Combination therapy of antioxidants prevent cognitive deficits caused by intracerebroventricular administration of streptozotocin in rats
INTRODUCTION

Alzheimer’s disease (AD) is a neurological disorder with increasing prevalence in the Western world. The etiology of sporadic AD, the most common form of this disease, is multifactorial (Hardy, 1997). Pathological oxidation has been proposed to be among the most important factors in its pathogenesis (Mark et al., 1996; Markesbery, 1997; Multhaup, 1997). Brain is especially vulnerable to oxidative stress as a result of its high oxygen consumption as well as high concentrations of easily oxidizable lipids and transition metal ions, which are capable of producing reactive oxygen species (Spector, 1977; Bush, 2000).

These data imply that pretreatment with antioxidants might delay the development of AD. a-Tocopherol (the main form of vitamin E) is considered as a major lipophilic antioxidant and is essential for the normal brain function (Vatassery, 1998). It has been shown to accumulate in the brain and thereby decrease peroxidation of brain lipids in animals (Clement and Bourre, 1997; Meydani et al., 1988). The antioxidative efficiency of a-tocopherol can be considerably increased by a co-supplementation with ascorbate (vitamin C), which is a co-antioxidant for the former (Stocker, 1994). a-Tocopherol also reduces the neurotoxicity of β-amyloid, a major component of senile plaques, in neuronal cell culture (Behl et al., 1992).

There is good evidence that oxidative stress and reactive oxygen species participate in the modulation of learning/memory in many species. Studies report that stress-induced lipid peroxidation affects learning and memory performances in the rat (Abidin et al., 2004) and that a mutant of C. elegans with increased oxidative stress shows impairment of learning-associative behavior (Murakami & Murakami, 2005). Conversely, antioxidants have proved to prevent memory impairments in various experimental conditions, supporting a role for oxidative stress in memory modulation (Haydas et al., 2005; Bickford et al., 2000; Silva et al., 2004).

In recent years, the antioxidative role of selenium (Se) against free radical production has also been extensively studied (Delmas-Beauvieux et al., 1996; Osamah et al., 1997). Se is popularly believed to be an antioxidant. But truly speaking, unlike most other antioxidants it does not function directly as a free radical scavenger. The specific function of selenium is that it is an integral part of the enzyme glutathione peroxidase (GPx). Selenium containing GPx (EC 1.11.11) catalyzes the oxidation of reduced glutathione to its oxidized form, glutathione disulfide (GSSG) and thereby reduces hydrogen peroxides and various lipid hydroperoxides to nontoxic compounds (Winklhofer-Roob et al., 1998).

Another line of evidence supporting the role of oxidative stress on cognition emerges from studies of vitamins with selenium combination. Vitamin E, a peroxyl radical trapping agent, improves cognitive function of patients with temporal lobe radionecrosis (Chan et al., 2004; Mecocci et al., 2004) and may be beneficial in lowering the incidence of atherosclerotic cardiovascular diseases in patients with high risk for oxidative stress (Halliwell, 2000). As from experimental preclinical studies in the rat, it has been shown that age-related motor learning and memory deficits can be reversed with antioxidant-rich (β-carotene, vitamins E and C) diets (Bickford et al., 2000) and that vitamins E and C treatment prevented deficits of learning/memory caused by homocysteine (Reis et al., 2002), however, Se plays an important role in providing protection against oxidative damage being an essential element in the mammalian diet (Burk, 1983).

There are several reports concerning the supplementation of human subjects with selenium compounds for the prevention of selenium deficiency diseases. Sodium selenite is a form of selenium commonly used as a dietary supplement to treat humans with selenium deficiency (Levander and Burk, 1994). Selenium, being a cofactor of the enzyme glutathione peroxidase, participates in the lipoxygenase pathway in organic antioxidant systems together with catalase,
superoxide dismutase, vitamin E, vitamin C and carotenoids (Levander and Burk, 1994; Apostolski et al., 1998; Chan et al., 1998). The antioxidant activity of selenium seems to be exerted synergistically with vitamin E and also with vitamin C (Schwarz and Polizo, 1999; Bartfay et al., 1998; Schwenke and Behr, 1998; Mahfouz et al., 1997). Thus, it is not yet known how vitamin E, vitamin C and selenium might interact with the treatment of diseases in humans and in animal models.

Considering that: (a) ICV-STZ infused might impair memory function, (b) Vitamin E+C and Se have antioxidant actions and so affects oxidative stress, a possible modulator of memory mechanisms and prevented memory impairment in other experimental models; we decided to evaluate the effect of ICV-STZ on spatial memory tasks in rats under the influence of CESe. The working hypothesis is that ICV-STZ will cause impairments in spatial tasks and those vitamins C, E and Se would prevent such effects.

In this study we investigated the effects of a combination of sodium selenite with vitamin C and vitamin E on ICV-STZ-induced cognitive impairments and that leads to several alterations in cellular, molecular and neuro-immunological disorders. Our results suggest that combinations of selenium with vitamins C and E were more effective to prevent cellular, molecular and neuroimmunological integrity in cognitive model.

MATERIAL AND METHODS

Drugs and Chemicals

(-)-Epinephrine, glutathione (oxidized and reduced), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), ascorbic acid (Vitamin-C), α-tocopherol (Vitamin E), sodium selenite, 2,4-dinitrophenylhydrazine (DNPH), guanidine, thiobarbituric acid (TBA) trichloroacetic acid (TCA) were purchased from Sigma-Aldrich Chemical private Ltd., India. Calcium test kit from Span diagnostic Ltd. and all other chemicals were of analytical grade.

Animals

The animals were used in accordance with the Animal Ethics of Jamia Hamdard (Hamdard University), New Delhi. Male Wistar rats (350-500 g) obtained from the Central Animal House of Hamdard University; aged 6-8 months were used. Rats were housed two per cage and maintained on a 12-h dark-light cycle. All rats were provided free access to pellet diet and water ad libitum. Rats were divided into two experimental groups as follows:

Experimental proceedings

Experimental proceedings were done by dividing experiment in two sets:

Experiment I

Experiment I was carried out to evaluate the pretreatment effect of vitamin C, vitamin E and Se (CESe) for two weeks on the contents of non-enzymatic markers thiobarbituric acid reactive substance (TBARS), as marker of lipid peroxidation, reduced glutathione (GSH), protein carbonyl (PC), Calcium, H₂O₂, and anti oxidant enzymes, glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), catalase (CAT), and acetylcholine esterase (AChE).

