Original Article

Neuroprotection of brain permeable Forskolin ameliorates behavioral, biochemical and histopathological alterations in rat model of intracerebral hemorrhage

Rajesh Dudi[1], Sidharth Mehan[2*]

[1]Department of Pharmacology, Rajendra Institute of Technology and Sciences, Sirsa, Haryana, India, [2]Department of Pharmacology, ISF College of Pharmacy, Moga, Punjab, India

Correspondence:
Dr. Sidharth Mehan,
Department of Pharmacology,
ISF College of Pharmacy, Moga - 142 001, Punjab, India. Phone: +91-8059889909/91-9461322911.
E-mail: sidh.mehan@gmail.com

How to cite this article:
Dudi R, Mehan S. Neuroprotection of brain permeable Forskolin ameliorates behavioral, biochemical and histopathological alterations in rat model of intracerebral hemorrhage. Pharmaspire 2018;10(2):68-86.

Source of Support: Nil,
Conflicts of Interest: None declared.

ABSTRACT

Mitochondrial complexes enzymes' (I, II, IV, and V) dysfunction increases neuroinflammatory cytokines, oxidative stress, and alterations of brain metabolic enzymes may be key pathological hallmarks of cerebral hemorrhage. Here, the first time in the history of this intracerebral hemorrhage (ICH) animal model, we extensively examined the huge range of behavioral, biochemical, neuropathological, morphological, and histopathological effects of direct adenylyl cyclase activator Forskolin (FSK) in adult rats' brain tissue homogenate, serum, and urine. Intraventricular injection of autologous blood in rats caused impairment in memory, grip strength posture, and cognitive function. Biochemical analysis of brain homogenate, serum, and urine samples in ICH-treated rats showed an increase in altered mitochondrial complexes activities, inflammatory cytokines, oxidative stress, and lipid biomarkers. Neurohistological alterations of hippocampus, basal ganglia, and cerebral cortex of ICH-treated rats exhibit severe neuronal space, irregular damaged cells, and dense pyknotic nuclei-associated marked focal diffused gliosis. FSK (20, 40, and 60 mg/kg, p.o) once daily treatment for a period of 22 days significantly improved motor performance and cognitive behavior task. Further, FSK treatment significantly improved mitochondrial complexes' enzyme activity, attenuated inflammatory, and oxidative damage of rat brain. In present research work, neuroprotective effects of direct AC activator FSK responsible for activation of cyclic adenosine monophosphate/protein kinase further leads to CREB activation, and through the repairing in the basal ganglia, cortex, and hippocampus functioning associate with mitochondrial dysfunctioning in cerebral hemorrhage.

Keywords: Cerebral hemorrhage, neuroinflammation, cyclic adenosine monophosphate, forskolin

INTRODUCTION

Neurological disorders are a heterogeneous group of diseases of the nervous system having different etiologies. They represent illnesses of the selective regions of the brain and nervous tissues which control vital physiological functions such as learning and memory, posture, and coordination of movements of nerves/muscles.[1] A variety of CNS disorders, including Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, Amyotrophic lateral sclerosis (ALS), brain abscess, multiple sclerosis, spinal cord injury, and cerebral stroke, traumatic brain injury, are characterized primarily by neurodegeneration and neuroinflammation.[2] Although intracerebral hemorrhage (ICH) characterized such as oxidative stress, excitotoxicity, neurotransmitter deficits, mitochondrial energy failure, and neuronal cell death that leads to behavioral and motor dysfunctions.[3] Stroke is an acute cerebrovascular disease which occurs as a result of sudden interruption of blood supply[4] to a part of brain typically by a thrombus or embolus occlusion or hemorrhage due to rupture of blood vessels[5] and age-related progressive, irreversible chronic neurodegenerative

Access this article online

Website: www.isfcppharmaspire.com | P-ISSN: 2321-4732 E-ISSN: XXXX-XXXX

disorder. Patients who have had a stroke are at increased risk of neurodegenerative complications as compared to the people of the same age group and the risk is posing major health challenge in India because of its high prevalence rate.[6] Stroke, which results from acute insult to the brain[7] and remains the third most common cause of death in industrialized nations, is placed at the seventh position among the ten leading causes of death in the developed countries.[8] It is estimated that, by 2050, the number of patients with cerebral stroke could be as high as 25 million.[9]

ICH having a common final finding, i.e., cognitive and motor impairment and this may occur as a result of multineurotransmitter deficits, decreased availability of intracellular molecules, and axonal transporters[10] through which the different neurons communicate with each other to maintain neuronal excitation and cognitive functioning.[10] At cellular levels, the storage of short-and long-term memory is associated with gene expression, *de novo* protein synthesis, and formation of new synaptic connections.

Intracellular molecules also known as secondary messengers such as cyclic nucleotides, i.e., cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) play a critical role in neuronal signaling and synaptic plasticity by activation of several pathways such as cAMP/protein kinase (PKA)/CREB, cGMP/PKG/CREB, and factors such as brain-derived neurotrophic factor (BDNF), semaphorins, netrin-1 and 16, nerve growth factor, and neurotrophins 3,4,5-inhibitory factors associated with myelin, and myelin-associated glycoprotein.[11] These pathways and factors are well known to help in neuronal survival and neurogenesis and protect neurons from injury.[12] Elevation of cAMP causes both short- and long-term increase in synaptic strength[13] and stimulates cholinergic neuronal cells to release acetylcholine.[14] However, the levels of cAMP and cGMP are reported to be decreased in neuropathological conditions including cerebral stroke and AD.[15]

It has been reported that cerebral ischemia-induced energy failure also leads to a reduction in the levels of key signaling molecules such as cAMP and cGMP and results in disruption of cAMP/PKA/CREB[15] and cGMP/PKG/CREB signaling pathways.[16] On the other hand, also reported to impair hippocampal long-term potentiation (LTP), a neurophysiological correlate of memory, by inhibiting the activation of both cAMP/PKA CREB as well as cGMP/PKG/CREB pathways in ICH pathology. The pyramidal CA1 neurons of hippocampus, involved in learning and memory, become a vulnerable target in cerebral stroke.[17]

Further, cAMP or cGMP dependent CREB phosphorylation has too been reported to induce long-term memory (LTP) and inhibit apoptotic and necrotic cell death.[18] CREB is a transcriptional factor responsible for the synthesis of proteins which are important for the growth and development of synaptic connections and increase in synaptic strength.[19]Thus, agents that enhance cAMP/PKA/CREB and cGMP/PKG/CREB pathways have potential for the treatment of stroke, AD, and other neurological diseases. cAMP and cGMP, mediate signaling of several neurotransmitters including serotonin, acetylcholine, glutamate, and dopamine which play an important role in cognitive functioning.[20] The activation of the cAMP-dependent protein kinase (PKA) significantly inhibits tumor necrosis factor-alpha (TNF-α) and inducible nitric oxide synthase in astrocytes and macrophages which are implicated in neuroinflammation.[21] and oxidative stress, respectively.[22] cAMP system is closely involved in the regulation of BDNF expression too[23] which play an important role in neuronal survival,[21] synaptic plasticity,[24] learning, and memory.[23] Further elevation of cAMP and cGMP levels is known to restore the energy levels, reduce excitotoxic damage, prevent Aβ mediated neurotoxicity, enhance biosynthesis and release of neurotransmitters,[25] and inhibit apoptotic and necrotic cell death, leading to improvement in cognitive functioning.[26]

Central administration of cAMP and cGMP has been reported to enhance neuronal survival[27] and memory performance.[26] In view of the above, the enhancement and prolongation of cAMP and cGMP signaling can thus be helpful in dealing with neurodegenerative disorders including ICH. This can be accomplished by activating the adenylyl cyclase enzyme which metabolizes these cyclic nucleotides. Forskolin (FSK), a major diterpenoid isolated from the roots of coleus forskohlii, directly activates the enzyme adenylyl cyclase, thereby increasing the intracellular level of cAMP and leading to various physiological effects.

