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Role of ambrisentan (selective endothelin-A receptor antagonist) on cigarette smoke exposure induced cognitive impairment in *Danio rerio*

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ABSTRACT

Background: Cigarette smoke is exogenous modifiable factors to changes the neurovascular complication. The chronic exposure of cigarette smoke enhances neurocognitive dysfunction.
Aims: The present study is focused on evaluating the role of ambrisentan (selective endothelin-A receptor antagonist) on cigarette smoke-induced cognitive impairment in *Danio rerio*.
Main methods: The cognitive dysfunction was developed by cigarette smoke exposure (CSE; 10 min in 25 ml of CSE per day) for five days. The selective endothelin-A receptor antagonist *i.e.*, ambrisentan (2.5 to 5 mg/kg; *i.p.* for five consecutive days) was used for testing of CSE induced cognitive dysfunction. In addition, treatment of reference drug *i.e.*, donepezil (10 mg/kg; *i.p.* for five consecutive days) was used for this cognitive function study. The cognitive functions were assessed by light and dark chamber; color recognition; partition preference; horizontal compartment; and T-Maze tests. Further, the CSE induced biomarkers changes of the zebrafish brain samples were estimated.
Key findings: The treatment of ambrisentan showed a potential ameliorative effect against the CSE induced cognitive functions along with attenuation of biochemical changes. The results are comparable to donepezil-treated groups.
Significance: Therefore, ambrisentan can be considered for the attenuation of CSE induced impairment neurocognitive functions due to its reduction of free radical scavenging and neuroinflammatory actions as well as regulation of cholinergic neurotransmitter functions.

1. Introduction

Cigarette smoke is modifiable factors to alter the neurovascular functions. Now a day, cigarette smoking in public place is offense due to toxic exposure cause the neurological and respiratory system damage to non-smoker population [1]. A literature survey expressed that world-wide six millions of peoples are dying every day by cigarette smoke exposure. The chronic cigarette smokers are shown lung cancer, ischemic heart disease, atherosclerotic diseases leads to produce the fatal morbidities [2]. And, it also causes the neurocognitive function leads to decreased quality of life. Thus, the strong interconnection between smoking and neurovascular complications like vascular dementia, multiple sclerosis, Alzheimer's disease, and neurodevelopmental damage are observed. Moreover, cigarette smoke triggers the blood-brain barrier and neuroimmune functions [3]. The cigarette smoke has numerous cytotoxic chemicals like carbon monoxide, nitrosamines, aryl hydrocarbon, phenolic and polynuclear aromatic compounds [4]. It is known to cause the neuroendocrine alteration and initiation of the neurodegenerative process [5]. Furthermore, cigarette smoke causes the neuronal death by the accumulation of free radicals, activation of neuroglial cells, sustained ion channels opening and enhancement of neuronal plasticity [6]. Hence, it causes critical complications to neurovascular systems.

Neurovascular systems are controlled by various peptides. Endothelin peptide is major peptides for the neurovascular function and it is generated by activation of endothelin converting enzymes. Endothelin peptide has a diversifiable function in the neurovascular system via regulation of endothelin-A and endothelin-B receptors [7]. There are numerous reports are documented that, the administration of endothelin-A receptor antagonist possess the neurovascular protective

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actions and enhancement of memory functions [8]. The exposure of cigarette smoke also accelerates the neurovascular complication via activation of endothelin receptors. However, the role of selective endothelin-A receptor antagonist in cigarette smoke-induced neurovascular complications and memory dysfunction is not explored yet. Donepezil is an acetylcholine esterase (AChE) inhibitors and it regulates the cholinergic neurotransmitters functions. It is widely used medicine for cognitive disorders. And, it has free radical scavenging, reduction of peroxidation of membrane lipids, anti-inflammation and neurovascular protective actions [9]. Therefore, the present research work designed to explore the role of ambrisentan (selective endothelin-A receptor antagonist) in CSE-induced memory impairment in *Danio rerio*.

2. Materials and methods

2.1. Animals

The eight-month-old male adult *Danio rerio* were apprenticed in this neurocognitive function study. All the zebrafishes were kept in 10 l housing tank. The tank was maintained with an aerator; temperature *i.e.*, 25 ± 2 °C; and light and dark cycles (11 to 12 h) of photoperiod. Animals acclimatized for 2 weeks before the testing of the animal in the newer testing chamber. All the behavioral observations were performed between the 09.00 AM to 13.00 PM to avoid the neuroendocrine associated neurobiological interaction and neurobehavioral abnormalities.

