

Original Research Article

Chrysin administration protects against collagenase-induced stroke in ratsRajwinder Kaur¹, Nitin Bansal^{1*}¹Department of Pharmacology, ASBASJSM College of Pharmacy, Bela (Ropar)-140111, Punjab, India***Corresponding author**

Dr. Nitin Bansal

Email: nitindsp@rediffmail.com

Abstract: The present study is an effort to demonstrate the role of Chrysin in the management of Collagenase-ICV induced brain stroke in rats. Wistar rats either sex 200-250 g. Animals were divided into 6 groups (n=6). Chrysin was administered daily in 3 doses (10, 20 and 40 mg/kg; *p.o.*) to rats for 28 successive days. Brain stroke was induced in rats by intracerebroventricular (ICV) injection of collagenase (10 µl/ml) using stereotaxic apparatus. Behavioural assessment of rats was carried out with the help of Elevated plus Maze, Novel objective recognition and locomotor activity were carried out. After behavioural evaluation, the animals were sacrificed and their brains were isolated for estimating brain TBARS, GSH, Nitrite, Catalase and protein levels. Brain stroke (induced by collagenase, 10 µl/ml) significantly increased the transfer latency (TL) of rats in elevated plus maze studies and showed significant difference in exploration of the novel and familiar object in retention trial of rat in novel objective recognition task as compared to sham control group. Collagenase treated rats showed significant decrease in brain GSH, catalase, protein levels and significant increase in brain TBARS and nitrite levels as compared to sham control group. Administration of Chrysin (10, 20 and 40 mg/kg; *p.o.*) significant ($p < 0.05$) reduced the TL of rats during elevated plus maze session, significant increases the time spent exploring the novel compared familiar object as compared to sham control group. Chrysin treated rats showed ($p < 0.05$) an increase in GSH, catalase, protein levels and ($p < 0.05$) decrease in brain TBARS and nitrite levels. Thus, Chrysin may prove to be a useful remedy for the management of brain stroke owing to its possible neuroprotective and antioxidant properties.

Keywords: Chrysin, intracerebral hemorrhage (ICH), collagenase, stroke, oxidative stress, antioxidant.

INTRODUCTION

The human brain, one of the most complex living organs in the universe, with 1,000 trillion synaptic connections. It is susceptible to many types of damage and disease. It continuously receives and analyzes sensory information, responding by controlling all bodily actions and functions. It is also centre of higher-order thinking, learning and memory, gives the power to think, plan, speak, imagine, dream, reason and experience emotions. Cerebrovascular disease (CVD) or a stroke is heterogeneous group of diseases arises due to dysfunction of the blood vessels supplying blood to the brain and include ischemic stroke and hemorrhagic stroke [1]. These disorders cause 2 million deaths each year and are a major cause of disability. Stroke has been ranked third most common cause of death world-wide and cerebrovascular diseases are considered to be the second most frequent cause of projected deaths [2]. Most strokes (~ 85%) are ischemic; that is, they result from occlusion of a major cerebral artery by a thrombus or embolism, result in reduced blood flow and a major decrease in the supply of oxygen and nutrients to the affected region. The rest of strokes are hemorrhagic,

which are caused by the rupture of a blood vessel either in the brain or on its surface [3]. The World Health Organization (WHO) estimates that 15 million patients worldwide suffer from stroke annually. Approximately one third of these cases die, one third are left disabled and one third has a good outcome. High blood pressure is a contributing factor in more than 12.7 million strokes annually worldwide. Generally, intracerebral hemorrhage (ICH) accounts for ~10% of all strokes and is associated with a 50% case fatality rate [4]. In recent years, many studies focus on the mechanism of secondary inflammation that can cause brain edema and this may provide new therapy targets for ICH. Dexamethasone can reduce cerebral cell apoptosis and inhibit inflammation [5], and deferoxamine (DFX) provides new therapy target [6]. Various neuroprotective agents currently in development include Maxipost (BMS-204352) (Ion channel modulators) [7], Naloxone (Opiate antagonism) [8], Edaravone (Free radical scavenger) [9], Selfotel (Glutamate antagonists) [10], IL-1 receptor antagonist (Inhibition of cytokines) [11].