Despite substantial research into neuroprotection, treatment options are still limited to supportive care and the management of complications. Currently available drugs provide symptomatic relief but do not stop the progression of disease.[6]Thus, the development of new therapeutic strategies remains an unmet medical need. Failure of current drug therapy may be due to their action at only one of the many neurotransmitters involved or their inability to upregulate signaling messengers reported to have an important role in neuronal excitability, neurotransmitter biosynthesis and release,[28] neuronal growth and differentiation, synaptic plasticity, and cognitive functioning.[29]

Phytochemicals drugs have been used since ancient times as medicines for the treatment of a range of diseases. Medicinal plants have played a key role in world health. In spite of the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care. Medicinal plants are distributed worldwide, but they are most abundant in tropical countries. Over the past decade, interest in drugs derived from higher plants, especially the phytotherapeutic ones, has increased expressively. It is estimated that about 25% of all modern medicines are directly or indirectly derived from higher plants. Phytomedicines are a standardized herbal preparation consisting of complex mixtures of one or more plants which are used in most countries for the management of various diseases. Other characteristics of phytochemicals are their wide therapeutic use and great acceptance by the population. In contrast to modern medicines, phytochemicals are frequently used to treat chronic diseases. Phytochemicals are normally marketed as standardized preparations in the form of liquid, solid, or various preparations. Compared with well-defined synthetic drugs, phytochemicals exhibit some marked differences, namely:

- The empirical use in folk medicine is a very important characteristic.
- They have a wide range of therapeutic use and are suitable for chronic treatments.

- The occurrence of undesirable side effects seems to be less frequent with herbal medicines, but well-controlled randomized clinical trials have revealed that they also exist.
- They usually cost less than synthetic drugs.

Therefore, as already mentioned above, one of the alternatives to enhance the levels of cAMP and cGMP secondary messengers or to enhance CREB phosphorylation can be achieved through activation of an enzyme adenylyl cyclase by herbal phytochemical FSK which are meant to increase these cyclic nucleotides. Based on the important and versatile role of cAMP signaling in the regulation of neuronal functions, the focus of the present study was directed to investigate the role of direct adenylyl cyclase activator FSK, cAMP selective enhancement in experimental models of ICH.

MATERIALS AND METHODS

Experimental animals

Male Wistar rats (weighing 220–250 g, aged 8–9 months) obtained from the animal house of the institute were employed in the studies. The animals were kept in polyacrylic cages with wire mesh top and soft bedding. They were kept under standard husbandry conditions of 12 h reverse light cycle with food and water ad libitum, maintained at 22 ± 2°C. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) (RITS/IAEC/2016/05/05) as per the guidelines of the committee for the purpose of control and supervision of experiments on animals, Government of India. Animals were acclimatized to laboratory conditions before experimentation.

Drugs and chemicals

FSK was provided as ex-gratia sample from BAPEX, Rajasthan. All other chemicals used in the study are of analytical grade. Solutions of the drugs and chemicals were freshly prepared before use. Autologous blood from rat was immediately collected in EDTA tubes and administered intracerebrally at a dose of 30 µl/10 min for continuous 8 days. FSK was dissolved in water (with 2% ethanol) and administered by oral route (p.o.).

Experiment protocol [Figure 1]

In experiment protocol schedule, there was total 23 day study. Behaviroal parameters (memory, cognition and motor activities) were performed on different-different time interval and at the end of protocol schedule biochemical estimations were analyzed in brain homogenate, blood serum and urine [Figure 1].

In the present investigation, the rats were randomly divided into six groups. Each group comprised of six rats. Where, groups were normal, FSK only, ICH Control, FSK20 + ICH, FSK40 + ICH, FSK60 + ICH. In the normal, FSK and ICH group, rats were treated with normal saline, FSK (60 mg/kg/d, orally) only for 22 days and ICH (30 µl/10 min/day) only for 08 days. Rats in the latter three groups were subjected to FSK (20, 40, 60 mg/kg/d, orally) administration prior to ICH (30 µl/10min/day) for 22 days.

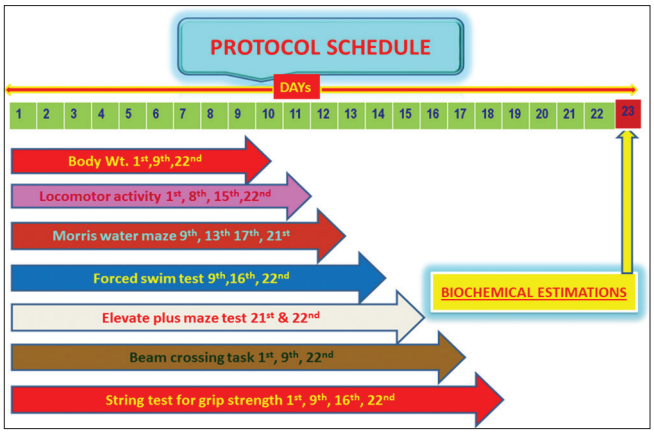

Figure 1: Experimental protocol schedule (Behavioral and Biochemical estimations)

Experimental animal models of hemorrhage-associated brain injury

ICH-associated brain injury has been modest and caused in part by limitations of model systems to reproduce the clinical situation. Cerebral hemorrhage has been modeled in the rat, rabbit, cat, dog, swine, and primate.[30] Significant technical and species differences in hemostasis question whether outcomes of model studies can be related to the human condition. These model systems have been used to examine the impact of hemorrhage on brain tissues: (1) Models mimicking primary hemorrhage involves induced cerebral artery rupture, stereotactic injection of blood into selected brain regions (e.g., basal ganglia), or stereotactic injection of matrix proteases (e.g., collagenase);[31-34] and (2) models of focal cerebral ischemia in which hemorrhagic transformation naturally occurs (e.g., primates and select rodent strains).[30] Hemorrhagic transformation has been modeled in the dog, cat, primate, rabbit, and rat, in which single arterial occlusion generates focal ischemia[35] and consistently produce hemorrhages encapsulated within structural boundaries (e.g. putamen, globus pallidus, and claustrum). Hemorrhagic infarction accompanies focal cerebral ischemia in the non-human primate in a manner similar to ischemic stroke.[36]

Few studies have been reported using rodent species. Primate and rodent studies have demonstrated the importance of platelet function for protection from hemorrhagic transformation. They also indicate that observations of hemorrhage in ischemic stroke are not uniformly mimicked in focal ischemia models. For modeling of ICH, intraparenchymal injection of autologous blood in rodent is the most commonly used method for pathophysiologic, biochemical, and behavioral studies.[37] Rat models involve blood infusions in the striatum and have focused solely on gray matter injury. Blood infusion models can induce ventricular rupture during the infusion, thereby potentially producing only ventricular hemorrhages. From these observations, the development of appropriate animal model systems for ICH and hemorrhagic transformation must be a priority. Models are needed that mimic human brain injury from hemorrhage [Figure 2].

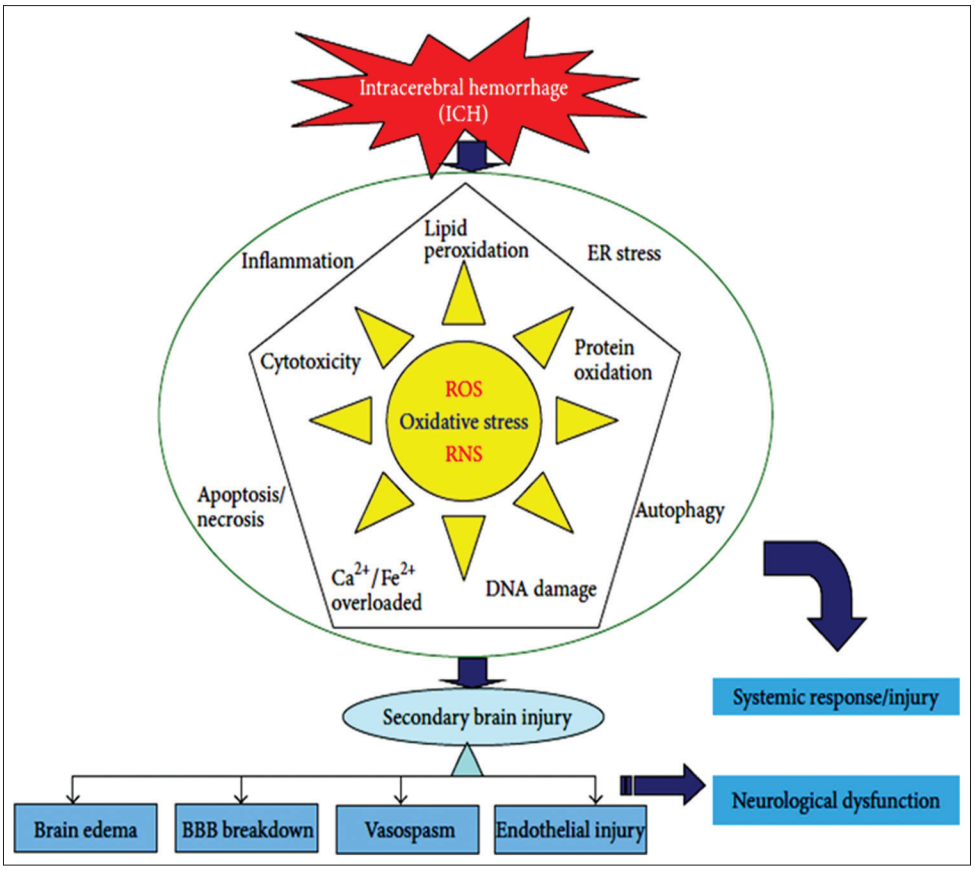

Figure 2: Schematic representation of major intracellular pathway during ICH. In the role of reactive oxygen species radicals in hemorrhagic stroke. ROS: Reactive oxygen species; RNS: Reactive nitrogen species; ICH: Intracerebral hemorrhage; ER stress: Endoplasmic reticulum stress; BBB: Blood brain barrier