2.2. Preparation of CSE

The production of CSE was carried out as described method of Yoon et al. with minor modification [10]. Briefly, the cigarettes (Gold Flake packet, Mysuru, India) were purchased from the market. Each cigarette piece consists of 12 mg of tar and 1.2 mg of nicotine. Six pieces of cigarettes were fixed in the smoke-pump machine. The cigarette smoke was pumped to 100 ml of pre-warmed distilled water. The saturated CSE solution was passed through a 0.22 μm pore filter (Millipore Corporation, Maharashtra, India) to separate the large particulate materials and bacteria. This solution was used for the cognitive dysfunction in *Danio rerio*.

2.3. Induction of cognitive dysfunction by CSE exposure

The cognitive dysfunction to *Danio rerio* was induced by CSE as described by Folkesson et al. [11] and Massarsky et al. [12]. Briefly, the cognitive dysfunction was induced by exposure of CSE (10 min in 25 ml of CSE per day). This procedure was continued for a further five consecutive days.

2.4. Experimental protocol

In this study, the experiments were designed with five groups of adult male zebrafishes (n = 20). Group-I was employed as a normal control group. The group-II was employed as a CSE challenged group (CSE; 10 min/day; for 5 days). Group-III and IV animals were received ambrisentan (2.5 and 5 mg/kg, *i.p.*) for 5 days. The group-V animals were received donepezil (10 mg/kg, *i.p.*) for 5 days. The behavioral variables were evaluated on the 6th day; whereas, T maze training was performed on the 6th day and the assessment of memory functions were performed on day 7. On the 7th day, animals were euthanized and brain samples collected for biochemical estimation.

2.5. Assessment of cognitive functions

Danio rerio were employed for the evaluation of neurobehavioral functions after the CSE exposure. The behavioral functions were evaluated based on light and dark chamber; color recognition; partition preference; horizontal compartment; and T-Maze tests. All animals allowed acclimatizing the test chamber for 5 min, before performing the behavioral assessments to avoid the biological error. All the animal movement patterns were tracked by a USB camera (12 Megapixel USB Camera, Intex, India).

2.6. Light and dark chamber test

Light and dark chamber assessment is used for the assessment of spatial learning and memory functions. This method was described by Dubey et al. [13]. Briefly, the test chamber *i.e.*, 20 L × 10 W × 23 H (cm) was maintained with 12 cm of water level. And, the chamber was divided (vertically) by two equal (10 cm) portions *i.e.*, one part was covered with black paper (dark chamber); another part was covered with transparent paper with 50 lx natural light (50 lx). The cognitive functions were assessed with the term “number of entry in dark chamber” (NEDC) and “time spent in light chamber” (TSLC). The preference of light chamber was considered as an improvement of memory function.

2.7. Color recognition test

Color recognition was assessed for spatial memory functions. This method was described by Dubey et al. [13] with modification of Rishitha and Muthuraman [14]. Briefly, the chamber *i.e.*, 20 L × 10 W × 10 H (cm) was maintained. The chamber was separated, two equal vertical parts *i.e.*, one part of the chamber was covered with red color transparent glass. And, this side chamber was considered as punishment side. Another half of the chamber was covered with green colored glass. And this side chamber considered as reward-chamber. The 5 cm height was covered with tap water. The cognitive functions were assessed with the term “number of entries into the red chamber (NERC)” and “time spent in green chamber (TSGC)”. The preference of green chamber was considered as an improvement of memory function. If the animal not preferred the green chamber indicates animal has a poor memory.

2.8. Partition preference test

The partition preference of animal was assessed for working and spatial memory function. This test was described by Dubey et al. [13] with a minor modification of Rishitha and Muthuraman [14]. Briefly, the chamber *i.e.*, 20 L × 10 W × 10 H (cm) was maintained. The chamber divided into two equal vertical parts with slit movement and the 1 cm gap was maintained. The 5 cm height was covered with tap water. Right side chamber was considered as home chamber and left side chamber was considered as a target chamber. The learning and memory functions were evaluated with the term “time spent in target chamber (TSTC)” and “percentage entry to target chamber” (% ETC)”. The preference of animals towards target chamber was considered a good memory function. If animal not entered within 60 s to home chamber; the animal was guided for finding target chamber and close the entry path with a vertical glass plate. And, allow to keeping in a target chamber for 20 s. The preference of both chambers was considered a good memory function. Animal remains in home chamber were considered as lack of memory function.