The rats were divided into four groups of 8 animals each. Group 1: sham (S) operated vehicle treated control; group 2: Lesioned (L), ICV-STZ induced rats; group 3: Lesioned (L) pretreated with vitamins C, E and Se (CESe+L); group 4: Vitamins+ Se (i.e. CESe) pretreated rats.

Experiment II

Experiment II was carried out to evaluate the pretreatment effect of vitamins C, E and Se (CESe) for two weeks on the expression of ChAT immunohistochemical reactivity and histopathological changing in the hippocampus. The rats were divided into four groups as in experiment I.
Induction of Cognitive Impairments (Alzheimer's disease)

After 2 weeks pretreatment with CESe the rats were anesthetized with chloral hydrate (400 mg/kg body weight in distilled water, i.p.) and placed in a stereotaxic frame. The head was positioned in a stereotaxic frame and a midline incision on the head with sterilized scalpel knife was made in the scalp. Burr holes were made in the skull on both the sides over the lateral ventricles using the coordinates of 0.9 mm posterior to bregma; ±1.5 mm lateral to sagittal suture; 4.0 mm beneath the surface of the brain. Streptozotocin (3 mg/kg body weight) was injected ICV bilaterally 5x1 injection/site to one group called lesioned group (L) and vitamins C, E and Se was given to another group for 15 days followed by streptozotocin (3 mg/kg b. wt) called CESe+L group. In the sham and CESe group, physiological saline (0.9%) was injected (5 μl on each site) on the same way as in the L group.

Post-operative care

Recovery of anesthesia took approximately 4—5 h. The rats were kept in a well-ventilated room at 25 ± 3 °C in individual cages till they gained full consciousness and then were housed together in a group of two animals per cage. Bedding of the cage was changed daily and the handling to the animal was carefully so that they could feel relaxed and not feel any pain without any physical trauma due to overhead injury. Food and water were provided every day as usual.

Behavioral Analysis

Effect of CESe on learning and memory

Passive avoidance task

One trial of passive avoidance test was carried out on days 14—15 after the lesioning according to the methods of (Nakahara et al., 1998; Sharma and Gupta, 2003) as described by us previously (Ishrat et al., 2006). Briefly, the apparatus consisted of two compartments, one illuminated and one dark, both equipped with a shock scrambler, grid floor. The two compartments were separated by a guillotine door. In the acquisition trial, each rat was placed in the illuminated compartment. After 60 s of habituation a guillotine door separating the lighted and dark chambers was opened, and the initial latency (IL) to enter the dark chamber was recorded. Rats that had an initial latency time of more than 60 s were excluded from further experiment. When the animal entered the dark compartment, the door was closed and an electric foot shock (50 V, 0.2 mA, 50 Hz) was delivered to the floor grids for 3 s. The rat was removed from the dark chamber 5 s later and placed back into its home cage. Retention latency (RL) time was measured 24 hr later in the same way as in the acquisition trial, but foot shock was not delivered, and the latency time was recorded to a maximum of 600 s.

Morris Water maze

About two weeks after the surgery (i.e. from day 16th), the Morris' water maze (Morris, 1984; Gallagher, 1993) was selected as a test of spatial learning and memory. A circular water tank (132 cm diameter, 60 cm height) was filled to a depth of 40 cm with water at 27±1°C, and the tank was divided virtually into four equal quadrants such as south-west (SW), south-east (SE), north-east (NE) and north-west (NW). The water was made opaque by the addition of white nontoxic water soluble paints. A platform (10 cm x 5 cm) was placed in one of the four maze quadrants and submerged 1-2 cm below the water surface.

The water tank from the pool wall was surrounded with white curtain up to the top of the camera. Four light bulbs (100 W) were attached from the ceiling for sufficient lighting and were focused directly on the pool. A computerized digital tracking system (Columbus Instruments, Videomex-ONE, Ohio, USA) to record escape latencies and path length during each trial was used. Rats were randomly selected from one of the four groups. For each individual rat, the position of the platform was fixed during the entire
experiment. The rats were trained for 4 trials per day for 6 consecutive days to locate and escape onto the platform. A different starting position for each rat was used on each trial. The rats were allowed to swim freely to find the hidden platform within 60 s, and after reaching the platform they were allowed to stay on the platform for 30 s, and then returned to the cage, and to await their next trial. There was a 10 minutes intertrial interval. If a rat failed to locate the platform within 60 s it was then placed on it for the same interval of time. Latency times and distance traveled (path length) by the animals to reach the platform were recorded for each trial.

Probe trial

One day after the last training session, each rat was subjected to a probe trial (60 s), in which there was no platform present. The time spent in the quadrant of the former platform position and the correct annulus crossing, i.e., the number of times animals passed through the circular area that formerly contained the submerged platform during acquisition were taken as measures for spatial memory.

Tissue preparation for biochemical studies

After behavioral study, the animals were sacrificed on day 21 and their brains were taken out to dissect hippocampus quickly and stored at -80 °C until biochemical estimation was carried out. Tissues were homogenized in phosphate buffer (10 mM, 5% w/v, pH 7.0), followed by centrifugation at 10,000 g for 15 min at 4 °C to obtain postmitochondrial supernatant (PMS), used for the estimation of various parameters related to antioxidant enzymes (GPx, GR, GST, SOD, CAT) and non-enzymatic parameters (TBARS, GSH, PC, H₂O₂).

BIOCHEMICAL ESTIMATION

1. Non-enzymatic marker

(i) Determination of Lipid peroxidation

The method of Utely et al. (1967) was modified for the estimation of lipid peroxidation. Briefly, 0.5 ml homogenate (5% w/v) was pipetted in a 15 X 100 mm test tube and incubated at 37 ± 1°C in a metabolic water bath shaker for 60 min., another 0.5 ml was pipetted in a centrifuge tube and placed at 0 °C and marked as 0 hours incubation. After 1 hours of incubation, 0.5 ml of 5% TCA (chilled) and 0.5 ml of 0.67% thiobarbituric acid was added in both samples (i.e. 0 hours and 37 °C). The reaction mixture was centrifuged at 3000 g for 10 min. The supernatant was transferred to another tube and placed in a boiling water bath for 10 min. Thereafter, the tubes were cooled and the absorbance of the color was read at 535 nm. The rate of lipid peroxidation was expressed as nmol TBARS formed/ν g tissue using molar extinction coefficient of 1.5 x 10⁵ M⁻¹ cm⁻¹.