Experimental procedure of ICH in rats

All rats in the experimental groups were anesthetized with ketamine (100 mg/kg, i.p) and xylazine (10 mg/kg, s.c.). A 1 cm long midline incision will be made in the scalp, beginning midway between the eyes and terminating behind the lambda. A cotton swab was used to clear away the soft tissue covering the skull. A Hamilton syringe was mounted onto the injection pump and the needle positioned directly over the bregma. The needle then positioned at the entry point, 0.5 mm posterior, and 1.0 mm lateral of the bregma to the right. A small cranial burr hole was drilled through the skull at the entry point. The rat tail was disinfected with 70% alcohol and immersed in warm, sterile water (40 uC) for 2 min. Rat tail artery was punctured with sterile needle (25G) and 30µl blood was drew in Hamilton syringe. The needle was slowly inserted into the right ventricle to a depth of 2.5 mm below the surface of the skull, and the blood was injected at a rate of 5 µL/min. The needle was left in place for 10 min and then removed at a rate of 1 mm/min to prevent the reflux of blood. The burr hole was filled with dental cement, and outer skin will be sutured.

Parameters evaluated

Measurement of body weight

Body weight was noted on the first and last days of the experiment. Percentage change in the body weight was calculated in comparison with the initial body weight on the 1st day of the experimentation.

$$\frac{\text{Bidyweight}(1^{st}\text{ day-}23^{rd}\text{ day})}{1^{st}\text{ day body weight}} \times 100$$

Measurement of brain weight

Brain weight was noted on the last days of the experiment after scarification of the animals.

Behavioral parameters

Spatial navigation task in Morris water maze

Spatial learning and memory of animals were tested in a Morris water maze on day 9th, 13th, 17th, and 21st day of protocol schedule (Morris, 1984). Before the training started, the rats were allowed to swim freely into the pool for 120 s without a platform. Animals received a training session consisting of four trials per session (once from each starting point) for 4 days (day 1, 2, 3, and 4) before final trail, i.e. on 9th, 13th, and 17th day according to 22-day protocol schedule, each trial having a ceiling time of 120 s and a trial interval of approximately 120 s. The time taken to locate the hidden platform time spent in target quadrant (TSTQ) zone was also measured. 24 h after the acquisition phase, a probe test (day 21), was conducted by removing the platform. Rats were allowed to swim freely in the pool for 120 s and the TSTQ, which had previously contained the hidden platform, was recorded. The time spent in the target quadrant

indicated the degree of memory consolidation which had taken place after learning.[38]

Spontaneous locomotor activity

Each animal was tested for spontaneous locomotor activity on day 1st, 8th, 15th, and 22nd. Each animal was observed in a square closed arena equipped with infrared light-sensitive photocells using a digital actophotometer for 5 min and values expressed as counts per 5 min.[39]

String test for grip strength

The rat was allowed to hold with the forepaws a steel wire (2 mm in diameter and 35 cm in length) and placed at a height of 50 cm over a cushion support on day 1st, 9th, 16th, and 22nd. The length of time the rat was able to hold the wire was recorded. This latency to the grip loss is considered as an indirect measure of muscle grip strength.[40]

Elevated plus maze (EPM) test

The EPM served as the behavioral model (wherein the stimulus existed outside the body) to evaluate learning and memory in rats. EPM was conducted for 2 days, i.e., 21st and 22nd day of protocol schedule. Each animal was kept at the end of an open arm, facing away from the central platform on 21th day. Transfer latency (TL) was recorded on 21th day, i.e., acquisition trial. Finally, TL was again examined 24 h after the first trial on 22nd day of protocol schedule, i.e., retention latency. TL was taken as the time taken by the animal to move into any one of the covered arms with all its four legs.[41]

Beam crossing task

This task requires an animal to walk on across a narrow wooden beam, measuring its motor coordination ability. To adapt to the elevated beam, a rat was allowed to explore it for 5 min before training on day 1st, 9th, and 22nd of protocol schedule. A training trial started by placing the rat on the platform at one end. When a rat walked across the beam from end to the other end, slipping of its feet occurred. A number of slip in each trial was recorded, and additionally, motor performance of rats was scored on a scale ranging from 0 to 4. A score of 0 was assigned to animal that could readily transverse the beam. Scores 1, 2, and 3 were given to animals demonstrated mild, moderate, and severe impairment, respectively. Score 4 was assigned to the animals completely unable to walk on beam.

Force swim test

The forced swim test (FST) is the most widely used test of antidepressant action and also used to infer "depression-like" behavior. In the Porsolt test, the first exposure is for 15 min and the second is performed 24 h after the first, with an exposure period of 5 min. The test for mice consists of a single 6 min exposure, with the first 2 min serving as a habituation period and the last 4 min consisting of the test itself, which yields the duration of immobility.[33]

Estimation of biochemical parameters

Preparation of homogenate

On 22nd day of protocol schedule, animals were scarified by decapitation; brains were removed and washed with ice cold isotonic saline solution. The brain tissue sample was then homogenized with 10 times (w/v) ice-cold 0.1 M phosphate buffer (7.4). The homogenate was centrifuged at 10,000×g for 15 min, supernatant separated, and aliquots used for biochemical estimation.

Estimation of biochemical parameters in serum and tissue homogenate

Protein estimation

The protein content was measured using Agappe protein estimation kit (Biuret method)

Lactate dehydrogenase (LDH) assay

A diagnostic kit (Trans Asia, India) was used to measure LDH activity in rat brain homogenate and serum it is expressed as IU/L.[42]

Estimation of malondialdehyde (MDA) levels

The quantitative measurement of MDA – end product of lipid peroxidation – in brain homogenate was performed according to the method of Wills.[43] The amount of MDA was measured, after its reaction with thiobarbituric acid, at 532 nm using spectrophotometer. The concentration of MDA was expressed as nM/mg protein.

Estimation of glutathione levels

Reduced glutathione in brain was estimated according to the method described by Ellman *et al*. 1 ml supernatant was precipitated with 1 ml of 4% sulfosalicylic acid and cold digested at 4°C for 1 h. The samples were centrifuged at 1200×g for 15 min. To 1 ml of the supernatant, 2.7 ml of phosphate buffer (0.1M, pH 8) and 0.2 ml of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were added. The yellow color that developed was measured immediately at 412 nm using a spectrophotometer. The concentration of glutathione in the supernatant expressed as μM/mg protein.[44]

Estimation of superoxide dismutase (SOD) activity

SOD activity was measured according to the method described by Misra and Frodvich (1972), by following spectrophotometrically the auto-oxidation of epinephrine at pH 10.4. In this method, supernatant (0.2 ml) of the brain homogenate was mixed with 0.8 ml 50 mM glycine buffer, pH 10.4, and the reaction was started by the addition of 0.02 ml of epinephrine. After 5 min, the absorbance was measured spectrophotometrically at 480 nm. The activity of SOD was expressed as % control.[45]

Estimation of catalase activity

Catalase activity was measured by the method of Aebi (1974). 0.1 ml of supernatant was added to the cuvette containing 1.9 ml of 50 mM

phosphate buffer (pH 7.0). Reaction was started by the addition of 1.0 ml of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm. The activity of catalase was expressed as μM/H_2O_2 decomposed/min activity.

Estimation of biochemical parameters in serum and urine

Estimation of total urea

A diagnostic kit (Trans Asia Biomedicals ltd.) was used to measure urea activity in rat blood as well as urine (urease method).[46]

Estimation of uric acid

A diagnostic kit (Transasia Bio-Medicals ltd.) was used to measure uric acid activity in rat blood as well as urine (modifier Trinder method, end point).[47]

Estimation of biochemical parameters in tissue homogenate for mitochondrial complex activity

Preparation of crude mitochondrial fraction from rat whole brain homogenate

The crude mitochondrial fraction was prepared by differential centrifugation following the procedure where the pellet formed during preparation of PMS was mixed with 0.1 M sodium phosphate buffer (pH 7.4) in 1: 10 proportion by stirring gently at 4°C for 60 min. The mixture was centrifuged immediately at 16.000 × g at 0°C for 30 min and the pellets were re-suspended in the same buffer containing additional sucrose at a concentration of 250 m mole/L. This step of centrifugation and re-suspension was repeated thrice, and finally, the crude mitochondrial fraction in buffered sucrose solution so obtained was used for further analysis.