Chrysin (5, 7-dihydroxyflavone or 5, 7-dihydroxy-2-phenyl-4H-chromen-4-one) belongs to the flavone class of the ubiquitous 15-carbon skeleton natural polyphenolic compounds collectively called flavonoids [12]. Flavonoids are also present in many medicinal plants and account for the different pharmacological benefits reported. Chrysin and its derivatives have been shown to be the principal constituents of the well-known medicinal plant, *Radix scutellariae*. Other common sources of chrysin, which attracted much scientific attention in recent years from the pharmacological point of view, are propolis and honey [13]. Moreover, many fruits, passion flowers such as *Passiflora incarata* Linn and oyster mushroom, *Pleurotus ostreatus*; [14] are known to be good sources of chrysin. While chrysin-containing plants have been widely used for medicinal purposes, synthetic chrysin is used for large scale uses. Chrysin has been shown to be a very active flavonoid exerting a vast number of pharmacological properties such as anti-inflammatory activity via blocking histamine release and pro-inflammatory cytokine expression [15], anti-asthmatic activity through suppression of inducible nitric oxide synthase (iNOS) and nuclear factor- κ B (NF- κ B) [16], anticancer activity by endorsing the cell death induced by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and increasing TRAIL-induced degradation of caspases 3 and 8 [17], preventing metastatic progression in breast cancer cells [18], anti-hypercholesterolemic activity [14], cardioprotective activity via improving post-ischaemic functional recovery [19], prevention of osteoporosis by activation of estrogen receptor (ER)/mitogen activated protein kinase (MAPK) [20].

MATERIAL AND METHODS

Animals

Wistar rats (either sex), weighing between 200-250 g, were procured from CPCSEA registered approved breeder. The animals were kept in quarantine section till monitoring of health status of animals and subsequently transferred to the housing area. The animals were acclimatized for seven days to the housing conditions of Central Animal House Facility of ASBASJSM College of Pharmacy, BELA prior to experiments. Animals were housed in polypropylene cages with dust free rice husk as a bedding material and maintained under standard laboratory conditions with controlled temperature ($23 \pm 2^\circ\text{C}$), humidity ($40 \pm 10\%$) and natural (12 h each) light-dark cycle. The animals were fed with standard rodent pellet diet (Ashirwad Industries, Mohali) and water *ad libitum*. The experiment was carried out between 09:00 and 18:00 h. The care of laboratory animals was done following the guidelines of CPCSEA, Ministry of Forests & Environment, Government of India.

Drugs and Chemicals

Chrysin and Collagenase was purchased from Sigma Aldrich. Edaravone was purchased from Lipin

Ltd. DTNB, Thiobarbituric acid purchased was from Himedia laboratories, Mumbai. Reduced glutathione, 5,5'-dithiobis (2-nitrobenzoic acid), thiobarbituric acid, trichloroacetic acid, sodium nitrate, copper sulfate, sodium potassium tartarate, tri-sodium citrate, Folin's phenol reagent, sodium hydroxide procured from Himedia.

Induction of Brain Stroke

Stereotaxic apparatus is used to directly inject the drug in brain by locating the coordinates. An experimental rat model was developed using Collagenase administered intracerebroventricularly (ICV) by Hamilton syringe. Rats were anaesthetized with chloral hydrate (400 mg/kg; i.p.). The head was positioned on the stereotaxic frame and middle sagittal incision was made in the scalp. A hole was drilled in the skull by using the following coordinates:

- 0.2 mm anterior to bregma
- 3.0 mm lateral to sagittal suture
- 6.0 mm below the surface of the skull [21].

Collagenase was administered at a dose of 10 $\mu\text{l/ml}$. Collagenase (0.1 mg) was dissolved in 9000 μl of saline + 1000 μl of CaCl_2 and was administered slowly in the volume of 10 μl .

Experimental Design

Wistar rats of either sex were used in this study. Animals were divided into 6 groups with at least 6 animals ($n=6$) in each group. Brain stroke was induced in rats by ICV administration of collagenase to rats using stereotaxic apparatus, after anaesthetising with Chloral hydrate (400 mg/kg, i.p.). Chrysin was administered through oral route to rats into 3 doses (10, 20, 40 mg/kg) daily for 28 days by dissolving in saline [22]

Group 1- Sham control: ACSF, 10 μl was administered via intracerebroventricular (ICV) injection to rats.