(ii) Determination of hydrogen peroxide (H₂O₂)

Hydrogen peroxide in brain was estimated colorimetrically by the method of Jiang et al. (1992). In brief, 0.1 ml of tissue homogenate (5 % w/v, phosphate buffer 10 mM, pH 7.0) was treated with 0.9 ml of Fox reagent (88 mg butylated hydroxytoluene, 7.6 mg xylenol orange and 9.8 mg ammonium ion sulphate were added to 90 ml of methanol and 10 ml 250 mM sulphuric acid) and incubated at 37°C for 30 min. The color developed was read at 560 nm colorimetrically. The value was expressed as mM/g tissue.

(iii) Determination Reduced Glutathione

Reduced glutathione in brain parts was determined by the method of Jollow et al. (1974) with slight modification. In brief, 0.5 ml postmitochondrial supernatant of hippocampus was precipitated with 0.5 ml of 4% sulfosalicylic acid. The samples were kept at 4 °C for 30 mins. Thereafter, the mixture was centrifuged at 4000 g for 10 mins and 0.1ml supernatant was transferred to another tube. To this 0.2 ml 5,5'-dithiobis-2-nitrobenzoic acid (4 mg/ml in phosphate buffer, 0.1 M, pH 7.4) and 2.7 ml 0.1 M.
phosphate buffer (pH 7.4) was added and vortexed. The yellow color developed was read immediately at 412 nm, the results were expressed as nm of GSH/g tissue using a molar extinction coefficient of 13.6 x 10^3 M^-1 cm^-1.

(iv) Determination of protein carbonyl (PC)
Protein carbonyl content was assayed according to the method of Levine et al. (1990) with slight modification. The tissue homogenate (0.25 ml) was reacted with equal volume of 20% TCA for precipitation. Thereafter, 0.25 ml of 10 mM 2, 4-dinitrophenylhydrazine (DNPH) in 2 M HCl was added and allows standing at room temperature for 1 hr, with vortexing every 10-15 min. Then 0.5 ml of 20% TCA was added and it was centrifuged at 11,000 g for 3-5 min. The supernatant was discarded and pellet was washed 3 times with 1 ml of ethanol-ethyl acetate (1:1) to remove free reagent, allowing the sample to stand for 10 min before centrifugation and the supernatant was discarded each time. Precipitated protein was redissolved in 0.6 ml guanidine hydrochloride solution within 15 min at 37-50 °C and then centrifuged at 11,000 g for 3 min to remove any insoluble material. The carbonyl contents were measured spectrophotometrically at 370 nm. The results were expressed as nmol of DNPH incorporated/mg protein using molar extinction coefficient of 22x10^3 M^-1 cm^-1.

(v) Determination of Calcium level
Calcium level in the blood serum was measured with Span diagnostic Test Kit according to the manufacturer's specifications (Span diagnostic Kit Ltd, Surat, India. Lot No. 6007).

2. Enzymatic biomarkers

(i) Determination of Glutathione peroxidase (GPx)
GPx (EC 1.11.1.9) activity was measured according to the procedure described by Mohandas et al. (1984). The reaction mixture consisted of 1.44 mL phosphate buffer (0.05 M, pH 7.0), 0.1 mL of EDTA (1mM), 0.1 mL of sodium azide (1mM), 0.05 mL of GR (1eu/mL), 0.1 mL of glutathione (1mM), 0.1 mL of NADPH (0.2 mM), 0.01mL of hydrogen peroxide (0.25mM) and 0.1 mL PMS (10% w/v) in a final volume of 2.0 mL. The disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was calculated as nmol NADPH oxidized/min/ mg protein using a molar extinction coefficient of 6.22 x 10^3 M^-1 cm^-1.

(ii) Determination of Glutathione Reductase (GR)
GR (EC 1.6.4.2) activity was assayed by the method of Calberg and Männervik (1975) as modified by Mohandas et al. (1984). The assay mixture contained of 1.65 mL phosphate buffer (0.1 M, pH 7.6), 0.1 mL NADPH (0.1mM), 0.1mL EDTA (0.5mM), 0.05mL oxidized glutathione (1mM) and 0.1 mL PMS (10% w/v) in a total volume of 2.0 mL. The enzyme activity was quantitated at room temperature by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized /min/ mg protein using a molar extinction coefficient of 6.22 x 10^3 M^-1 cm^-1.

(iii) Determination of Glutathione-S-transferase (GST)
GST (EC 2.5.1.18) activity was determined by the method of Habig et al. (1974) as described by Athar et al. (1989). The reaction mixture consisted of 1.47 mL phosphate buffer (0.1M, pH 6.5), 0.2 mL reduced glutathione (1mM), 0.025 mL CDNB (1mM) and 0.30 mL PMS (10% w/v) in a total volume of 2.0 mL. The change in absorbance was recorded at 340 nm and the enzyme activity was calculated as nmol CDNB conjugate formed /min / mg protein using a molar extinction coefficient of 9.6 x 10^3 M^-1 cm^-1.

(iv) Determination of Superoxide dismutase (SOD)
SOD (EC 1.15.1.1) activity was measured by the method of (Steven et al., 2000) spectrophotometrically at 480 nm monitoring
by the auto-oxidation of (-)-epinephrine at pH 10.4 for ~5 min. The reaction mixture contained 0.8 ml of 50 mmol/l glycine buffer, pH 10.4, and 0.2 ml enzyme (PMS). The reaction was initiated by the addition of 0.02 ml of a 20 mg/ml solution of (-)-epinephrine. Due to its poor solubility, (-)-epinephrine (40 mg) was suspended in 2 ml water and was solubilized by adding 2–3 drops of 2N HCl. SOD activity was expressed as nmol of (-)-epinephrine protected from oxidation by the sample compared with the corresponding readings in the blank cuvette. The molar extinction coefficient of 4.02 mmol \cdot l^{-1} \cdot cm^{-1} was used for calculations.

(v) Determination of Catalase (CAT)

CAT (EC 1.11.1.6) activity was assayed by the method of Claiborne (1985). Briefly, the assay mixture contained 1.9 ml phosphate buffer (0.05 M, pH 7.0), 1 ml hydrogen peroxide (0.019 M) and 0.1 ml PMS in a total volume of 3.0 ml. Change in absorbance was recorded at 240 nm. CAT activity was calculated in terms of nmol H_{2}O_{2} consumed/min/mg protein using a molar extinction coefficient of 43.6 x 10^{3} M^{-1} \cdot cm^{-1}.

