18:3–10. https://doi.org/10.2174/1871253616661708071830001

Katz DL, Doughty K, Ali A (2011) Cocoa and chocolate in human health and disease. Antioxid Redox Signal 15:2779–2811. https://doi.org/10.1089/ars.2010.3697

Kumral A, Yesilirmak DC, Akyan S, Genc S, Tugyan K, Cilaker S, Akhisaroglu M, Aksu L, Sutcuoglu S, Yilmaz O, Duman N, Ozkan H (2010) Protective effects of methylxanthines on hypoxia-induced apoptotic neurodegeneration and long-term cognitive functions in the developing rat brain. Neonatology 98:128–136. https://doi.org/10.1159/000278840

Lee RHC, Lee MHH, Wu CYC, Couto E, Silva A, Possoit HE, Hsieh TH, Minagar A, Lin HW (2018) Cerebral ischemia and neuroregeneration. Neural Regen Res 13:373. https://doi.org/10.4103/1673-5374.228711
Levy JH, Bailey JM (2000) Phosphodiesterase inhibitors: the inro-
tropes of choice for the new millennium? J Cardiothorac Vasc Anesth 14:365–366. https://doi.org/10.1053/jcana.2000.7919
Li M, Meng N, Guo X, Niu X, Zhao Z, Wang W, Xie X, Lv P (2020)
Di-3-n-Butylphthalide promotes remyelination and suppresses
inflammation by regulating AMPK/SIRT1 and STAT3/NF-κB
signaling in chronic cerebral hypoperfusion. Front Aging Neuro-
sci 12:137. https://doi.org/10.3389/fagi.2020.000137
Liu H, Zhang J (2012) Cerebral hypoperfusion and cognitive impair-
ment: The pathogenic role of vascular oxidative stress. Int J Neu-
rosci 122:494–499. https://doi.org/10.1111/j.1940-1604.2012.06865.
43
Liu T, Zhang L, Joo D, Sun SC (2017) NF-κB signaling in inflam-
mation. Signal Transduct Target Ther 2:17023. https://doi.
org/10.1038/srep14474
Liu T, Zhang L, Joo D, Sun SC (2017) NF-κB signaling in inflam-
mation. Signal Transduct Target Ther 2:17023. https://doi.
org/10.1038/srep14474
Okahawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in
animal tissues by thiobarbituric acid reaction. Anal Biochem
95:351–358. https://doi.org/10.1016/0003-2697(79)90738-3
Oñatiabia-Astibia A, Franco R, Martinez-Pinilla E (2017) Health ben-
efits of methylxanthines in neurodegenerative diseases. Mol Nutr
Food Res 61:1600670. https://doi.org/10.1002/mnfr.201600670
Oymada N, Sone M, Miyashita K, Park K, Taura D, Inuzuka M,
Sonoyama T, Tsujimoto H, Fukunaga Y, Tamura N, Itoh H,
Nakao K (2008) The role of mineralocorticoid receptor expres-
sion in brain remodeling after cerebral ischemia. Endocrinology
149:3764–3777. https://doi.org/10.1210/en.2007-1770
Park JH, Hong JH, Lee SW, Ji HD, Jung JA, Yoon KW, Lee JI, Won
KS, Song B-II, Kim HW (2019) The effect of chronic cerebral
hypoperfusion on the pathology of Alzheimer’s disease: A posi-
tion emission tomography study in rats. Sci Rep 9:14102. https://
doi.org/10.1038/s41598-019-05681-4
Parle M, Dhirang D (2003) Ascorbic acid: A promising memory-
enhancer in mice. J Pharmocol Sci 93:129–135. https://doi.
org/10.1254/jphs.93.129
Pedata F, Pugliese AM, Coppi E, Dettori I, Maruola G, Celli L, Mel-
ani A (2014) Adenosine A2A receptors modulate acute injury
and neuroinflammation in brain ischemia. Mediators Inflamm
2014:805198. https://doi.org/10.1154/2014.805198
Rajesh V, Riju T, Venkatesh S, Babu G (2017) Memory enhanc-
ing activity of Lawsonia inermis Linn. leaves against scopol-
amine induced memory impairment in Swiss albino mice. Orient
Pharm Exp Med 17:127–142. https://doi.org/10.1007/
s13596-017-0268-8
Rehni AK, Shri R, Singh M (2007) Remote ischaemic preconditioning
and prevention of cerebral injury. Indian J Exp Biol 45:247–252
Resende RR, Adhikari AA (2009) Cholinergic receptor pathways in-
volved in apoptosis, cell proliferation and neuronal differentiation.
Cell Commum Signal 7:20. https://doi.org/10.1186/1478-811X-7-20
Saggu R, Schumacher T, Gerich F, Rakers C, Tai K, Delekate A,
Petzold GC (2016) Astroglial NF-κB contributes to white mat-
er damage and cognitive impairment in a mouse model of vas-
cular dementia. Acta Neuropathol Commun 4:76. https://doi.
org/10.1186/s40478-016-0350-3
Salihovic M, Husieinovic S, Siptovic-Halilovic S, Osmanovic A,
Dedic A, Asimovic Z, Zavrsknik D (2014) DFT study and biologi-
cal activity of some methylxanthines. Bull Chem Technol Bosnia
Herzeg 42:31–36
Sastry KVH, Moudgal RP, Mohan J, Tyagi JS, Rao GS (2002) Spec-
trophotometric determination of serum nitrite and nitrate by
copper-cadmium alloy. Anal Biochem 306:79–82. https://doi.
org/10.1016/s0003-2697(01)00507-6
Saxena AK, Abdul-Majeed SS, Gurtu S, Mohamed WMY (2015)
Investigation of redox status in chronic cerebral hypoperfusion-
induced neurodegeneration in rats. Appl Transl Genomics 5:30–
32. https://doi.org/10.1016/j.ajt.2015.05.004
Shively CA, White DM, Blauh JL, Tarka SM (1984) Dominant lethal
testing of theobromine in rats. Toxicol Lett 20:325–329. https://
doi.org/10.1016/0378-4274(84)90016-x
Shojai-Zarghani S, Yari Khorosrshahia A, Rafraf M (2021) Oncop-
reventive effects of theanine and theobromine on dimethylhy-
drazine-induced colon cancer model. Biomed Pharmacother
134:111140. https://doi.org/10.1016/j.biopharm.2020.111140
Soria G, Tudela R, Marquez-Martín A, Camon L, Batalle D, Munoz-
Moreno E, Eixarch E, Puig J, Pedraza S, Vila E, Prats-Galino
A, Planas AM (2013) The ins and outs of the BCCAo model for
chronic hypoperfusion: A multimodal and longitudinal MRI
approach. PLoS ONE 8:e74631. https://doi.org/10.1371.journal.
pone.0074631
Sorond FA, Lipsitz LA, Hollenberg NK, Fisher ND (2008) Cerebral
blood flow response to flavanol-rich cocoa in healthy elderly
Wang XX, Zhang B, Xia R, Jia QY (2020) Inflammation, apoptosis and autophagy as critical players in vascular dementia. Eur Rev Med Pharmacol Sci 24:9601–9614. https://doi.org/10.26355/eurrev_202009_23048

