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Neuroprotective Effects of Theobromine in Permanent Bilateral Common Carotid Artery Occlusion Rat Model of Cerebral Hypoperfusion

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Research Article

Keywords: Theobromine, Cerebral hypoperfusion, GABA, Inflammation, Memory, Oxidative stress

Posted Date: February 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1163958/v1

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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.

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 hypocholesterolaemia agents (Wei et al., 2021; Jang et al., 2020; Franco et al., 2013; Martinez-Pinilla et al., 2015; Camps-Bossacoma et al., 2018; Katz et al., 2011). 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 indicated that theobromine inhibits amyloid-β_{40-42} deposition (Sugimoto et al., 2019; Cova et al., 2019), mTOR (mammalian target of rapamycin) and nuclear factor-kappa B (NF-κB) 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 vagosympathetic 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 (Shojaei-Zarghani et al., 2021; Jang et al., 2020; Mendiola-Precoma 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-homogenate 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 $\varepsilon = 1.36 \times 10^4$ /M/cm. 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 O_2 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 H_2O_2 decomposed per min per mg protein of brain) was computed using ε = 43.6 /M/cm at λ_{max} = 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 μM per mg protein. The lactate dehydrogenase activity (μmol NADH oxidized/min/mg protein) was scrutinized by the procedure of Horecker and Kornberg (1948) using $\varepsilon = 6220\ M^{-1}cm^{-1}$ at λ_{max} = 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 $\varepsilon = 1.36 \times 10^4$ /M/cm at λ_{max} = 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-1β, 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 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 sections (5.0 μm) were carved out using a rotary microtome and applied with hematoxylin and eosin (H&E) dye. Permanent slides were scrutinized using light microscopy at × 40 magnifications.

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. Box and whisker plots (Tukey) display mean (+), median (bold horizontal line), quartiles (box), and total range (whisker). NDS and rotarod 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. 2A). 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. 3A). 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. 3C). 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 DI 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 (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 brain antioxidants ($p < 0.05$) in reference to theobromine dose 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. 5A) 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 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. 6A), 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 in rats exposed to CH. Furthermore, CH triggered a substantial rise ($p < 0.001$) in the brain NF-κB levels with respect to sham-operation. However, theobromine (dose 100 mg/kg) decreased ($p < 0.001$) [$F_{(4,20)} = 17.40$, $p < 0.001$] the NF-κB level in rats rendered to CH when compared to rats that received CH and vehicle treatments only (Fig. 6D). These results showed that post-treatment with theobromine for 14 days reduced levels of pro-inflammatory cytokines in the brain of rats that received CH on day 1.

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.

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 (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. Hence, it can be inferred that oral administration of theobromine attenuated CH triggered neurodegeneration in the brain (Fig. 7).

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 A_2, 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 A_2, 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-*κ*B 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-1*β*, 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-1*β*, 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-*κ*B, 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-*κ*B 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-*κ*B levels that might have contributed to lowering inflammatory cytokines (TNF-*α*, IL-1*β*, 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; Gnatek 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 depreciation can be a factor for overt glutamatergic excitatory transmission (Asomugha 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 (Bacigaluppi 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.

Conclusion

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.

Declarations

Acknowledgments

The authors appreciate the organization of Swift School of Pharmacy, Ghaggar Sarai (Rajpura), for facilitating the required research services.

Funding

The present research received no specific funding.

Conflicts of interest

None declared.

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.

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.

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).

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Figures

Figure 1

Experimental protocol.

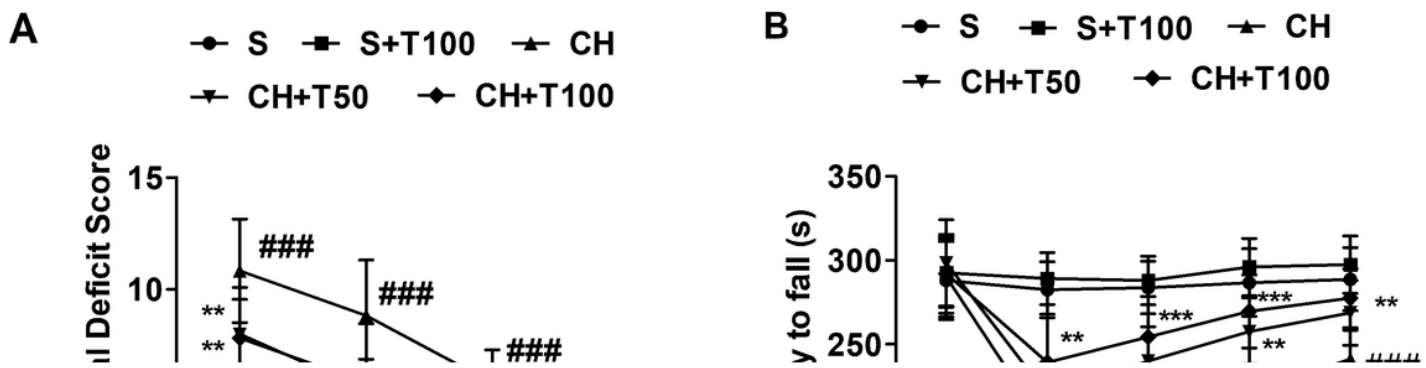

Figure 2

Theobromine improves neurological (A) and sensorimotor performance (B) against cerebral hypoperfusion. (n = 6), ### (p < 0.001) *vs.* Sham-operated (S) group; * (p < 0.05), ** (p < 0.01), *** (p < 0.001) *vs.* CH group