(vi) Determination of Acetylcholine esterase (AChE) activity

AChE (EC 3.1.1.7) activity was determined by a modified method of Ellman et al. (1961). Briefly, the reaction mixture consisted of 2.6 ml of PB (0.1 M, pH 8.0), 0.1 ml of buffered Ellman’s reagent (DTNB 10 mM, Na_{2}CO_{3} 15 mM) and 0.04 ml of tissue homogenate. The reaction mixture was taken in a cuvette and incubated for 5 min at room temperature and zero OD was adjusted with these mixture. Thereafter, the reaction was initiated by the addition of 0.04 ml of acetylthiocholine iodide as a substrate and optical density at 412 nm was recorded for 3 min after every 1 min interval. AChE activity was expressed as nmol thiocholine formed min^{-1} mg^{-1} protein using a molar extinction coefficient of 1.36 x 10^{4} M^{-1} \cdot cm^{-1}.

(vii) Determination of phospholipase A_{2} (PLA_{2})

The assay was performed as described by Kim et al. (1995). PLA_{2} activity was assayed by measuring the hydrolysis of [1-^{14}C] AA from 1-stearoyl-2-[1-^{14}C] arachidonyl-sn-3-glycerophosphocholine ([1-^{14}C] AA-GPC). The standard incubation medium (100 μL) contained 50 μL Tris-HCl (200 mM, pH 8.3), 20 μL CaCl_{2} (20 mM), and 10 μL radioligand [1-^{14}C] AA-GPC (1:15.66 dilution with Tris-HCl). The reaction was carried out at 37 °C for 30 min and stopped by adding 400 μL modified Dole’s reagent (n-heptane: isopropanol: 0.5M of sulfuric acid: 20:5:1 by v/v). The [^{14}C] AA released was extracted as follows: 1.2 ml heptane and 1 ml water was added and the sample was vortex and centrifuged at 1,000 g for 10 min. Then 1ml of the upper layer (organic phase) was transferred to another test tube, to which ~200 mg of silica gel and 2 ml of n-heptane were added. The samples were vortex and centrifuged again for 10 min, after which 2 ml of supernatant with scintillation fluid was mixed and counted for radioactivity in WALLAC-1410 Liquid β-Scintillation Counter. The activity was expressed as pmol FA formed/min/mg protein.

Protein Estimation

Protein was determined by the method of Lowry et al. (1951). 20 μL of PMS, 0.98 mL IN NaOH, 5.0 mL copper reagent (2% sodium carbonate, 1% copper sulfate and 2.68% of sodium potassium tetrarate in 98:1 ratio). After 10 min 0.5 ml Folin’s reagent was added. The tubes were thoroughly vortex after each addition of reagent. Samples were incubated for 30 min at room temperature. BSA (0.1 mg/ml) was used as standard to calculate the amount of unknown samples. The peptide bonds form a complex with alkaline copper sulphate reagents, which gives a blue color with Folin’s reagent, was measured at 660 nm.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis was performed by using Origin Scientific Graphic and Analysis software,
Experimental Protocol II

(1) Immunohistochemistry for Choline acetyl transferase (ChAT)

After completion of behavioral testing on day 21, rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with 100 ml filtered PBS at ambient temperature, followed by 4% paraformaldehyde in PBS (pH 7.4). The movement of the tail followed by the stiffness should be observed. The whole brain with skull was removed immediately after perfusion and placed overnight in a fixative containing 4% paraformaldehyde in PBS at 4°C. Thereafter, brain was removed from the skull and placed in a fresh 4% paraformaldehyde and allow to post-fix overnight at 4°C, and then brain was transferred to 30% sucrose solution at 4°C for 24-48 hr to cryoprotect prior to sectioning.

Coronal sections (10 µm) of the hippocampus were cut on cryostat and the sections were picked up directly on to gelatin coated slides. The sections were initially incubated in 0.3% H2O2 in methanol for 30 min, washed three times (5 min each) in PBS, and blocked either with a diluted normal blocking serum (Vectastain ABC Elite kit) or PBS containing 0.5% BSA and 0.1% Triton X-100 for 1 h. Hippocampal sections were incubated for 18-36 h with primary antibody (70 µl) for choline acetyltransferase (ChAT), Chemicon, Temecula, CA; dilution-1:250. Sections were washed three times for 5 min in PBS (0.1M, pH 7.0). Thereafter, washing sections were then incubated at room temperature for 1 h in diluted biotinylated secondary antibody (70 µl) (Vectastain Elite ABC kit, Burlingame CA; 1:600). After 3 washing each of 5 min, sections were incubated for 30 min at room temperature with streptavidin–horseradish peroxidase (70 µl) 1:100 dilution. Sections were then washed with PBS three times 5 min each, and then incubated in peroxidase substrate solution, diaminobenzidine (DAB) (50µl) until desired characteristic brown color developed. Sections were rinsed in tap water and counterstain, clear and mounted was done. Finally slides were observed under microscope and photographs were captured at desired intensity. Sections were prepared from four animals from each group.

(2) Histopathological studies

For histopathological study, the brains of all control and experimental groups in cognitive impairment model of Alzheimer’s disease were perfused transcardially as mentioned earlier in Immunohistochemistry for ChAT. The tissues were cut into 5 mm thickness, so that fixative readily penetrates throughout the tissue in short time. The tissue block was passed through a series of solvents as per schedule for dehydration, clearing and paraffin infiltration allowing desired time (1h) at each stage. During the process of embedding in the paraffin, the blocks were oriented so that sections 4-5 mm thick were cut in desired plane of tissues and stained with hematoxylin and eosin (HE).

RESULTS

Body weight

Effect on body weight after ICV-STZ induction and recovery by CESe.

Body weight (BW) was significantly affected ICV-STZ induced animals at the end of experiment and recovered by pretreatment with CESe. Post hoc comparisons revealed that on day 21 from the induction of ICV-STZ rats i.e. L group was observed significantly (p<0.001) decreased body weight as compared with S group (Mean±SEM of L and S was 335.62±6.2 and 502.5±2.5 respectively). However, pretreated L group with CESe i.e. (CESe+L) was significantly (p<0.001) recovered body weight as compared with L group. On the other hand CESe group was shown significantly (p<0.001) increased body weight as compared to S group. (Table 1)
Table I: Effect of CESe on the body weight after ICV-STZ induction in rats