2.9. Horizontal compartment test

The horizontal compartment was used for evaluation of memory function. This method was described by Dubey et al. [13] with modification of Rishitha and Muthuraman [14]. Briefly, the test chamber *i.e.*, 20 L × 10 W × 24 H (cm) was maintained. The chamber was divided three horizontal (7 × 7 × 7 cm) segments with outside marking. The 21 cm height was covered with tap water. One day before the horizontal compartment testing, separate training was given for 120 s time period to acclimatize the test environments. During this session, all fishes allowed swimming in every compartment; if the animal does not

prefer any compartments, animals were guided to swim with a guiding tool. Next day, cognitive function was assessed with the term "time spent in upper segment (TSUS)"; and "time spent in lower segment (TSLS)". The animal was allowed to swim in test chamber and observe the preference of upper and lower segments. The preference for swimming in the upper segment within 15 s was considered as an improvement of memory function. Animal remains in middle and lower chambers were considered as lack of memory function.

2.10. T-maze test

The T-maze apparatus was employed for evaluation of memory functions. This method was described by Colwill et al. [15] with modification of Kim et al. [16]. Briefly, the T maze device was maintained with two short arms *i.e.*, 10 cm length, 6 cm width, and 10 cm height with the different colors. One arm covered with red color transparent paper, and another arm covered with green color transparent paper. Both arms were connected with one long arm chamber. The dimension of long arm was constructed with 20 L × 10 W × 24 H (cm). The terminal part of this long arm was made small home chamber environment. The home chamber environment was made with 5 cm length, 6 cm width and 10 cm height of the non-transparent glass plate. The cognitive function was assessed with the term "transfer latency (TL)" and *percentage of target* (*green*) *chamber preference* (% TCP). The TL was defined as time taken to reach one of the short arm corners from the starting of the home chamber. The shorter time of transfer latency and preference to the green chamber was considered as an improvement of memory functions. Animal remains in long arm and not prefer the red chamber were considered as lack of memory function.

2.11. Estimations of biomarker changes

On the 7th day, all the *Danio rerio* were sacrificed and brain samples were isolated immediately. The samples were stored at −4 °C. Next day, samples were homogenized with phosphate buffer solution and a clear aliquot was collected by centrifugation (1372 *g*-force for 15 min) process. Biochemical estimations like acetylcholinesterase (AChE) activity; lipid peroxidation (LPO); reduced glutathione (GSH); and total protein levels were performed in the aliquot samples.

2.11.1. Estimation of AChE level

The level of AChE activity was guesstimated by spectrophotometric method. This method was narrated by Ellman et al. [17] with modification of Rishitha and Muthuraman [14]. Briefly, 500 µl of the aliquot was added in 0.25 ml (0.001 M) DTNB solution and incubated with room temperature (37 °C) for 10 min. The variation of absorbance was recorded from spectrophotometer (DU 640B Spectrophotometer, Beckman Coulter Inc., CA, USA). The 420 nm wavelength was fixed for the assessment of absorbance changes. The absorbance changes vary with respect to change in acetylthiocholine hydrolyzed by AChE activity. The total AChE activity levels were calculated with standard formula *i.e.*, R = δO.D. × Volume of the assay (3 ml) / ε × mg of protein. R represents rate of enzyme activity *i.e.*, 'n' acetylthiocholine iodide (mol) hydrolyzed/min/mg of protein. The symbol of δO.D. represents a change of absorbance per minute. The symbol epsilon (ε) represents the extinction coefficient *i.e.*, 13,600 per mol per centimeter. Results were noted as acetylthiocholine (µM) hydrolyzed/mg of protein/min.

2.11.2. Estimation of lipid peroxidation level

The lipid peroxidation (LPO) product was guesstimated by a spectrophotometric method. This method was narrated by Ohkawa et al. [18]. with modification of Rishitha and Muthuraman [14]. Briefly, aliquot (0.2 ml) was added in 0.2 ml of sodium dodecyl sulfate (SDS, 8.1% w/v), 1.5. ml of acetic acid (30%; pH 3.5), 1.5 ml of thiobarbituric acid (0.8% w/v). The distilled water was used for the maintaining of test tube volume (4 ml). Then tubes were incubated in warm (95 °C) water bath for 1 h. Thereafter, test tubes were cooled with tap water. Further, distilled water and 15% v/v of n-butanol-pyridine mixture (1:5) was added. After 10 min, tubes were ultracentrifuged for 15 min with relative centrifugal force *i.e.*, 1372 *g* of relative centrifugal force. The color intensity of pink color chromogen was analyzed by spectrophotometer (DU 640B Spectrophotometer, Beckman Coulter Inc., CA, USA). The spectrophotometer was fixed with 535 nm wavelength for assessment of changes of absorbance. Result of TBARS was recorded as nanomole (nM)/mg of protein.