Complex-II activity was measured spectrophotometrically. The method involves oxidation of succinate by an artificial electron acceptor and potassium ferricyanide. The reaction mixture contained 0.2 M phosphate buffer pH 7.8, 1% BSA, 0.6 M succinic acid, and 0.03 M potassium ferricyanide. The reaction was initiated by the addition of mitochondrial sample and absorbance change was followed at 420 nm for 2 min.

Complex-1 activity (NADPH dehydrogenase)

Adult rat brain fragments were homogenized mechanically and complex I activity was measured spectrophotometrically at 37°C during 3 min by the rate of NADH oxidation at 340 nm in an assay medium. Reactions were performed in the absence and the presence of 2 μM rotenone, and the rotenone-sensitive activity was attributed to complex I.

Complex II activity (succinate dehydrogenase/SDH)

SDH is a marker of impaired mitochondrial metabolism in brain. The quantitative measurement levels in brain were performed, where 0.3 ml of sodium succinate solution was mixed with the 50 μl of gradient fraction of homogenate. Moreover, the absorbance at 490 nm was determined with spectrophotometer (Shimadzu, UV-1700). Results were calculated using molar extinction coefficient of chromophore (1.36 × 104 M−1 cm−1) and expressed as INT reduced μmol/mg protein.

Complex IV activity (cytochrome oxidase)

Cytochrome oxidase activity was assayed in brain mitochondria according to the method of Sottocasa *et al*.[48] The assay mixture contained 0.3 mM reduced cytochrome c in 75 mM phosphate buffer. The reaction was started by the addition of solubilized mitochondrial sample, and the absorbance change was recorded at 550 nm for 2 min.

Complex V activity (ATP)

Aliquots of homogenates were sonicated immediately in ice cold perchloric acid (0.1 N) to inactivate ATPases. After centrifugation (14.000 g, 4°C, and 5 min), supernatants containing ATP were neutralized with 1N NaOH and stored at −80°C until analysis. ATP level in supernatants was quantified using a reverse-phase HPLC (Perkin Elmer). The detection wavelength was 254 nm and reference solution of ATP was prepared according to dissolving standard (Sigma, St. Louis.MO, USA).

Estimation of acetylcholinesterase (AChE) levels

The quantitative measurement of AChE activity in brain was performed according to the method described by Ellman *et al*. The assay mixture contained 0.05 ml of supernatant, 3 ml of 0.01 M sodium phosphate buffer (pH 8), 0.10 ml of acetylthiocholine iodide, and 0.10 ml of DTNB (Ellman reagent). The change in absorbance was measured immediately at 412nm spectrophotometrically. The enzymatic activity in the supernatant was expressed as μM/mg protein.[49]

Determination of protein carbonyl (PC)

Protein carbonyl content was assayed according to the method of Levine *et al.* with slight modification. The tissue homogenate (0.25 ml) was reacted with equal volume of 20% TCA. The carbonyl contents were measured at 370 nm in a spectrophotometer (Shimadzu-1601, Japan). The results were expressed as nanomoles of carbonyl per milligram of protein using a molar extinction coefficient of 22 × 10^3/M/cm.[50]

Estimation of nitrite levels

The accumulation of nitrite in the supernatant, an indicator of the production of NO, is determined by a colorimetric assay using Greiss reagent (0.1% N-(1-naphthyl) ethylene diamine dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid) as described by Green *et al*. The concentration of nitrite in the supernatant is determined at 540 nm spectrophotometrically compared from sodium nitrite standard curve and expressed as μM/mg protein.[51]

Estimation of biochemical parameters in serum

Estimation of complete blood count (CBC)

Determination of different hematological parameters such as red blood cells (RBC), white blood cells (WBCs), hemoglobin (HB), hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), RBC distribution width (RDW), neutrophils%, lymphocytes%, monocytes%, eosinophils%, basophils%, mean platelet volume (MPV), platelet distribution Width (PDW)%, plateletcrit (PCT)%, and platelets (PLTs) was measured in rat serum or blood sample through Balaji lab, Sirsa, Haryana, India.

Estimation of blood glucose levels

A diagnostic kit (Trans Asia Biomedicals ltd.) was used to measure blood glucose in rat (GOD-POD method).

Estimation of triglyceride levels

A diagnostic kit (Transasia Bio-Medicals Ltd.) was used to measure triglycerides activity in rat serum (Trinder method, end point).

Estimation of total cholesterol levels

A diagnostic kit (Transasia Bio-Medicals ltd.) was used to measure total cholesterol activity in rat serum (phototungstic acid method, end point).

Estimation of serum C-reactive protein (CRP) levels

A diagnostic kit (SPINREACT) was used to measure serum CRP level in rat (CRP-TURBI).

Inflammatory parameters in tissue homogenate

Animals were sacrificed by decapitation, brains removed, weighed, and rinsed with ice cold isotonic saline. Brain tissue samples were then homogenized with ice cold extraction buffer (1mM phenylmethylsulfonyl fluoride, 1µg/ml a protein, 0.05% Tween 20, and PBS to make up to volume to 4 ml/g of tissue). The homogenate was centrifuged at 5000 × g for 10 min and aliquots of supernatant separated and used for the estimation of inflammatory parameters.[52]

Enzyme-linked immunosorbent assay (ELISA)

Animals were sacrificed by decapitation, brains removed, weighed, and rinsed with ice cold isotonic saline. Brain tissue samples were then homogenized with ice cold extraction buffer. The homogenate was centrifuged at 5000 × g for 10 min and aliquots of supernatant separated and used for estimation of inflammatory parameters.

Estimation of TNF-α

The assay was performed using Quantikine TNF-α (R and D KIT system, Cat. No.RTA00; Becton Dickinson Biosc., India). Samples and all the reagents were prepared as described in the kit. The optical density of the reaction mixture was determined at 450 nm in the microtiter plate.

Interleukin-1β (IL-1β)

The assay was performed using Quantikine RAT IL-1β (R and D KIT system). Reagents, standard curve dilutions, and samples were prepared as directed in the R and D kit system. Optical density was determined using microplate reader (Thermo Multiscan Spectrum) set at 450 nm.

Estimation of IL-6

The assay procedure was carried out using Quantikine RAT IL-6 (R and D KIT system). All the reagents, standard dilution, control, and samples were prepared as described in the R and D kit system. After gently tapping the plate for mixing thoroughly the contents of all wells, the optical density of each well was determined at 450 nm.

Estimation of IL-10

The assay procedure was carried out using Quantikine RAT IL-10 (R and D KIT system). According to the kit system, all the reagents and samples were taken out of the deep freezer and allowed to reach room temperature and prepared as described in the kit system. To stop the reaction after 30 min, stop solution was added and the optical density determined at 450 nm within 30 min.

Estimation of biochemical parameters in urine

Urine output

At 21th day, rats were placed in metabolic cages for fasting at 6:30 PM for the collection of urine sample and next day (at 22nd day) urine will be collected and measured volume.

Urine dipstick test

Estimation of urine specific gravity, urine pH, protein, and glucose can be done through strip testing after the collection of urine at 22nd day (Siemens test).

Histophathological and morphological studies

The brain samples were fixed in 10% formalin solution before embedding in paraffin wax. The tissue was then processed and sectioned (10 µm 290 thick) using a rotary microtome. The sections were H and E (hematoxylin 291 and eosin stain) stained. The slides were then mounted with DPX 292 (Mixture of 10 g of distyrene 80, 5ml of dibutyl phthalate, and 35 ml of 293 xylene) and viewed under the light microscope (higher-power views, 294×400) and photographed using a digital camera (Nikon, Japan).

Morphologically, whole rat brain was isolated to measure the brain size and shape, meninges' cell surface damaging, and injury site.

Statistical analysis

All the results were expressed as mean and standard error mean (SEM). Data were analyzed using two-way ANOVA followed by Post hoc test Bonferroni and one-way ANOVA followed by *post hoc* test Tukey's multicomparison test. $P<0.05$ was considered as statically significant.

RESULTS AND DISCUSSION

Effect of FSK on body weight in ICH-treated rats

Chronic, ICH treatment (-17.83±0.95) caused a significant decrease in body weight as compared to normal (11.22±1.27) rats. Treatment with FSK *per se* (9.78±0.89) did not show any change in body weight as compared to normal animals. Pretreatment with FSK 20, 40, and 60 mg/kg, p.o., (-15.33±0.95, -10.98±0.83, and -9.01±0.81) in ICH-treated rats significantly attenuated the ICH induced loss in body weight. FSK 60 mg/kg, p.o. treatment was found to be most effective in curbing ICH-induced weight loss, as compared to FSK 20 and 40 mg/kg, p.o. treated rats [Figure 3].