<table>
<thead>
<tr>
<th>Weighed on different days</th>
<th>Sham (S)</th>
<th>Lesion (L)</th>
<th>CESe+L</th>
<th>CESe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randomly selected animals (0 day) (450-500 g)</td>
<td>486.25±6.25</td>
<td>488.12±6.26</td>
<td>491.25±2.95</td>
<td>485.62±6.90</td>
</tr>
<tr>
<td>Dosing period (1-15 days) on the last day of the dosing</td>
<td>501.25±2.26</td>
<td>497.5±1.33</td>
<td>513.12±2.82</td>
<td>515±2.11</td>
</tr>
<tr>
<td>ICV-STZ / Normal saline (NS) infusion (On day 16)</td>
<td>500±2.83 (NS)</td>
<td>495.62±1.75 (ICV-STZ)</td>
<td>515±1.89 (ICV-STZ)</td>
<td>516.25±1.56 (NS)</td>
</tr>
<tr>
<td>On day 21 from the day of ICV-STZ/NS induction</td>
<td>502.5±2.5</td>
<td>356.6±6.2 (-33.20%)a</td>
<td>500.62±5.85 (+32.95%)b</td>
<td>553.12±2.9 (+9.15%)c</td>
</tr>
</tbody>
</table>

Data are expressed as mean±S.E. Values in the parentheses show the percentage increase or decrease with respect to their control. *p < 0.001 S vs L; **p < 0.001 L vs CESe+L.
*p < 0.001 S vs CESe; On '0' day not significant among all the groups.

On day last of the dosing (Day on 15): S vs L: n.s. significant (p>0.05);

Behavioral analysis

Effect of Learning and memory (Cognitive deficit) on behavioral activity and its restoration by CESe

Step-through passive avoidance test

Learning and memory was tested in one-trial passive avoidance paradigm on day 14 to 15 after the start of ICV-STZ infusion. The mean initial latency on day 14 did not differ significantly between the sham (S), Lesioned (L), CESe+L, and CESe groups. The initial latencies were 14.25±0.47 s, 18.75±0.47 s, 16±0.40 s and 13.75±0.47 s respectively.

On day 15th the mean retention latency in ICV-STZ infused rats i.e. lesioned group was significantly decreased (P<0.001) as compared to the sham group, the retention latency being 237.5±16.13 s and 537.5±16.13 s, respectively. On the other hand, the group L that was treated with CESe (CESe+L) has shown significantly increased (P<0.01) of transfer latency as compared to L group. The mean retention latency in CESe+L group was 387±29.75 s. However, the effect was significant on CESe administration, which was significantly higher than ICV-STZ infusion in L group indicating improved acquisition or retention of memory (Figure 3.1). No significant effect was found in the step-through latencies or number of trial among CESe group as compared to sham group.

Figure 3.1 Effect of pretreatment of CESe on passive avoidance paradigm in rats. Values are expressed as mean ±S.E.M. *P<0.001, retention latency of L group rats vs retention latency of S group rats; **P<0.01, L group vs CESe+L group.
Morris water maze

Spatial memory was assessed using Morris water maze (MWM) for 6 days starting from day 16. The results were divided into three headings, viz. escape latency, path length and the probe trail (% time spent in the correct quadrant).

Escape latency

Figure (3.2 and 3.3) show the potential effect of the combined antioxidants, Vitamin C, E and selenium (CESe) on escape latency and path length in ICV-STZ induced neurodegeneration (cognitive deficit) in S and L groups and restoration afforded by CESe. A significant (p<0.001) increase in escape latency was observed in L group, as compared to S group, while treatment with CESe in L group i.e. (CESe+L) group has significantly reduced the escape latency as compared to L group. No significant difference was observed in CESe group as compared to S group (Figure 3.2).

Path length

The path length covered by the L group was significantly (p<0.001) longer as compared to S group while CESe treated L group i.e. (CESe+L) has shown a significant reduction in the path length. The CESe group has shown better response as compared to S group. (Figure 3.3)

Probe trial (% time spent in the correct quadrant)

Data from the probe trial of the Morris water maze navigation task indicate a significant in learning performance and platform location during the six days of training of the animals between different groups (Figure 3.4A-B). The L group rats spent significantly (p<0.01) less time in the correct quadrant zone as compared the S group. On the other hand, the animal pretreated with CESe i.e. (CESe+L) group, spent significantly (p<0.05) more time in the target quadrant than the L group animals in the probe trail. Where as, no significant change in CESe group rat was observed as compared to S group rat.

BIOCHEMICAL ESTIMATIONS

Effect of learning and memory (Cognitive impairments) on the contents of lipid biomarkers, thiobarbituric acid reactive substance (TBARS) and its prevention/
There was a significant increase \((p<0.01)\) in the TBARS content following ICV-STZ as compared with sham-operated animals respectively \((17.54 \pm 0.39; 9.74 \pm 0.24)\). Pretreatment with CESe to the L (CESe+L) group animal resulted in a significantly \((p<0.05)\) reduction in the free radical-mediated lipid peroxidation as indicated by a decrease in the TBARS levels. In CESe-pretreated groups, a reduction in TBARS levels \((from 17.54\pm0.39 to 11.27 \pm 0.35)\) was observed (Figure 3.5).

**Effect of learning and memory (Cognitive impairments) on hydroperoxide \((\text{H}_2\text{O}_2)\) and its prevention / restoration by pretreatment with combined antioxidants CESe.**

Figure (3.6) the effect of cognitive impairments on hydroperoxide another biomarker of lipid peroxidation and protection by CESe. The level of hydroperoxide was elevated significantly \((p<0.05)\) in L group as compared to S and its level was increased significantly \((p<0.05)\) in CESe+L group as compared to L group.

**Effect of Cognitive impairments on the contents of reduced glutathione (GSH) and their protection by CESe**

The effect of cognitive impairments on reduced glutathione and protection by CESe shown in Figure (3.7). The level of GSH was depleted significantly \((p<0.01)\) in L group as compared to S and its level was increased significantly \((p<0.01)\) in CESe+L group as compared to L group. No significant change was observed in CESe as compared to S group.

**Effect of cognitive impairments on the activity of antioxidant enzymes and their protection by CESe**

Table 2 shows the effect of cognitive impairments of AD model and protection by CESe on the activity of GPx, GR and GST.
was observed in CESe group as compared to S group.

**Effect of Cognitive impairments on the activity of antioxidant enzymes, SOD and CAT and its protection by CESe**

Figure 3.8 and 3.9 shows the activity of SOD and catalase respectively in the hippocampus which was significantly (p<0.01) decreased in L group as compared to S group and it was restored significantly (p<0.05) in CESe+L groups as compared to L group. No significant change was observed in CESe groups as compared to the S group.

**Protection of phospholipase A2 (PLA2) activity by CESe in Cognitive impairments**

As compared with the controls, the activity of PLA2 was significantly (p<0.001) increased in L group as compared to S group. On the other hand, its activity was significantly (p<0.001) decreased in CESe+L group as compared with L group. No significant change was observed in the CESe group as compared to S (Figure 3.10).