2.11.3. Estimation of GSH level

The GSH level was guesstimated by spectrophotometric method. This method was described by Ellman [19] with modification of Rishitha and Muthuraman [14]. Briefly, 0.5 ml aliquot was added in disodium hydrogen phosphate solution (0.3 M) and freshly prepared DTNB solution (0.001 M) mixture (2:2.5). The color intensity yellow chromogen was noted by using a spectrophotometer (DU 640B Spectrophotometer, Beckman Coulter Inc., CA, USA) at 412 nm wavelength. Results of GSH were recorded as micromole (µM) of GSH/mg of protein.

2.11.4. Estimation of total protein level

The total protein level was guesstimated by a spectrophotometric method as described by Lowry et al [20]. Briefly, 300 µl aliquot was mixed with 700 µl of distilled water. Further, 5 ml of Lowry's reagent was mixed in test samples. The mixture was incubated at 37 °C for 15 min. Then, Folin-Ciocalteu reagent solution (0.5 ml) was mixed slowly and vortexed vigorously for 30 min. The color intensity of purple chromogen was noted by using spectrophotometer (DU 640B, UV-Spectrophotometer, Beckman Coulter Inc., CA, USA). The 750 nm wavelength was fixed for guesstimation of total protein level. Results were recorded as mg of protein per ml of supernatant.

2.12. Statistical analysis

Results were recorded mean plus or minus (±) standard deviation (SD). Data secured from all behavior tests and tissue biomarkers were scrutinized by one-way analysis of variance (ANOVA). Further, Tukey's test was considered for Post-hoc analysis. The Graph pad prism Version-5.0 software was used for statistical analysis of all data. A probability value of $p < 0.05$ was contemplated as statistically significant.

3. Results

3.1. Role of ambrisentan in light and dark chamber test

The CSE produced the significant ($p < 0.05$) increase NEDC and the significant decrease the TSLC values, comparison to a normal control group. It indicates that it produces spatial memory impairment. The treatment of ambrisentan (2.5 and 5 mg/kg; *i.p.*) shown neuroprotective action and amelioration of CSE associated memory impairment with a different dose level of treatments. Moreover, the administration reference compound, *i.e.*, donepezil (10 mg/kg) produced the significant amelioration of CSE induced cognitive impairment. It indicates that the treatment of ambrisentan possesses the neuroprotective and cognitive enhancing effects against CSE induced neurotoxicity. The results were illustrated in Fig. 1(A and B).

3.2. Role of ambrisentan in the color recognition test

The CSE produced the significant ($p < 0.05$) raising of NERC and the significant decrease the TSGC values, comparison to a normal control group. It indicates that it produces spatial memory impairment. The treatment of ambrisentan (2.5 and 5 mg/kg; *i.p.*) shown neuroprotective action and amelioration of CSE associated memory impairment with a different dose level of treatments. Moreover, the

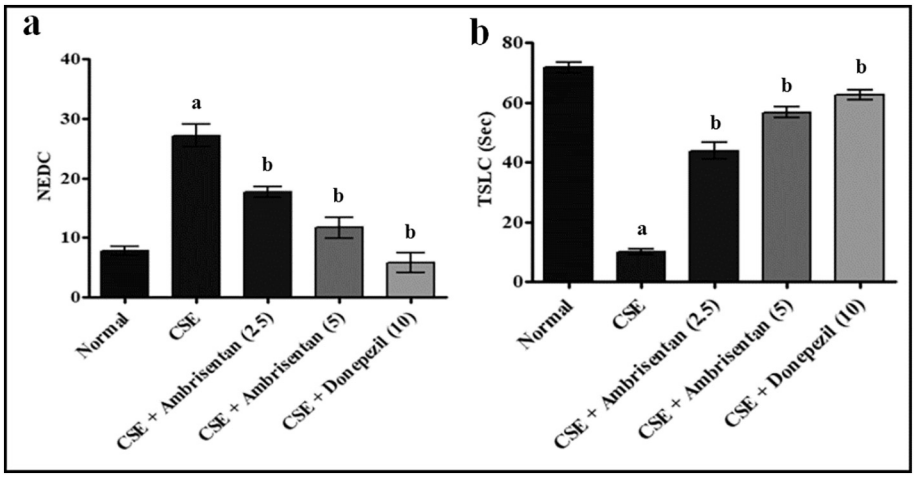

Fig. 1. Role of ambrisentan in CSE induced changes in light and dark chamber test. Digits in parenthesis indicate dose mg/kg. Data were expressed as mean ± SD, n = 6 zebrafish per group. $^{a}p < 0.05$ versus normal group. $^{b}p < 0.05$ versus CSE exposure group. Abbreviation: CSE, cigarette smoke extract; and NEDC, number of entries to the dark chamber; TSLC, time spent in the light chamber.