Warner DS, Sheng H, Batinić-Haberle I (2004) Oxidants, antioxidants and the ischemic brain. J Exp Biol 207:3221–3231. https://doi.org/10.1242/jeb.01022

Wei D, Wu, Liu J, Zhang X, Guan X, Gao L, Xu Z (2021) Theobromine ameliorates nonalcoholic fatty liver disease by regulating hepatic lipid metabolism via mTOR signaling pathway in vivo and in vitro. Can J Physiol Pharmacol 99:775–785. https://doi.org/10.1139/cjpp-2020-0259

Winterbourn CC, Hawkins RE, Brian M, Carrell RW (1975) The estimation of red cell superoxide dismutase activity. J Lab Clin Med 85:337–341. https://doi.org/10.5555/uri:pii:0022214375904394

Woodcock T, Morganti-Kossmann MC (2013) The role of markers of inflammation in traumatic brain injury. Front Neurol 4:18. https://doi.org/10.3389/fneur.2013.00018

Yanpallewar S, Rai S, Kumar M, Chauhan S, Acharya SB (2005) Neuroprotective effect of Azadirachta indica on cerebral post-ischemic reperfusion and hypoperfusion in rats. Life Sci 76:1325–1338. https://doi.org/10.1016/j.lfs.2004.06.029

Zhao Y, Gong CX (2015) From chronic cerebral hypoperfusion to Alzheimer-like brain pathology and neurodegeneration. Cell Mol Neurobiol 35:101–110. https://doi.org/10.1007/s10571-014-0127-9

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