**Effect of Cognitive impairments on the protein oxidation (protein carbonyl) measurement and its restoration by CESe**

Homogenate of the hippocampus was analyzed for protein carbonyl, a marker of oxidative damage of proteins. A significant (p<0.001) increase in oxidized proteins in L group was observed as compared S group. One way ANOVA followed by Tukey – kramer multiple comparison test on protein carbonyl measures indicated a significant effect of treatment with CESe in the case of L group i.e. (CESe+L). These results indicate that pretreatment with CESe significantly (P<0.05) restored the amount of oxidized brain proteins in CESe+L group (Figure 3.11).

**Effect of Cognitive impairments on the level of calcium and its restoration by CESe**

Figure 3.12 shows the effect of CESe on calcium level on cognitive impairment. The calcium level in serum was significantly (P<0.05) elevated in L group as compared S group. However, the level of calcium was restored significantly (p<0.05) in CESe+L group compared with L group. The insignificant change was observed between CESe group and S group.

**Effect of Cognitive impairments on the activity of cholinergic marker,**
acetylcholine esterase (AChE) and its restoration by CESe

AChE activity in the hippocampus was not changed significantly in S group and CESe group but it was decreased significantly \((p<0.01)\) in L group as compared to S group. The CESe has significantly \((p<0.05)\) attenuated its activity in CESe+ L group as compared to L group (Figure 3.13).

**Figure 3.7** The effect of CESe for 2 weeks pretreatment on reduced glutathione (GSH) content in hippocampus in rat lesioned by an ICV-STZ induced and sham received normal saline as a vehicle. Each bar represents the mean±SE of eight animals. \(*p<0.01\) S vs L, \(^{**}p<0.05\) S vs CESe + L.

**Figure 3.8** Superoxide dismutase (SOD) activity in hippocampus was restored by the pretreatment with CESe for 2 weeks. Animals were lesioned by an ICV-STZ induced and sham received normal saline as a vehicle. Each bar represents the mean±SE of eight animals. \(*p<0.01\) S vs L, \(^{**}p<0.05\) L vs CESe + L.

**Figure 3.9** Catalase (CAT) activity in hippocampus was protected by the pre-treatment with CESe for 2 weeks. Animals were lesioned by an ICV-STZ induced and sham received normal saline as a vehicle. Each bar represents the mean±SE of eight animals. \(*p<0.01\) S vs L, \(^{**}p<0.05\) S vs CESe + L.

**Effect of Cognitive impairments on choline acetyltransferase (ChAT) immunohistochemistry and protection by CESe**

To assess whether ChAT is associated with changes in hippocampus cholinergic neurons, immunohistochemistry for choline acetyltransferase (ChAT) expression was used as a cholinergic marker in hippocampus sections. Figure 3.14 (A-D) illustrates that significantly decreases in ChAT immunoreactive expression positive cells in ICV-STZ infused (L) group as compared with S group. By contrast, animal treated with CESe (CESe+L) had increased ChAT expression as compared L group. Drug control, CESe, showed almost similar expression as S group.

**Histopathological study**

Figure 3.15 (A-F) shown the histopathological changes in CA1 hippocampus region of ICV-STZ infused model of Alzheimer’s disease (cognitive impairments) and pretreatment with CESe. The neurodegeneration was observed after ICV-STZ infusion. The neurons shown shrunk size with dense and hyperchromatic nuclei and each neuron have
Chapter III

now acquired a clear space around itself due to retraction of the cell body as compared to S group. Pretreatment with CESe (CESe+L) markedly reverse these changes as compared to L group.

Table II: Effect of CESe on the activity of antioxidant enzymes in hippocampus of ICV-STZ rats

<table>
<thead>
<tr>
<th>parameters</th>
<th>Sham (S)</th>
<th>Lesion (L)</th>
<th>CESe+L</th>
<th>CESe</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPs (nmol NADPH oxidized/min/mg protein)</td>
<td>626.14±88.06</td>
<td>317.01±25.45&lt;sup&gt;a&lt;/sup&gt; (&lt;span&gt;49.37&lt;sup&gt;%&lt;/sup&gt;&lt;/span&gt;)</td>
<td>560.15±43.05&lt;sup&gt;b&lt;/sup&gt; (&lt;span&gt;76.69&lt;sup&gt;%&lt;/sup&gt;&lt;/span&gt;)</td>
<td>604.01±47.44</td>
</tr>
<tr>
<td>GR (nmol NADPH oxidized/min/mg protein)</td>
<td>531.35±72.15</td>
<td>308.94±23.91&lt;sup&gt;a&lt;/sup&gt; (&lt;span&gt;41.85&lt;sup&gt;%&lt;/sup&gt;&lt;/span&gt;)</td>
<td>470.36±13.25&lt;sup&gt;b&lt;/sup&gt; (&lt;span&gt;52.24&lt;sup&gt;%&lt;/sup&gt;&lt;/span&gt;)</td>
<td>558.46±29.84</td>
</tr>
<tr>
<td>GST (nmol CDNB conjugate formed/min/mg protein)</td>
<td>567.13±46.48</td>
<td>305.39±25.30&lt;sup&gt;a&lt;/sup&gt; (&lt;span&gt;46.15&lt;sup&gt;%&lt;/sup&gt;&lt;/span&gt;)</td>
<td>488.91±67.95&lt;sup&gt;b&lt;/sup&gt; (&lt;span&gt;60.09&lt;sup&gt;%&lt;/sup&gt;&lt;/span&gt;)</td>
<td>446.29±22.58</td>
</tr>
</tbody>
</table>

Animals pre-treated with CESe for 15 days were injected ICV-STZ bilaterally. Sham received normal saline as a vehicle. ICV-STZ-lesioned group differ significantly from S group (<span>p<0.01</span>) and CESe+L group differ significantly (<span>p<0.05</span>) from L group.

Figure 3.10 The effect of CESe for 2 weeks pretreatment on the activity of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) in hippocampus in rat lesioned by an ICV-STZ induced and sham received normal saline as a vehicle. Each bar represents the mean±SE of eight animals. <sup>*p<0.001 S vs L, ^p<0.001 L vs CESe</sup>.

Figure 3.11 The effect of CESe for 2 weeks pretreatment on protein carbonyl (PC) contents in hippocampus in rat lesioned by an ICV-STZ induced and sham received normal saline as a vehicle. Each bar represents the mean±SE of eight animals. <sup>*p<0.001 S vs L, ^p<0.05 L vs CESe + L</sup>. 