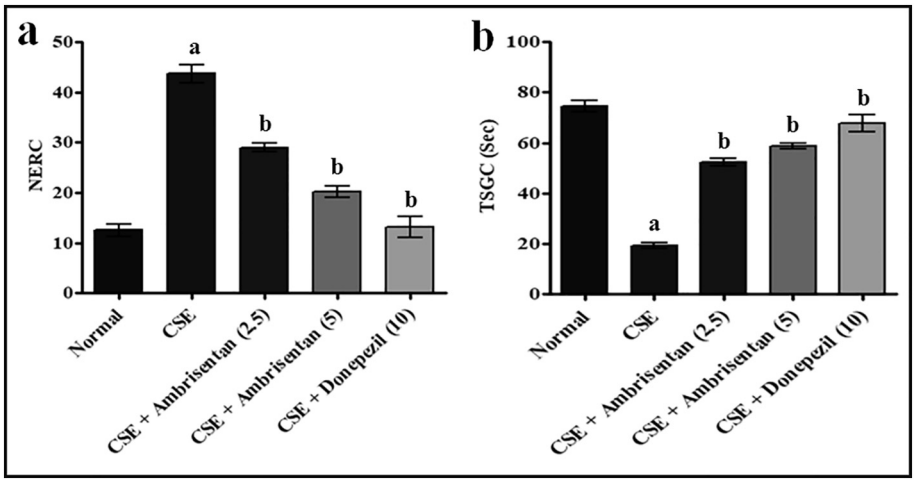

Fig. 2. Role of ambrisentan in CSE induced changes of a color recognition test. Digits in parenthesis indicate dose mg/kg. Data were expressed as mean ± SD, n = 6 zebrafish per group. $^{a}p < 0.05$ versus normal group. $^{b}p < 0.05$ versus CSE exposure group. Abbreviation: CSE, cigarette smoke extract; and NERC, number of the entry to the red chamber; TSGS, time spent in the green chamber. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

administration reference compound, *i.e.*, donepezil (10 mg/kg) produced the significant amelioration of CSE induced cognitive impairment. It indicates that the treatment of ambrisentan possesses the neuroprotective and cognitive enhancing effects against CSE induced neurotoxicity. The results were illustrated in Fig. 2(A and B).

3.3. Role of ambrisentan in a partition preference test

The CSE produced the significant ($p < 0.05$) decrease TSTC and percentage of ETC values comparison to a normal control group. It indicates that it produces spatial memory impairment. The treatment of ambrisentan (2.5 and 5 mg/kg; *i.p.*) shown neuroprotective action and amelioration of CSE associated memory impairment with a different dose level of treatments. Moreover, the administration reference compound, *i.e.*, donepezil (10 mg/kg) produced the significant amelioration of CSE induced cognitive impairment. It indicates that the treatment of ambrisentan possesses the neuroprotective and cognitive enhancing effects against CSE induced neurotoxicity. The results were illustrated in Fig. 3(A and B).

3.4. Role of ambrisentan in horizontal compartment test

The CSE produced the consequential ($p < 0.05$) decrease TSUS and the significant raising TSLS values, comparison to a normal control group. It indicates that it produces cognitive impairment. The treatment of ambrisentan (2.5 and 5 mg/kg; *i.p.*) shown neuroprotective action and amelioration of CSE associated memory impairment with a different dose level of treatments. Moreover, the administration reference compound, *i.e.*, donepezil (10 mg/kg) produced the significant amelioration of CSE induced cognitive impairment. It indicates that the treatment of ambrisentan possesses the neuroprotective and cognitive enhancing effects against CSE induced neurotoxicity. The results were illustrated in Fig. 4(A and B).