Group 2- COL control: Collagenase 10 μl was administered via intracerebroventricular (ICV) injection to rats.

Group 3- COL + CHR 10: Collagenase 10 μl was administered via intracerebroventricular (ICV) injection to rats. After 30 minutes chrysin (10 mg/kg; p.o) was administered to rats daily for 28 successive days.

Group 4- COL+ CHR 20: Collagenase 10 μl was administered via intracerebroventricular (ICV) injection to rats. After 30 minutes chrysin (20 mg/kg; p.o) was administered to rats daily for 28 successive days.

Group 5- COL+ CHR 40: Collagenase 10 μl was administered via intracerebroventricular (ICV) injection to rats. After 30 minutes Chrysin (40 mg/kg; p.o) was administered to rats daily for 28 successive days.

Group 6- COL+ ED: Collagenase 10 μl was administered via intracerebroventricular (ICV) injection to rats. After 30 minutes Edaravone (10 mg/kg; i.p) was administered to rats daily for 28 successive days.

BEHAVIORAL ASSESSMENT

ELEVATED PLUS MAZE

Procedure

The elevated plus maze consisted of two open arms (50 cm × 10 cm) extending from a central platform (10 cm²). The maze was elevated to a height of 50 cm from the floor. On the first day, each rat was placed at the end of either of the open arms, facing away from the central platform. The transfer latency was noted down. Transfer latency (TL) was defined as the time (in seconds) taken by the animal to move from the open arm into one of the covered arms with all of its four paws. The cut off time to reach the closed arm was 90 sec. In case the rat does not locate the closed arm in 90 sec, it was gently guided to one of the closed arm. The rat was allowed to explore the maze for 20 s and then return to home cage. Retention of this learned task (memory) was examined 24 hours after the learning trial. Significant reduction in TL value indicates improvement of memory [23].

Novel Objective Recognition (NOR)

Object Recognition (OR) may be performed in a typical apparatus has a 50cm high, 40 x 60cm box made of wood (or plastic) with a frontal glass wall, the inside of which is painted with clear colors. Usually the recognition objects are made of plastic or metal to allow easy cleaning between sessions with different animals. In the object recognition task, each rat was placed into the box and exposed to two identical objects (A1 and A2) for a period of 3 min. The rats were then returned to their home cage for a 1-min inter-trial interval, the entire box was cleaned, both objects removed and one replaced with an identical familiar copy and one with a novel object. Following the 1-min inter-trial interval, rats were returned to explore the familiar (A) and novel object (B) in the test box for a 3-min retention trial. Object exploration was defined as the rats sniffing, licking or touching the objects with forepaws whilst sniffing but not by leaning against, turning around, standing or sitting on the objects. The exploration time (s) of each object in each trial was recorded manually using two stopwatches and the following factors were calculated: $E1$ = the total exploration time of both objects in the acquisition trial ($EA1 + EA2$), $E2$ = the total exploration time of both objects in the retention trial ($EA + EB$), and H = the habituation of exploratory activity ($E1 - E2$). The index of habituation to the familiar object measures differences between the average time spent in exploring the objects in the acquisition and the retention trials. DI = discrimination index ($(EB - EA)/(EB + EA)$), the discrimination index represents the difference in exploration time expressed as a proportion of the total time spent exploring the two objects in the retention trial [24].

LOCOMOTOR ACTIVITY

The locomotor activity was recorded using actophotometer for a period of 5 min. Ambulatory

activities were recorded and the locomotor activity was expressed in terms of total photo beam counts for 5 minutes per animal [25].

BIOCHEMICAL ESTIMATIONS

Brain homogenate preparation

All animals were sacrificed at the end of study i.e. 28 days and brain was isolated. Tissue homogenates were prepared with 0.1 M phosphate buffer (pH 7.4) and supernatant of homogenates was employed to estimate thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), Catalase, nitrite level.

Measurement of lipid peroxidation

Brain homogenized with 0.1 M Phosphate buffer (pH 7.4) and supernatant was used for the measurement of thiobarbituric acid reactive substances (TBARS) at absorbance 532 nm by using U.V/Visible spectrophotometer (Shimadzu 1700, Singapore) [26].