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DISCUSSION

A variety of procedures and devices have been documented to study behavioral functions in experimental animals. There are many supported working hypothesis is that ICV-STZ induced cognitive impairment, a well-documented model of neurodegeneration, (Prickaerts et al., 1995; Terwel et al., 1995) shows great similarities with the sporadic form of AD (Blokland and Jolles, 1993; Duclie et al., 1994; Nitsch and Hoyer, 1991; Plaschke and Hoyer, 1993). ICV-STZ induced cognitive deficit provides a relevant example of endogenous chronic oxidative stress due to mitochondrial dysfunction, which has been reported in senile dementia of Alzheimer type (Kish et al., 1992; Mutsya et al., 1994; Hirai et al., 2001). Such mitochondrial abnormalities could be both, the cause as well as the result of oxidative stress and neurodegeneration. Although, STZ is a diabetogenic drug, infusion with ICV causes cognitive impairments by generation of ROS following scission of the peroxide as catalyzed by iron (Rush et al., 1985). It also potentiates mitochondrial damage and depletes ATP productions which in turn alter redox state of the cell (Adams et al., 1993; Castilho et al., 1995).

At the onset of the study, behavioral activity was analyzed through one-step passive avoidance and Morris water test. A single bilateral administration of ICV-STZ (3 mg/kg b wt) causes long term abnormalities in learning, memory and cognition abilities in the training and retest phase through disturbance in the neuronal insulin signal transduction cascade. STZ-infused animals revealed significantly decreased latencies in passive avoidance on day 15. There was no improvement in passive avoidance throughout the course of the whole investigation period in ICV-STZ administered rats. The deterioration after STZ damage may be due to the fact that these animals had made good experience not to receive a further foot-shock. Our results are consistent with other workers who have demonstrated cognitive impairments after ICV-STZ in rats (Blokland and Jolles, 1993; Hoyer et al., 1994; Lannert and Hoyer, 1998).
Figure 3.14 (A-D) Effect of 2 weeks of pretreatment of CESe on Cholineacetyl transferase (ChAT) expression in hippocampus of rats by an ICV-STZ injection and sham received normal saline as a vehicle. The expression of ChAT was reduced in lesioned group (B) as compared to sham group (A) where as lesioned group pretreated with CESe i.e. (CESe+L) group (C) has shown positive expression for ChAT. However, CESe group (D) shown positive expression as sham group. The magnification is 400 x.

The escape latency and distances traveled (path length) to find the hidden platform during the acquisition phase of the experiment were analyzed through Morris water maze test and are present in Figure 3.2 & 3.3. Significantly high path length and escape latency were observed in ICV-STZ infused rats as compared to those pretreated with CESe. The path lengths and escape latencies in all 3 groups i.e. sham, CESe+L, and CESe were similar on the first day of the first trial, which suggested that their motor performance (ability to swim) was unaffected by the ICV-normal saline (NS), ICV-STZ induction and ICV-NS respectively, whereas the ICV-STZ (L) group tended to use more time than controls in following trials. L group animals showed a lower ability to find the platform and learn its location on 6th day of training. The poor performance was partly restored by pretreatment with CESe, resulting the increase in path length and escape latency to find the platform on 4th and 6th days of training (P<0.01 and P<0.05, respectively).

Data from the probe trial of the Morris water maze study, which measures how well the animals have learned and consolidated the platform location during the six days of training, indicated significant differences between the groups (Figure 3.4A-B). Lesioned rats spent less time in the target quadrant than the control group (P<0.01). On the other hand, the rats pretreated with CESe spent significantly more time in the target quadrant than the L group in the probe test (P<0.05).

The exact mechanism of action of CESe in preventing learning and memory deficits are still in debate. It could be hypothesized that the individual components of the combined drugs activated each other, crossed the blood brain barrier and scavenged the free radicals in the brain which have been observed in this
Figure 3.15 (A-F) Representative photographs of coronal sections of hippocampus. A. Sham (control) operated group animal showing hippocampal region CA1 with well formed neuronal layer (100 X), B. sham operated group animal showing a portion of the CA1 region. All the neurons have large round vesicular nuclei with prominent nucleoli and amphophilic cytoplasm. Neuronal density was 22/ HPF (HE x 400), C. Lesioned (L) group showing a region CA1 of the hippocampus with an intact neuronal layer (HE x 100), D. Lesioned (L) group showing a portion of the CA1 region. The neurons appear to have shrunken in size with dense and hyperchromatic nuclei and each neuron have now acquired a clear space around itself due to retraction of the cell body. Neuronal density was 26/ HPF (HE x 400), E. CSE + L group showing a portion of CA1 region of the hippocampus pretreated with CSE to L group rat with an intact neuronal layer (HE x 100), F. CSE + L group showing a portion of CA1 region of the hippocampus pretreated with CSE to L group rat. While neuronal shrinkage is still present, vesicular nuclei are seen in many neurons. The retraction space around the neurons seen in STZ only is absent. Neuronal density was 23/ HPF. (HE x 400), G. CSE group showing a portion of CA1 region of the hippocampus with an intact neuronal layer (HE x 100), H. CSE group showing a portion of the CA1 region of the hippocampus. There was no neuronal shrinkage and vesicular nuclei were seen the neurons. Neuronal density was 26/ HPF (HE x 400).
study to prevent the loss of neurons. Earlier reports postulated that vitamins C and E (Arzi et al., 2004) improved cognitive function through their antioxidant activities and Se (Planas et al., 2004) supplementation prevented weight loss in mild AD.

The studies of antioxidant enzymes in AD have not shown a consistent pattern (Casetta et al., 2005). Here, the activity of glutathione dependent enzymes [glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase, (GST)] and other antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) is significantly decreased in hippocampus. In general mechanism, GSH is converted to GSSG by the enzyme GPx which is reconverted to GSH by GR, thus balancing the pool of GSH, which, in conjunction with reduced nicotinamide adenine dinucleotide phosphate (NADPH), can reduce the formation of lipid peroxidation, free radicals and hydrogen peroxidase (H₂O₂). The increase in the content of GSH and decrease in the level of TBARS and H₂O₂ as markers of lipid peroxidation following treatment with CESe, in our study, is in accordance with earlier reports (Sharma and Gupta, 2003; Ishart et al., 2006; Khan et al., 2006), where antioxidants have been used for the same model of cognitive impairment. Our findings are consistent with the earlier reports in that GST inactivates the toxic products of oxygen metabolism including 4-hydroxynonenal (HNE), a marker of lipid peroxidation (Danielson et al., 1987). Significant decrease in glutathione-S-transferase activity and other antioxidative enzymes was also described in hippocampus of patients with AD (Lovell et al., 1998).