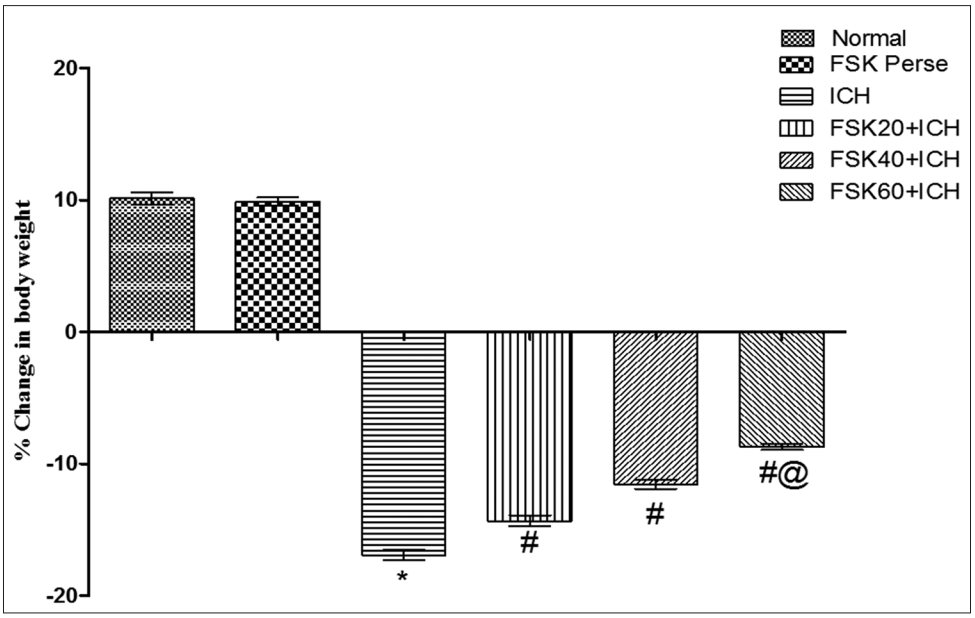

Figure 3: Effect of forskolin (FSK) on body weight in intracerebral hemorrhage (ICH) treated rats. Values are mean ± standard deviation ($n = 6$), *signifies $P < 0.05$ as compared to normal and FSK perse, $^{\#}P < 0.05$ versus ICH, $^{\#@}P < 0.05$ as compared to FSK (20+ICH) and FSK (40 + ICH)

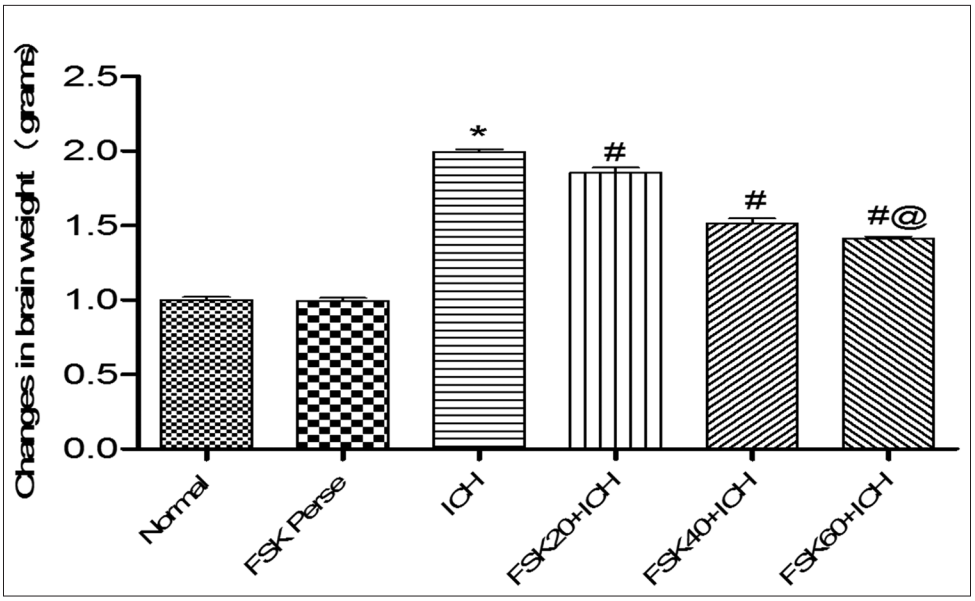

Figure 4: Effect of forskolin (FSK) on brain weight in intracerebral hemorrhage (ICH) treated rats. Values are mean ± standard deviation ($n = 6$), *signifies $P < 0.05$ as compared to normal and FSK perse, $^{\#}P < 0.05$ versus ICH, $^{\#@}P < 0.05$as compared to FSK (20+ICH) and FSK (40+ICH)

Effect of FSK on brain weight in ICH-treated rats

Chronic, ICH treatment (1.885±0.023) caused a significant increase in brain weight as compared to normal (1.120±0.021) rats. Treatment with FSK *per se* (1.009±0.02) did not show any change in brain weight as compared to normal animals. Pretreatment with FSK 20, 40, and 60 mg/kg, p.o., (1.94±0.026, 1.621±0.011, and 1.501±0.017) in ICH-treated rats significantly attenuated the ICH-induced increase in brain weight. FSK 60 mg/kg, p.o. treatment was found to be most effective in curbing ICH-induced weight increase, as compared to FSK 20 and 40 mg/kg, p.o. treated rats [Figure 4].

Behavioral parameters

To explore neuroprotective effects of FSK at the doses of 20, 40, and 60 mg/kg, p.o., behavioral parameters used were Morris water maze, EPM, locomotor activity, grip strength, beam crossing task, and FST.

Effect of FSK on rats in spatial navigation task using Morris water maze

On 9th, 13th, 17th, and 21stdays of protocol schedule, escape latency (ELT) was observed. On 9th, 13th, 17thday, there were significant

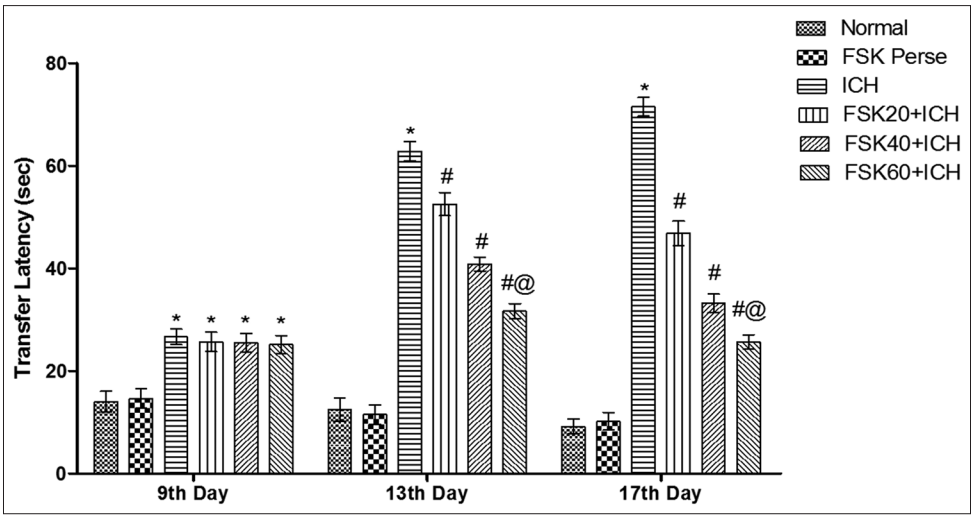

Figure 5: Effect of forskolin (FSK) on transfer latency of rats using Morris water maze, values are mean ± standard deviation ($n = 6$), *signifies $P < 0.05$ as compared to normal and FSK perse, $^{\#}P < 0.05$ versus intracerebral hemorrhage (ICH), $^{\#@}P < 0.05$as compared to FSK (20+ICH) and FSK (40+ICH)