Measurement of GSH

The GSH assay was performed by the method Ellman *et al.* Supernatant was used for the measurement of GSH at absorbance 412 nm by using U.V/Visible spectrophotometer. The concentrations were determined using a standard curve of reduced glutathione and the results were expressed as $\mu\text{M}/\text{mL}$ [27].

Measurement of catalase activity

The assay mixture consisted of 3 ml of H_2O_2 , phosphate buffer and 0.05 ml of supernatant of tissue homogenate (10%), and the change in absorbance was recorded at 240 nm. The results were expressed as micromoles of H_2O_2 decomposed per milligram of protein/min [28].

Estimation of nitrite level

The nitrite levels were estimated by the acidic Griess reaction after reduction of nitrate to nitrite by vanadium trichloride according to the method described by Green *et al.* The concentrations were determined using a standard curve of sodium nitrate and the results were expressed as $\mu\text{M}/\text{mL}$ [29].

RESULTS

Effect of Chrysin on Loco-motor activity using Actophotometer

In COL control group, loco-motor activity of rat was significantly reduce ($p < 0.05$) as compared to the sham control group. However, treatment of rats with chrysin (10, 20 and 40 mg/kg; *p.o.*) showed significantly ($P < 0.05$) prevented reduction of loco-motor activity when compared to COL control group. Treatment of rats with edaravone for 28 days also prevented the reduction ($p < 0.05$) in the loco-motor activity as compared COL control group.

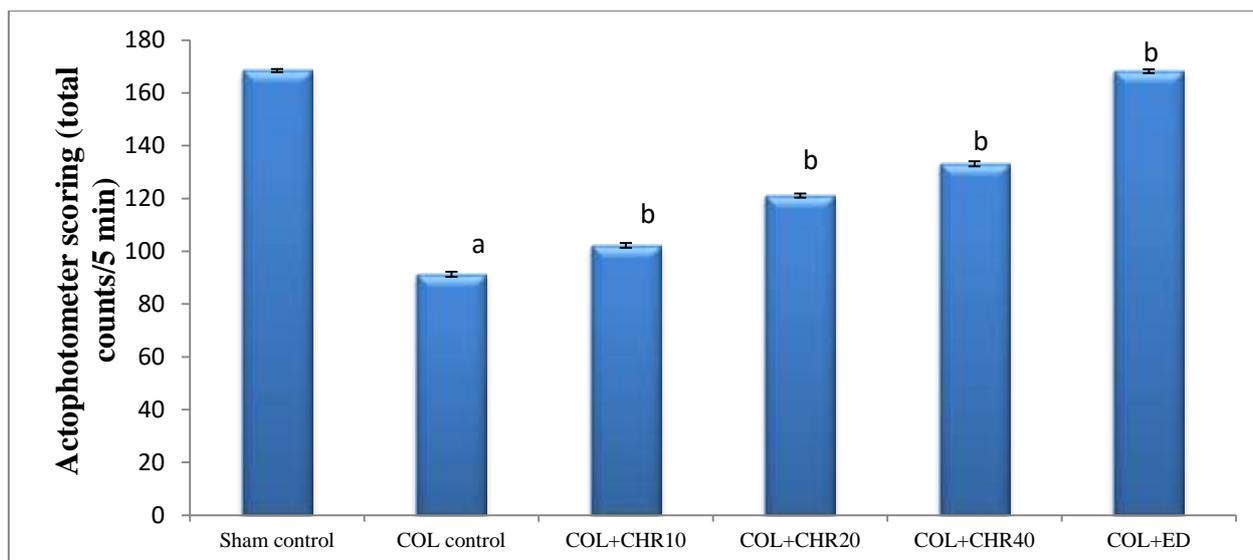


Fig-1: Effect of Chrysin on Loco-motor activity using Actophotometer

Values are expressed as mean \pm SEM (n=6), ^a denotes $p < 0.05$ compared to sham control group, ^b denotes $p < 0.05$ compared to COL control group (one way ANOVA followed by Tukey's test).