Figure 3

Theobromine improves spatial (A-C) and recognition memory (D) against cerebral hypoperfusion. (n = 6), * (p < 0.05), ** (p < 0.01), *** (p < 0.001)

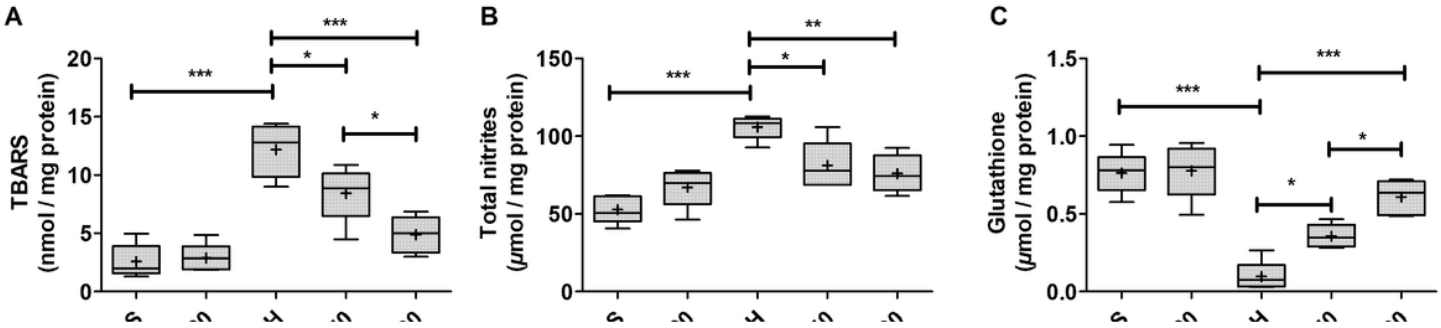

Figure 4

Theobromine decreases lipid peroxidation (A), total nitrites (B) and improved antioxidant levels (C-E) against cerebral hypoperfusion. (n = 5), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$)

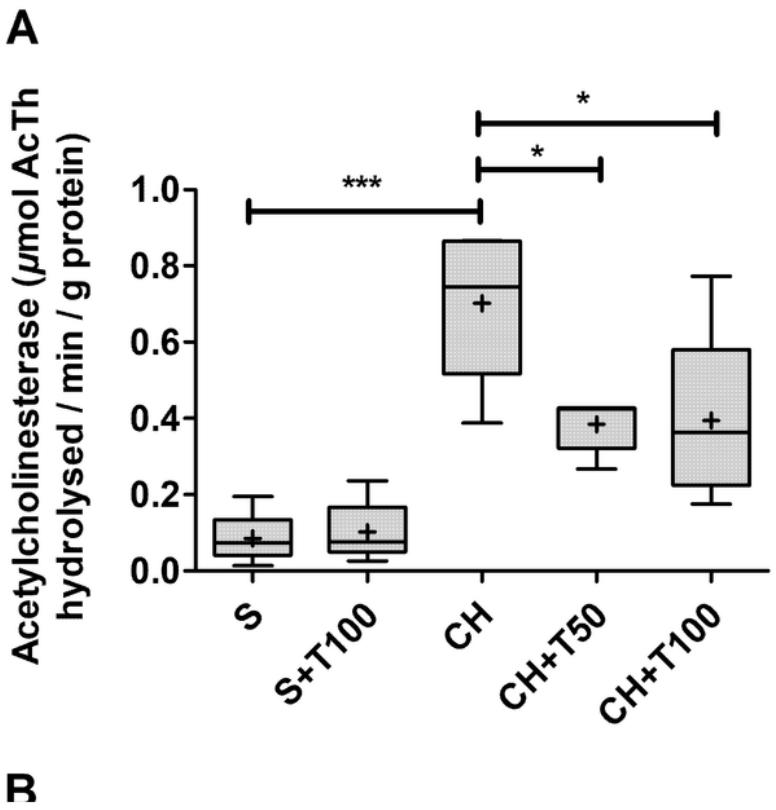

Figure 5

Theobromine ameliorated neurotransmitters acetylcholinesterase (A) and *γ*-aminobutyric acid (B) against cerebral hypoperfusion. (n = 5), * (p < 0.05), ** (p < 0.01), *** (p < 0.001)