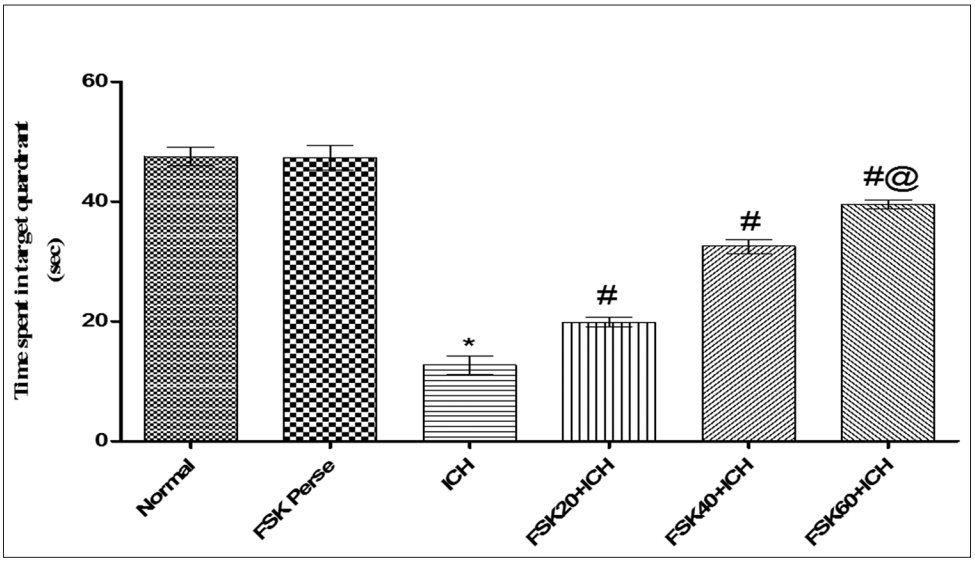

Figure 6: Effect of forskolin (FSK) on time spent in target quadrant of rats using Morris water maze. Values are mean ± standard deviation ($n = 6$), *signifies $P < 0.05$ as compared to normal and FSK perse, $^{\#}P < 0.05$ versus intracerebral hemorrhage (ICH), $^{\#@}P < 0.05$ as compared to FSK (20+ICH) and FSK (40+ICH)

changes observed in ICH (27.58±2.99, 68.76±4.21, and 77.41±4.23 s)-treated rats, when compared to normal (15.00±4.92, 13.31±4.98, and 10.00±4.01 s) and FSK *per se* governed (14.45±4.32, 12.70±5.00, and 11.07±4.25 s) rats. FSK *per se* administration did not show any significant changes when compared to normal rats.

On day 13th and 17th FSK at the doses of 20, 40, and 60 mg/kg, p.o., proved remarkable decreased (53.40±5.84, 48.53±5.43 s and 41.42±3.21, 35.63±4.78 s and 34.07±3.03, 24.09±3.28) in the ELT, when compared to ICH employed rats. FSK at the dose 60 mg/kg, p.o., at the day 13th and 17th was significantly decreased the ELT, when compared to FSK 20 and 40 mg/kg, p.o., treated rats, indicating remarkable improvement in learning [Figure 5].

On 22nd day of protocol schedule, TSTQ was performed. TSTQ in search of missing platform provided as an index of retrieval. ICH-treated rats showed remarkable decrease (13.57±3.45 s) in TSTQ when compared to normal (46.51±4.02 s) and FSK *per se*-treated (46.56±4.21 s) rats. In *per se* group of FSK, there were no changes during TSTQ when compared to normal group. FSK 20 and 40 mg/kg, p.o., administration showed a remarkable increase (20.52±2.02 and 33.42±3.09 s) in TSTQ when compared to ICH-treated rats. FSK 60 mg/kg, p.o., the administration indicated improvement (40.21±2.09 s) in memory function which shows markedly difference in between treatment doses of FSK 20 and 40 mg/kg, p.o. [Figure 6].

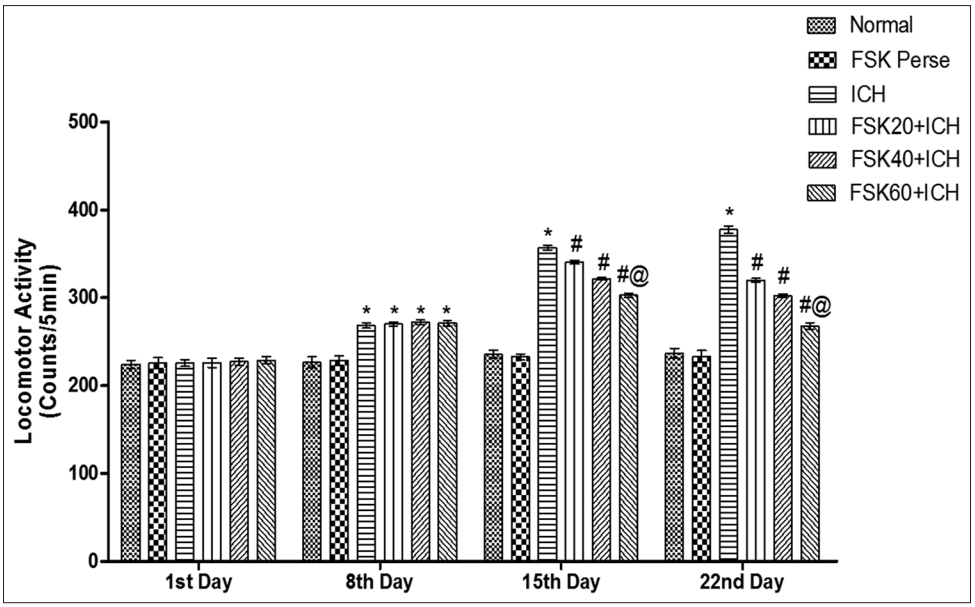

Figure 7: Effect of forskolin (FSK) on locomotor activity in intracerebral hemorrhage (ICH) treated rats. Values are mean ± standard deviation ($n = 6$), *signifies $P < 0.05$ as compared to normal and FSK perse, $^{\#}P < 0.05$ versus ICH, $^{\#@}P < 0.05$as compared to FSK (20+ICH) and FSK (40+ICH)

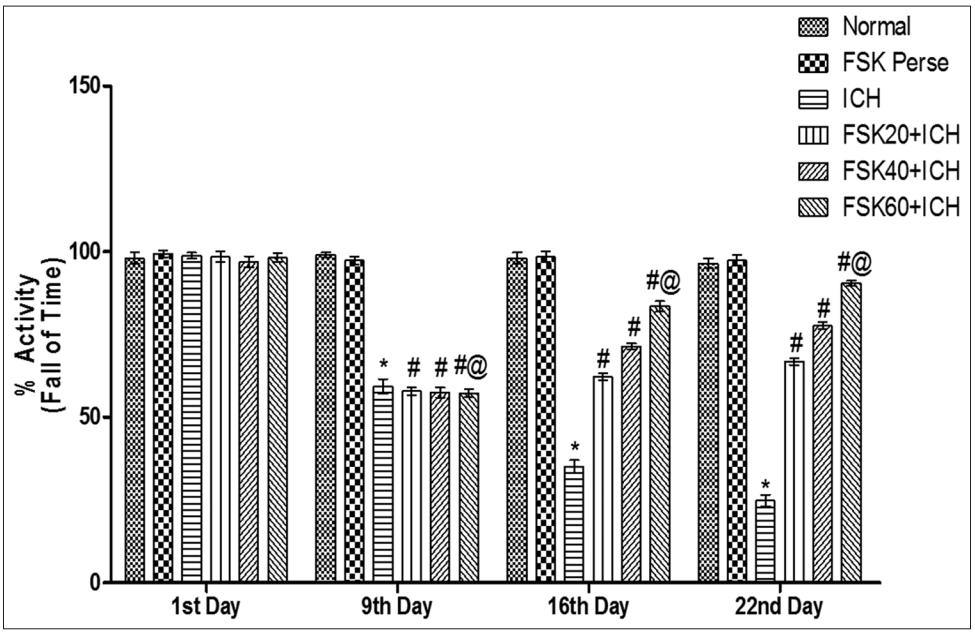

Figure 8: Effect of forskolin (FSK) on grip strength in intracerebral hemorrhage (ICH) treated rats. Values are mean ± standard deviation ($n = 6$), *signifies $P < 0.05$ as compared to normal and FSK perse, $^{\#}P < 0.05$ versus ICH, $^{\#@}P < 0.05$as compared to FSK (20+ICH) and FSK (40+ICH)

Effect of FSK on locomotor activity in ICH-treated rats

On 1st, 8th, 15th, and 22nd day of protocol schedule, locomotor activity was observed to rule out any interference in locomotor activity by treatment drugs. ICH employed rats revealed a significant change (232.7±10.08, 278.2±6.90, 364.4±5.90, and 387.8±10.04) in locomotor activity when compared to normal (234.2±10.51, 233.4±16.03, 229.4±10.15, and 235.2±12.94) and FSK *per se* (230.5±14.24, 238.4±12.99, 237.5±9.02, and 232.5±15.09) treated rats. FSK *per se* administration also did not show any considerable change in locomotor activity at 60 mg/kg, p.o. when compared to normal rats. FSK 20 mg/kg, p.o. (231.4±12.97, 276.4±5.21, 334.4±6.02, and 322.7±5.25), 40 mg/kg, p.o., (237.2±5.92, 282.3±6.01, 331.4±3.49, and 310.3±5.04), and 60 mg/kg, p.o., (230.5±10.21, 276.6±6.91, 321.4±5.91, and 259.2±10.07) administration shows significantly dose-dependent changes in locomotor activity of rats when differentiate to ICH-treated rats on day 1st, 8th, 15th, and 22nd, indicating there were significant effects on locomotor activity [Figure 7].