Effect of Chrysin on Transfer Latency (TL) using Elevated plus Maze (EPM)

Transfer latency (TL) on day 2 (46.27 ± 0.7) was significantly ($p < 0.05$) decreased in sham control group when compared with that on day 1 (63.83 ± 0.9) acquisition trial. In COL control group, TL of rats were significantly increased ($p < 0.05$) as compared to the

sham control group. However, treatment of rats with chrysin (10, 20 and 40 mg/kg; *p.o.*) showed significant ($P < 0.05$) decreased in TL when compared to COL control group. Treatment of rats with edaravone for 28 days also decreased ($p < 0.05$) in the TL as compared COL control group.

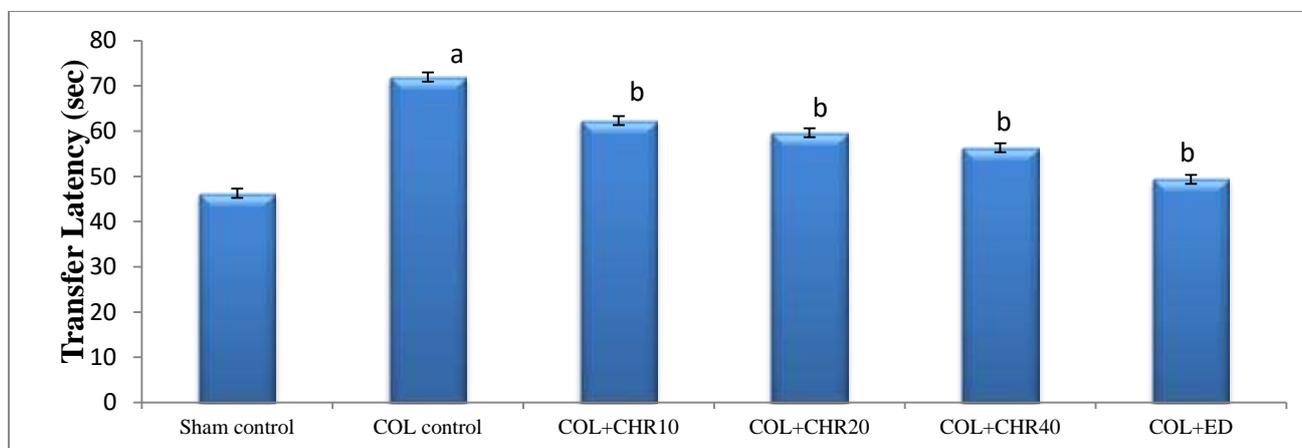


Fig 2: Effect of Chrysin on Transfer Latency (TL) using Elevated plus Maze (EPM)

Values are expressed as mean \pm SEM (n=6), ^a denotes $p < 0.05$ compared to sham control group, ^b denotes $p < 0.05$ compared to COL control group (one way ANOVA followed by Tukey's test).

Effect of Chrysin on Exploration time using Novel Object Recognition Task Acquisition trial

All groups of rats spent almost similar time exploring the identical objects (left and right) in the acquisition phase. Statistical analysis showed no significant difference in time spent exploring the identical objects in the acquisition phase in any group.

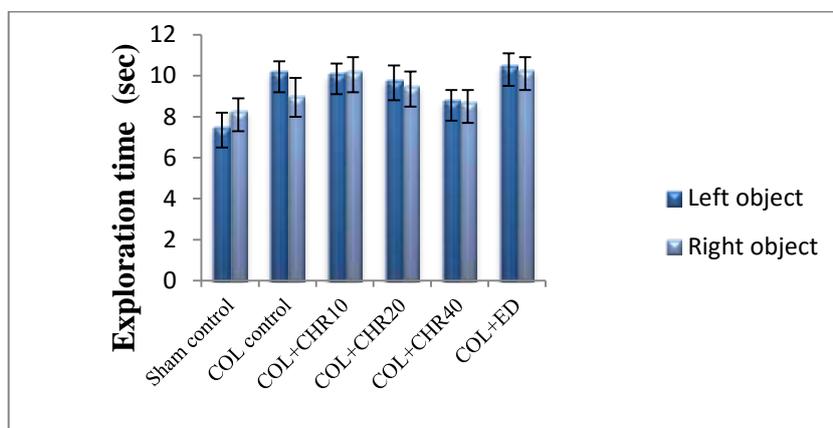
Retention trial

Rats treated with ACSF spent more time ($p < 0.05$) exploring the novel object compared with the familiar object. The ability to discriminate familiar and novel objects was abolished following COL treatment. Chrysin at doses (10, 20 and 40mg/kg; *o.p.*) and Edaravone significantly attenuated the COL control impairment such that a significant increase in time spent exploring the novel object compared with the familiar object was observed ($p < 0.05$).