3.5. Role of ambrisentan in T-maze tests

The CSE produced the significant ($p < 0.05$) increasing TL values and the significant decrease percentage of TCP values, comparison to a normal control group. It indicates that it produces cognitive impairment. The treatment of ambrisentan (2.5 and 5 mg/kg; *i.p.*) shown neuroprotective action and amelioration of CSE associated memory impairment with a different dose level of treatments. Moreover, the administration reference compound, *i.e.*, donepezil (10 mg/kg) produced the significant amelioration of CSE induced cognitive impairment. It indicates that the treatment of ambrisentan possesses the neuroprotective and cognitive enhancing effects against CSE induced neurotoxicity. The results were illustrated in Fig. 5(A and B).

3.6. Role of ambrisentan in biomarkers changes

The CSE produced the significant ($p < 0.05$) rise of brain AChE activity and LPO; and the significant decrement in the GSH levels, comparison to a normal control group. The treatment of ambrisentan (2.5 and 5 mg/kg; *i.p.*) shown attenuation of CSE induced biochemical changes with a different dose level of treatments. Further, the administration reference compound, *i.e.*, donepezil (10 mg/kg) produced a

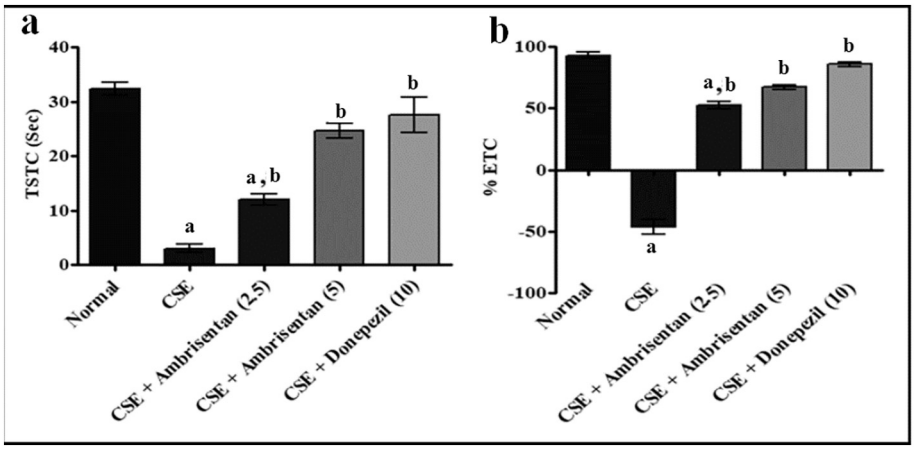

Fig. 3. Role of ambrisentan in CSE induced changes of partition preference test. Digits in parenthesis indicate dose mg/kg. Data were expressed as mean ± SD, n = 6 zebrafish per group. $^{a}p < 0.05$ versus normal group. $^{b}p < 0.05$ versus CSE exposure group. Abbreviation: CSE, cigarette smoke extract; and TSTC, time spent in the target chamber; % ETC, percentage entry to the target chamber.

significant ameliorative effect against CSE induced tissue biomarker changes. It indicates that the treatment of ambrisentan produces the neuroprotective action against CSE induced cognitive dysfunction via free radical scavenging; anti-lipid peroxidative; and reduction of acetylcholinesterase activity. The results appeared in Table 1.

4. Discussion

The data of the present study showed that, treatment of ambrisentan (2.5 and 5 mg/kg; *i.p.*) ameliorates the CSE exposure induced cognitive impairment. The ambrisentan shown cognitive improvement against CSE exposure induced decrease TSLC and increase NEDC values in light and dark chamber (Fig. 1A and B); decreased TSGC values and increased NERC values in color recognition test (Fig. 2A and B) (ii) decreased percentage of ETC and TSTC values in partition preference test (Fig. 3A and B) (iii) decreased TSUS values and increased TSLS values in horizontal compartment test (Fig. 4A and B) (v) increased TL and decreased percentage of TCP values in T-maze tests (Fig. 5A and B). In addition, ambrisentan also produces attenuation of CSE induced biomarkers changes. These results were similar to that of donepezil treatment.

Cigarette smoking plays a chief risk factor in various neurovascular complications [21]. The mechanism of CSE induced neurovascular complications are activates the immune cells like glial cells, dendritic cells, mast cells and so on [22]. In addition, it also alters the cellular metabolic pathways lead to generate abundant free radicals via mitochondrial dysfunction. Further, it alters the cytosolic ion concentration via open the ion channels and enhances the excitatory amino acid receptors functions [23]. These actions intensified the long-term-potentiation via neuronal firings [24]. Subsequently, it alters the nuclear, mitochondria, endoplasmic reticulum and cellular membrane proteins (lipid peroxidation) and minimizes the reduced glutathione level [25]. The exposure of cigarette smoke and their condensate has additional effect *i.e.*, alteration of neurotransmitters including acetylcholine levels via modulation of acetylcholinesterase (AChE) activity [26–27]. The raising AChE activity level by CSE induces memory dysfunction [26]. The exact molecular mechanism of CSE in AChE activity is not clear yet. However, CSE alters the amyloid peptide pathway with the interference of AChE activity leads to alleviating the Alzheimer's disease [28–29].