Effect of FSK on grip strength in ICH treated rats

On day 1st, there was no significant difference found in between all treatment groups in % activity (fall in time) using grip strength task. In ICH-administered group (60.23±5.12, 38.21±5.01, and 25.43±4.37), a significant loss in grip strength was recorded, measured by the reduction in time to hold the metal wire as compared to normal rats at the day 9th, 16th, and 22nd [Figure 8], However, chronic treatment with FSK 20 mg/kg, p.o. (59.23±3.14, 66.61±3.09, and 70.45±2.29), 40 (60.43±4.13, 73.56±2.45, and 76.72±2.42), 60 (59.73±3.76, 85.40±4.17, and 92.56±2.00) significantly improved ICH-induced loss in grip strength as compared to ICH control group in a dose-dependent fashion.

Effect of FSK on TL of ICH-induced rats using EPM

On 13th day of protocol schedule, acquisition latency was recorded. Retention was observed as TL on 14th day to evaluate learning and memory in rats using EPM. On 14th day, ICH-administered rats showed remarkable increase (14.67±4.19 s) in TL, when compared to normal (71.03±3.92 s) and FSK *per se*-treated rats (66.72±3.65 s). During the experiment, FSK *per se* administration did not reveal any change, when compared to normal rats in TL. Administration of FSK at the dose of 20, 40, and 60 mg/kg, p.o., exhibits notable decrease (32.36±3.27, 46.37±4.28, and 59.03±3.03 s) in TL, when compared to ICH-treated rats. There were expressively variation was found in between treatment doses of FSK 20, 40, and 60 mg/kg, p.o., indicating improved retention memory [Figure 9].

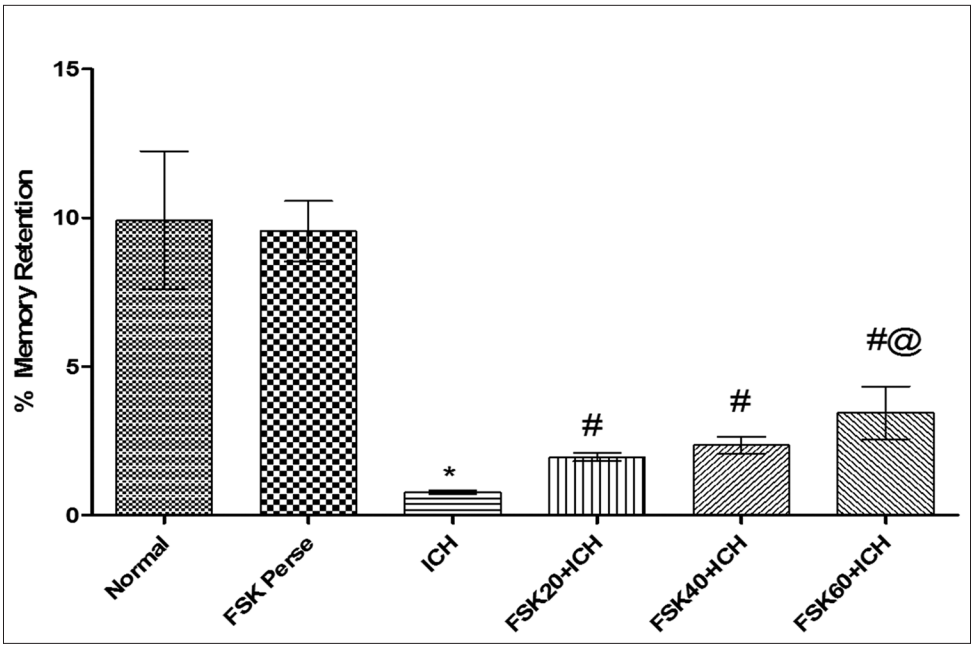

Figure 9: Effect of forskolin (FSK) on transfer latency of rats using elevated plus maze. Values are mean ± standard deviation ($n = 6$), *signifies $P < 0.05$ as compared to normal and FSK perse, $^{\#}P < 0.05$ versus intracerebral hemorrhage (ICH), $^{\#@}P < 0.05$as compared to FSK (20+ICH) and FSK (40+ICH)

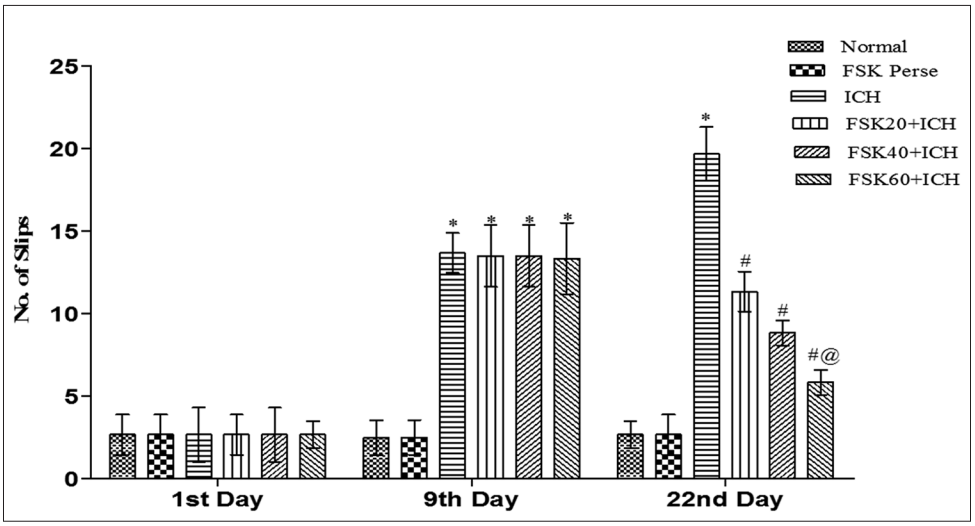

Figure 10: Effect of forskolin (FSK) on balance beam walking performance in intracerebral hemorrhage (ICH) treated rats. (Number of slips). Values are mean ± standard deviation ($n = 6$), *signifies $P < 0.05$ as compared to normal and FSK perse, $^{\#}P < 0.05$ versus ICH, $^{\#@}P < 0.05$ as compared to FSK (20+ICH) and FSK (40+ICH)

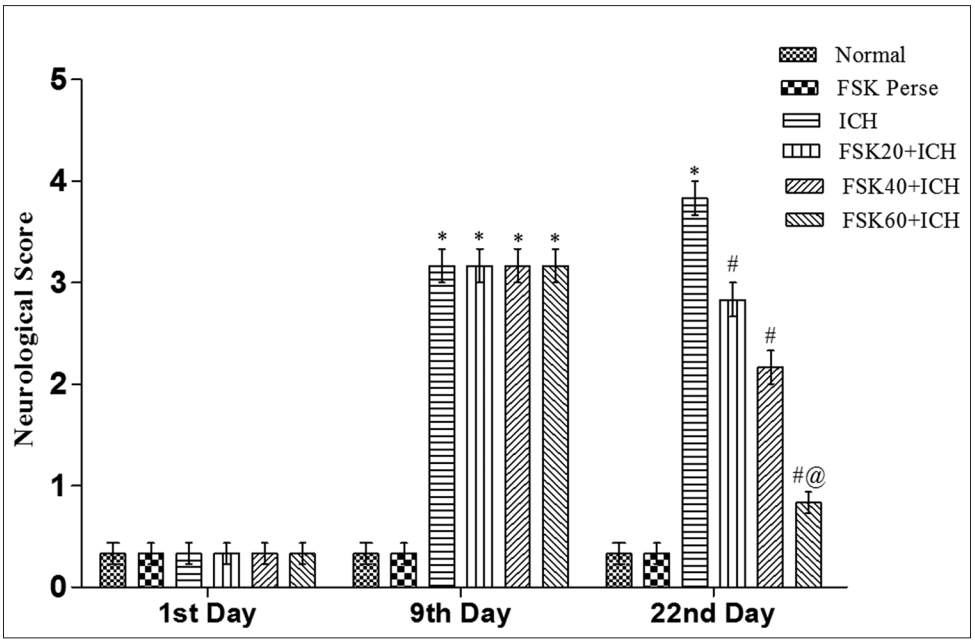

Figure 11: Effect of forskolin (FSK) on balance beam walking performance in intracerebral hemorrhage (ICH) treated rats. (Neurological score). Values are mean ± standard deviation ($n = 6$), *signifies $P < 0.05$ as compared to normal and FSK perse, $^{\#}P < 0.05$ versus ICH, $^{\#@}P < 0.05$as compared to FSK (20+ICH) and FSK (40+ICH)