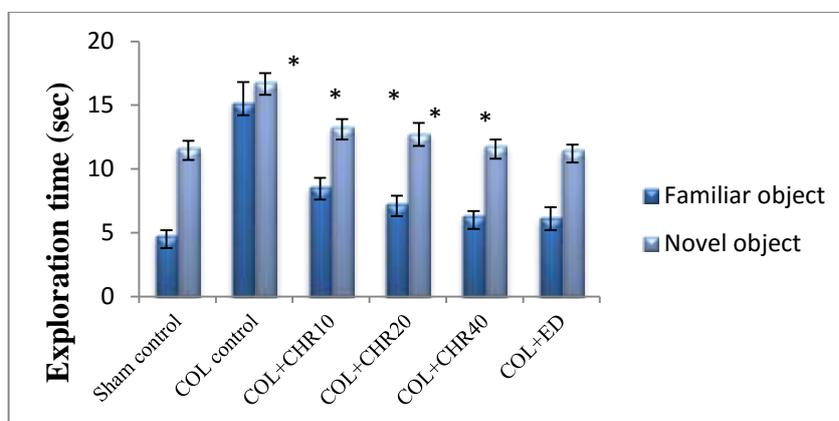
Discrimination Index (DI)

A one-way ANOVA revealed a significant effect of treatment on DI. The DI was reduced ($p < 0.05$) following Collagenase (i.c.v) - induced brain stroke.

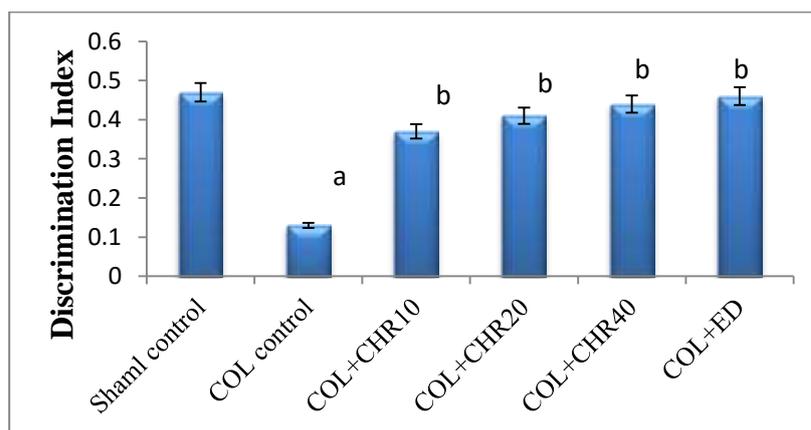
Chrysin (10, 20 and 40 mg/kg; p.o.) treatment resulted in dose dependent increase in DI as compared to COL control group.



a) Acquisition trial



b) Retention trial



c) Discrimination Index

Fig-3: Effect of Chrysin on Exploration time in a) Acquisition, b) Retention trial and c) Discrimination Index (DI) using Novel Object Task

Effect of Chrysin on brain TBARS, GSH, Nitrite levels and Catalase activity

In COL control group rats showed significantly higher brain TBARS and nitrite levels ($p < 0.05$) and decrease the GSH levels and catalase activity as compared to the sham control group,

indicating the rise in oxidative stress. The administration of Chrysin (10, 20 and 40 mg/kg; p.o.) for 28 successive days, prevented the rise in brain TBARS and nitrite levels and prevented the reduction in GSH levels and catalase activity in dose dependent manner and the results were statistically significant

($p < 0.05$) as compared to COL control group. Treatment with the standard drug edaravone for 28 days significantly attenuated the rise ($p < 0.05$) in the level of

brain TBARS levels, nitrite and prevented the reduction in GSH levels and catalase activity as compared to COL control group.