Recent reports revealed that CSE affects the endothelial cells of various tissues like kidney, liver, heart including brain [26,30]. The damage of brain endothelial cells enhances the progress of cognitive dysfunction due to the accumulation of toxic free bio-radicals and activation of inflammatory cytokines [26]. In addition, the CSE alters the basal and induced peroxidation of neuronal membrane lipids [31]. Further, it enforces the oxidant stress, activation of degradation of membrane lipids with peroxidation and disturbs the endogenous antioxidant defense system [32–34]. Moreover, CSE also alters the glutathione biosynthetic pathways via modulatory action on glutathione peroxidase (GSH-Px) and glutathione-S-transferase (GST) activity [26,34–35]. The direct attack of CSE also enhances the vascular dysfunction by releasing endothelial-derived contractility factors *i.e.*, endothelin peptides [36]. Endothelin peptides activate the endothelin-1 and endothelin-2 receptors. The endothelin-1 receptor has a role in the progress of neurodegeneration via reorganization of blood-brain barrier and neuroimmune function. The administration of selective endothelin 1 receptor antagonist *i.e.*, ambrisentan attenuates the L-methionine

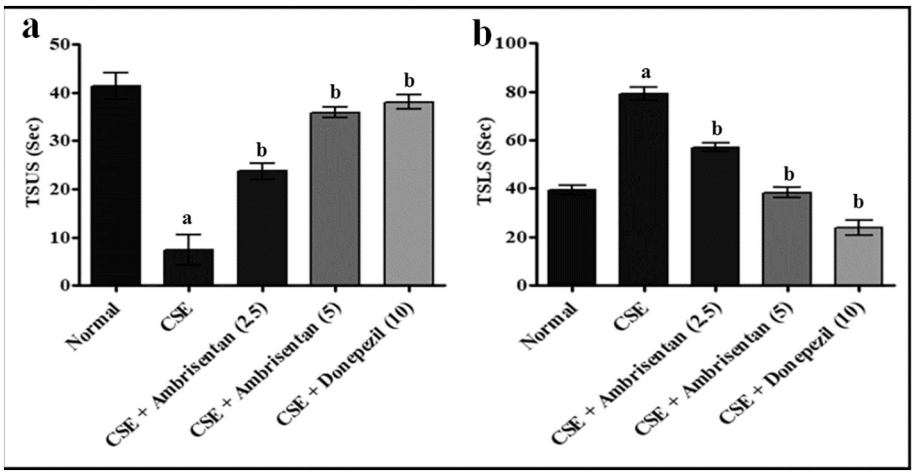

Fig. 4. Role of ambrisentan in CSE induced changes of horizontal compartment test. Digits in parenthesis indicate dose mg/kg. Data were expressed as mean ± SD, n = 6 zebrafish per group. $^{a}p < 0.05$ versus normal group. $^{b}p < 0.05$ versus CSE exposure group. Abbreviation: CSE, cigarette smoke extract; TSUS, time spent in the upper segment; and TSLS, time spent in the lower segment.