Damage to brain membrane phospholipids may play an important role in the pathogenesis of AD. Inconsistent reports were found with respect to the activity of PLA2 in the brain of AD patients. Enzyme PLA2 plays a significant role in the conversion of phospholipids to arachidonic acid, a precursor in the biosynthetic pathway of prostaglandins. Earlier reports showed that ICV-STZ increased free fatty acid and decreased phospholipids in rat brains (Mulier et al., 1998) due to phospholipase activity rather than peroxidative degradation. This interpretation was underscored by the observation that PLA2 is activated by intracellular depletion of an energy bond molecule ATP, and raises cytosolic calcium (Markellos and Garbus, 1975, Moskowitz et al., 1984, Sun et al., 1993). The earlier evidences (Stephenson et al., 1999; Farooqui et al., 2003) support our study that oxidative stress may upregulate PLA2 activity and its activation is considered a major underlying cause for abnormalities in a number of pathological conditions in AD. Thus, ICV-STZ induced cognitive impairment led to increased PLA2 activity and cytosolic calcium levels, and pretreatment with CESe to L group restored the activity of PLA2 activity and calcium level.

The tissue proteins are vulnerable to ROS attack (Oliver et al., 1984, 1987; Stadtmann and Oliver, 1991; Stadtmann, 1992) which undergo carbonyl modification and primarily aroused from iron-catalyzed oxidation. Increase in the protein carbonyl groups in different brain parts has been reported in the motor cortex of the hippocampus, the region contributing to memory function (Dubey et al. 1996; Bardgett and Henry, 1999; Suzuki and Clayton, 2000) and also regarded as the most vulnerable region of the AD brain (Aksenov and Markesbery, 2001). In the present study, the protein carbonyl was attenuated by pretreatment with CESe to ICV-STZ infused rats which was due to restoration of the protein oxidation in the hippocampus. The result demonstrated a synergistic effect of the combined antioxidants at lower doses in decreasing the carbonyl formation of proteins after administration to ICV-STZ i.e. lesioned group. Our study with ICV-STZ induced oxidative damage due to protein carbonyl induction is in good agreement with parallel studies involving lipid peroxidation in various regions of the brain (Bano and Parihar, 1997; Parihar et al., 1997).

Transmission of acetylcholine (ACh) to the hippocampus from its afferent sources plays an important role in normal learning and
memory function. Its synthesis depends on the availability of acetyl Co-A, provided by the breakdown of glucose and insulin, which controls the activity of ChAT, a synthesizing enzyme for ACh, and a marker of cholinergic function and the cognitive deficits (Blokland and Jolles, 1993). Immunohistochemical studies showed decreased ChAT positive cell expression in the lesioned group (Figure 3.14 B) which was restored by CESe pretreatment in ICV-STZ infused rats. On the other hand, activity of AChE, a hydrolyzing enzyme for ACh, was significantly decreased in the lesioned group, corroborating the earlier report (Davis et al., 1999). Pretreatment with CESe significantly attenuated AChE activity in ICV-STZ induced rats indicating a potential neuroprotective efficacy of CESe with synergistic effect on the cholinergic-neurons in ICV-STZ administered rats, resulting in decrease in central energy metabolism, possibly due to the ATP generating mechanism of action of combined CESe.

However, the present study appears to provide direct immunological (Figure 3.14) and histopathological evidence (Figure 3.15) of specific change and damage caused by ICV-STZ in neuronal body of hippocampus that is essential for learning and spatial memory. It therefore, seems likely that STZ does not induce learning deficits only by impairing glucose utilization (Plaschke and Hoyer, 1993) through an inferred action on brain insulin receptor function (Hoyer et al., 1996). The present study suggests that cholinergic neurons in the hippocampus are protected from lesion-induced degeneration by treatments with a combination of the antioxidants (CESe). We observed attenuation on the loss of ChAT-positive hippocampal cells which may be due to decrease level of ATP and local glucose following lesion-induced by ICV-STZ in the hippocampus. To the best of our knowledge, this is the first study to report protective effect of CESe on ChAT-immunoreactive neurons following ICV-STZ induced cognitive impairment in rat models.

The present study reflects our understanding on the basis of histopathological observation in the hippocampal area in different groups of rats. The neuronal layers in CA1 region of the hippocampus did not reveal any evidence of neuronal loss and the neuronal densities and did not differ in a significant manner between the groups. There was however, a distinct difference in the neuronal structures seen. The neurons in the sham group were large, conical in shaped with well delineated amphophilic cytoplasm and round vesicular nuclei with prominent nucleoli. In the ICV-STZ treated group, CA1 layer neurons showed pronounced shrinkage of the neuronal bodies with the nuclei losing their regular outlines and becoming hyperchromatic. The cell shrinkage also resulted in a clear space around each neuron. The group pretreated with CESe along with STZ showed similar changes in STZ group but significantly lower degree. Thus, while some neurons showed shrinkage, others retained their vesicular nuclei. The group treated with drug alone did not reveal any significant neuronal changes. Conclusively, ICV-STZ induction resulted in neuronal structural changes but did not cause any reduction in the number of neurons present in the CA1 region of the hippocampus. Pretreatment of CESe along with STZ was able to lower the intensity of these structural changes.

Summarizing the present study which demonstrates that administration of ICV-STZ leads to cognitive impairments and significant alterations in normal behavioral paradigms. The restoration of behavioral activity was observed with the pretreatment of CESe. Significant elevation of TBARS, hydroperoxide, protein carbonyl and calcium and significant depletion of GSH, GPx, GR, GST, SOD, CAT, AChE and glucose was observed. This effect is probably mediated by oxidative stress, because pretreatment with CESe restored the enzymatic and non-enzymatic biomarkers caused by ICV-STZ infusion. ChAT-immunological expression and histopathological evidences support our findings for the potential implication of CESe on cognitive impairments. Assuming the possibility that these phenomena may occur in the human pathology, our findings might be
relevant to explain, at least in part, the neurological dysfunction associated with AD (Cognitive impairments). Whether oxidative stress, excitotoxicity or other alterations elicited by ICV-STZ are the main factors responsible for the neurological dysfunction of cognitive impairments remain to be determined. Our results also suggest that the administration of antioxidants should be considered as an adjuvant therapy to specific diets or to other pharmacological interventions in cognitive patients. However, more studies must be conducted before the administration of antioxidants to the patients of AD.

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