Table-1: Effect of Chrysin on brain TBARS, GSH, Nitrite levels and Catalase activity

Groups	TBARS ($\mu\text{M/mL}$)	Conc.	GSH Conc. ($\mu\text{M/mL}$)	Nitrite ($\mu\text{M/mL}$)	Conc.	Catalase (Units ^A /mg protein)	Conc.
Sham control	12.18 \pm 0.67		148.33 \pm 0.4	21.72 \pm 0.4		0.64 \pm 0.01	
COL control	30.89 \pm 0.04 ^a		102.5 \pm 0.3 ^a	42.13 \pm 0.7 ^a		0.53 \pm 0.01 ^a	
COL+CHR10	23.67 \pm 2.1 ^b		124.16 \pm 0.3 ^b	37.55 \pm 0.8 ^b		0.56 \pm 0.01 ^b	
COL+CHR20	21.45 \pm 0.7 ^b		129.16 \pm 0.5 ^b	36.71 \pm 0.5 ^b		0.59 \pm 0.01 ^b	
COL+CHR40	16.44 \pm 2.3 ^b		134.16 \pm 0.7 ^b	35.04 \pm 0.5 ^b		0.61 \pm 0.02 ^b	
COL+ED	13.67 \pm 2.3 ^b		144.16 \pm 0.3 ^b	33.11 \pm 0.8 ^b		0.63 \pm 0.01 ^b	

Values are expressed as mean \pm SEM (n=6), ^a denotes $p < 0.05$ compared to sham control group, ^b denotes $p < 0.05$ compared to COL control group (one way ANOVA followed by Tukey's test).

DISCUSSION

Intracerebral hemorrhage is commonly produced in rat striatum by infusing bacterial collagenase [30]. Collagenase disrupts the basal lamina of cerebral blood vessels causing blood to leak into the surrounding brain tissue, which increases intracranial pressure and formation of hematomas (causes brain edema and BBB disruption) and release of clot derived factor (thrombin, hemoglobin, iron) [31]. This stroke like condition causes the impairment of memory [32]. In the present study, collagenase administration to animals caused the deficits in learning and memory as indicated by novel object recognition task and elevated plus maze study. Collagenase treated rats were not able to discriminate between novel and familiar objects; the discrimination index was significantly reduced as compared to sham control group. Furthermore, in EPM study, TL was significantly increased in collagenase control group suggesting impairment of memory as compared to the sham control group. In addition collagenase administration caused an increase in the TBARS, nitrite and decrease in level of brain GSH and catalase activity. Various studies have demonstrated that central administration of collagenase causes an increase in free radical generation and the subsequent oxidative stress. Oxidative stress is a condition in which the overproduction of free radicals, mainly reactive oxygen species (ROS), exceeds the antioxidant capacity and subsequently leads to cell injury via directly oxidizing cellular protein, lipid and DNA or participating in cell death signaling pathways. Collagenase induces a direct inflammatory response in the CNS that leads to free radical-induced neurotoxicity [33]. ROS produces malondialdehyde (MDA). In the present study, the extent of lipid peroxidation and nitrite levels in brain homogenate of hemorrhagic rats (Collagenase group) was elevated and GSH levels and catalase activity were found decreased as compared to sham control group.

Chrysin is a natural flavonoid generally extracted from passion flower *Passiflora incarnata* Linn (Passifloraceae), honey comb and Indian trumpet flower

[34]. Chrysin is reported to possess anti-oxidant, anti-microbial, anti-spasmodic, anxiolytic and anti-inflammatory activities [35, 36, 37]. Chrysin administration for 28 days protected the animals from development of memory impairment deficits as indicated by higher discrimination index compared to collagenase control animals. Furthermore, chrysin treated rats showed reduced TL as well. These results showed that chrysin restored the cognitive capabilities of animals and this may be because of antioxidant property of chrysin; as chrysin administration caused the decreased in lipid peroxidation (reduced brain TBARS and nitrite levels) and enhanced free radical scavengers (enhanced GSH level and catalase activity) in rats. These results in agreement with previous studies which demonstrated the antioxidant properties of chrysin [38].

Hence, chrysin may find the place as potential therapeutic option for the prevention or treatment of brain stroke and other neurodegenerative diseases of aging.

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