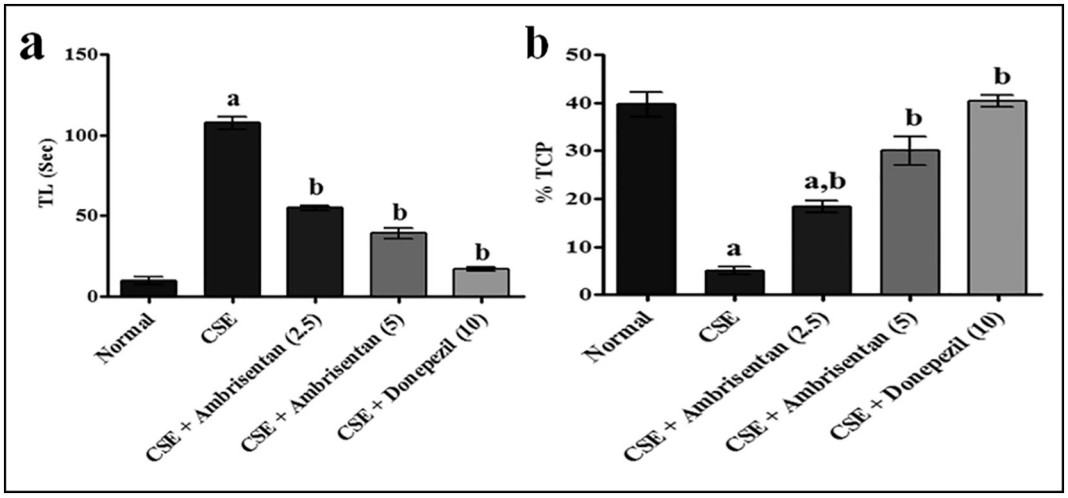

Fig. 5. Role of ambrisentan in CSE induced changes of T-Maze tests. Digits in parenthesis indicate dose mg/kg. Data were expressed as mean ± SD, n = 6 zebrafish per group. [a]$p < 0.05$ versus normal group. [b]$p < 0.05$ versus CSE exposure group. Abbreviation: CSE, cigarette smoke extract; TL, transfer latency; and % TCP, percentage of target (green) chamber preference. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Effect of ambrisentan in CSE induced zebrafish brain biomarkers changes.

Groups	AChE activity (μM/mg of protein/min)	GSH (μM/mg of protein)	LPO (nM/mg of protein)
Normal	19.8 ± 1.12	19.2 ± 1.27	2.4 ± 1.12
CSE	64.6 ± 0.31[a]	4.3 ± 1.09[a]	18.2 ± 1.08[a]
CSE + ambrisentan (2.5)	38.7 ± 1.02[b]	9.8 ± 1.25[b]	9.6 ± 0.34[b]
CSE + ambrisentan (5)	24.2 ± 0.76[b]	14.9 ± 1.39[b]	5.2 ± 0.42[b]
CSE + donepezil (10)	21.2 ± 1.05[b]	17.9 ± 1.23[b]	4.6 ± 0.23[b]

Digits in parenthesis indicate dose mg/kg. Data were expressed as mean ± SD, n = 6 zebrafish per group. Abbreviation: CSE, cigarette smoke extract; AChE, acetylcholine esterase; GSH, reduced glutathione; and LPO, lipid peroxidation.
[a] $p < 0.05$ versus normal group.
[b] $p < 0.05$ versus CSE exposure group.

induced cognitive dysfunctions [37]. The present study reports also shown the administration of ambrisentan ameliorates the CSE induced the neurotoxic effect. Clinically, it's also proved as neuroprotective agent for the neurovascular complication due to its regulatory property of blood-brain barrier and vascular integrity [38]. Furthermore, CSE induced vascular damage has been ameliorated by ambrisentan via regulation of NADPH oxidases [34,37]. The NADPH oxidases are the key factors for generation of univalent oxygen anion (superoxide), cytokines, and acceleration of AChE activity in brain neuronal cells. The treatment of endothelin receptor antagonists like ambrisentan exerts the anti-inflammatory actions by the reduction of leukocyte-endothelium interactions and attenuates the dysfunction of the endothelial cell barrier. Ambrisentan also attenuates the free radicals associated peroxidation of neuronal lipid membranes [37]. In addition, glutathione peroxidase is known to inhibit the formation of lipid peroxidative products *i.e.*, malondialdehyde [39]. Both, activity of NADPH oxidase and membrane lipid peroxidation are induced the cognitive dysfunction via reduction of glutathione and AChE activity levels [37,40].

The nootropic agents *i.e.*, donepezil is also shown significant contribution in the attenuation of free radical generation, activation of endogenous antioxidant defense system like GSH and lipid peroxidation via its pleiotropic actions [41]. In addition, donepezil showed the improvement of memory functions with the regulation of vascular integrity, endothelial functions, and neurovascular complications [37].

Hence, it may be concluded that ambrisentan may be useful medicine for CSE associated neurocognitive dysfunction due to its potential multi-targeted actions on the neurovascular system. However, more extensive studies are required to confirm the effect of ambrisentan usage for multiple neurovascular complications.

Conflict of interest

The authors declare that there is no conflict of interest in the present study.

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Author's contributions

Arunachalam Muthuraman: He has contributed to the design, interpretation of results, statistical analysis of data and critical evaluation of the final manuscript, and critical evaluation of manuscript preparation.

Nafisa Kamus: She has contributed to the collection of data and interpretation of results.

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