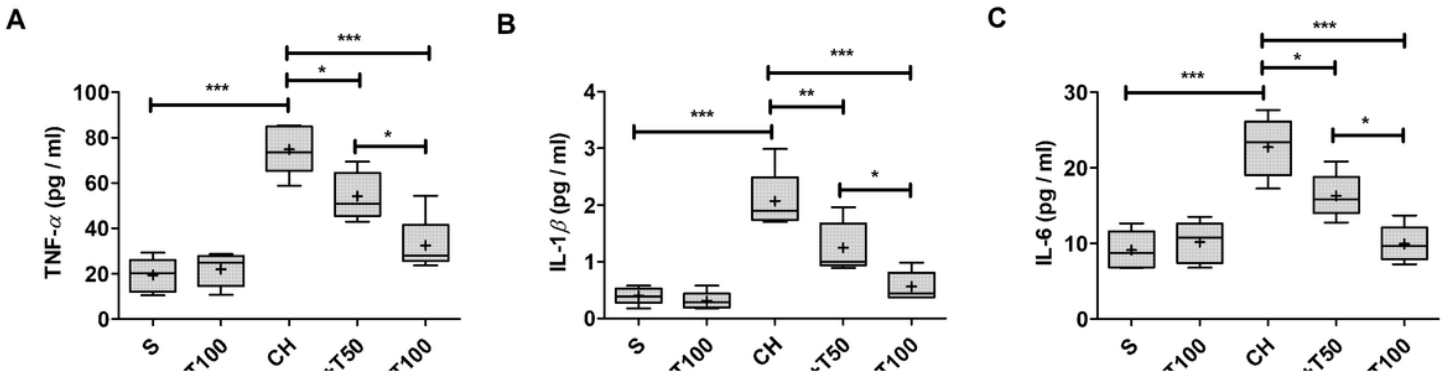

Figure 6

Theobromine attenuated expression of brain inflammatory molecules, transcription factor, and reduced biomarkers of cell death in cerebral hypoperfusion rat model. (n = 5), * (p < 0.05), ** (p < 0.01), *** (p < 0.001)

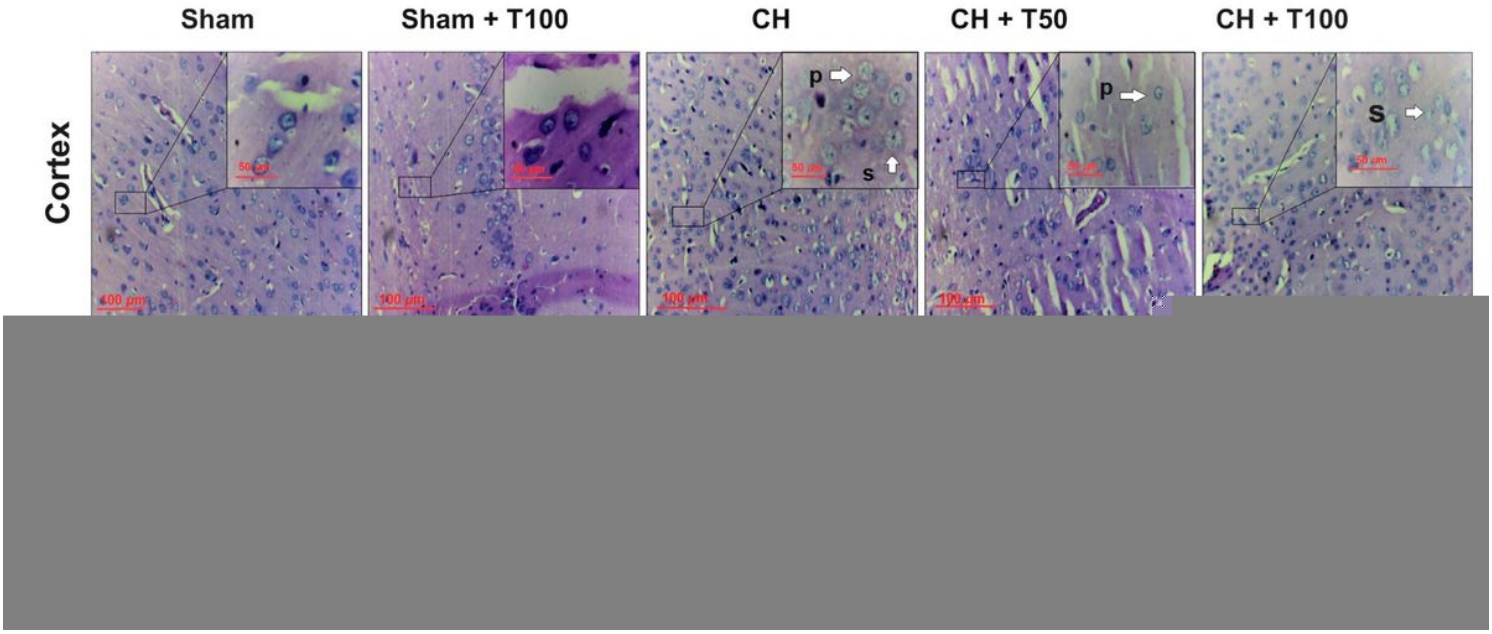

Figure 7

Theobromine attenuated cerebral hypoperfusion triggered neurodegenerative changes in the cortical (frontal lobe pyramidal neurons) and hippocampus (CA1 and CA3) regions (n = 1) (H&E stain, ×40, scale 50 μm and 100 μm). Pyknosis (p), bulging of plasma membrane (b), and swelling (s) were observed.