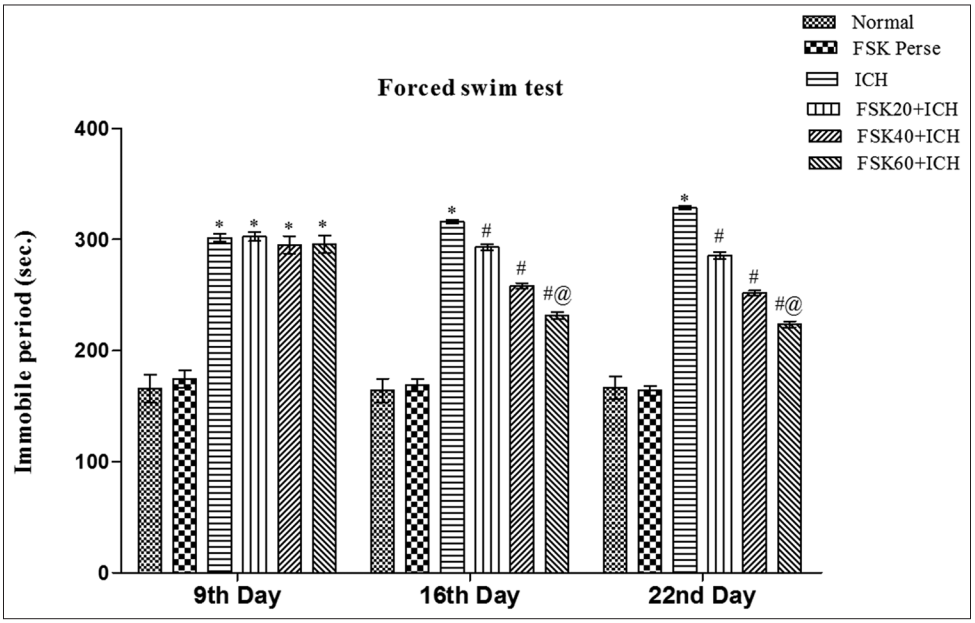

Figure 12: Effect of forskolin (FSK) on swimming performance in intracerebral hemorrhage (ICH) treated rats. Values are mean ± standard deviation ($n = 6$), *signifies $P < 0.05$ as compared to normal and FSK perse, $^{\#}P < 0.05$ versus ICH, $^{\#@}P < 0.05$ as compared to FSK (20+ICH) and FSK (40+ICH)

Effect of FSK on balance beam crossing task in ICH-treated rats

On day 1st, there was no significant difference found in between all treatment groups in the number of slips and in walking performance. In ICH control group, there was increase in number of slips (14.73±1.18 and 20.76±1.35) as well as impairment in beam walking performance (4.06±0.36 and 3.92±0.370) [Figure 10] on 9th and 22ndday when compare with normal (2.65±1.12 and 0.37±0.53) and FSK *per se* (2.78±1.19 and 0.32±0.57)-treated rats as indicated by neurological scoring. There was no significant difference found in between normal and FSK *per se*-treated rats. FSK 20, 40, and 60 mg/kg, p.o., treated rats shows significantly decrease the number of slips (14.06±1.73 and 11.95±1.15) and (13.47±1.79 and 8.36±0.59) and (13.35±2.65 and 4.94±0.54) as well as improve balance beam walking performance (3.65±0.69 and 2.34±0.63) and (3.64±0.73 and 2.63±0.60) and (3.26±0.63 and 0.83±0.25) on 9th and 22nd as compared to the ICH-treated group [Figure 11]. Moreover, FSK 60 mg/kg, p.o., was found more effective than FSK 20 and 40 mg/kg, p.o. in beam crossing task performance.

Table 1: Effect of FSK treatment against ICH induced bio-chemical changes (mitochondrial complex I, II, IV and V) in the rat brain homogenate

Treatment	Complex I (%control)	Complex II (nM/mg protein)	Complex IV (%control)	Complex V (μM/g tissue)
Normal	100.0±0.0	8.43±0.14	100.0±0.0	617.3±13.42
FSK perse	92.74±1.31	6.84±0.74	92.04±2.34	599.2±12.43
ICH control	26.35±2.54*	1.49±0.28*	31.34±2.67*	221.3±14.34*
FSK 20+ICH	35.05±3.91$^{\#}$	3.44±0.50$^{\#}$	44.58±3.14$^{\#}$	297.4±15.04$^{\#}$
FSK 40+ICH	49.04±7.18$^{\#}$	4.21±0.93$^{\#}$	62.04±3.29$^{\#}$	384.3±19.91$^{\#}$
FSK 60+ICH	71.25±6.83$^{\#@}$	5.48±0.22$^{\#@}$	79.14±2.96$^{\#@}$	476.6±20.42$^{\#@}$

Values are mean±SD (n=6), *signifies P<0.05 as compared to normal and FSK perse, $^{\#}P$<0.05 versus ICH, $^{\#@}P$<0.05 as compared to FSK (20+ICH) and FSK (40+ICH). FSK: Forskolin, ICH: Intracerebral hemorrhage, SD: Standard deviation

Table 2: Effect of FSK treatment against ICH induced bio-chemical changes (LDH and AChE levels) in the rat brain homogenate

Treatment	LDH (IU/L)	AChE (μM/mg protein)
Normal	411.2±11.83	11.23±0.47
FSK perse	401.5±12.22	12.04±0.30
ICH control	1204±23.93*	43.53±0.27*
FSK 20+ICH	866.2±21.34$^{\#}$	31.34±0.43$^{\#}$
FSK 40+ICH	693.3±14.06$^{\#}$	26.66±0.73$^{\#}$
FSK 60+ICH	455.3±16.18$^{\#@}$	19.92±0.84$^{\#@}$

Values are mean±SD (n=6), * signifies P<0.05 as compared to normal and FSK perse, $^{\#}P$<0.05versus ICH, $^{\#@}P$<0.05 as compared to FSK (20+ICH) and FSK (40+ICH). FSK: Forskolin, ICH: Intracerebral hemorrhage, SD: Standard deviation, LDH: Lactate dehydrogenase, AChE: Acetyl cholinesterase

Effect of FSK on FST in ICH-treated rats

In ICH-administered group (306.4±8.57, 321.4±4.05 and 331.5±4.73), a significant increase in immobility period of rats as compared to normal group at the day 9th, 16th, and 22nd. However, chronic treatment with FSK 20 mg/kg, p.o. (307.6±10.12, 296.0±6.62, and 282.2±7.06), FSK 40 (299.11±20.17, 252.2±5.91, and 255.7±5.68), FSK 60 (294.37±19.37, 234.3±8.00, and 229.3±6.06) significantly improved ICH-induced immobility as compared to ICH control group in a dose-dependent fashion [Figure 12].

Biochemical estimations

Estimations of biochemical parameters in brain homogenate

Effect of FSK on mitochondrial complexes I, II, IV, and V and enzyme activity of LDH and AChE in brain homogenates of rats

In comparison to normal and FSK *per se*-treated rats, a gradual decline in the brain mitochondrial complexes I, II, IV, and V content and increase in the level of LDH and AChE in individual brain homogenate samples (striatum, cortex, and hippocampus) when compared with ICH-treated rats. There was no significant difference found in between normal and SNL *per se*-treated groups.

Mitochondrial complexes I, II, IV, and V content was significantly increased in FSK 20 mg/kg, p.o., 40 mg/kg, p.o., and 60 mg/kg, p.o. as well as same significance results were found in LDH and AChE where these enzyme levels were remarkably decreased, respectively, when compared with ICH-treated rats. In treatment doses of FSK 60 mg/kg, p.o. was found effectively as compared with FSK 20 and 40 mg/kg, p.o. dose dependently in all individual samples of brain homogenate [Tables 1 and 2].

Effect of FSK treatment against ICH-induced biochemical changes (MDA, GSH, nitrite, SOD, and catalase levels) in the rat brain homogenate

During oxidative and nitrosative stress, autologous blood administration significantly decreased the GSH, SOD, and catalase and increase the MDA, protein carbonyl, and nitrite levels when compared to normal and FSK *per se* in brain homogenate of rats. FSK *per se* administration did not reveal any considerable change in any biochemical activity when collated to normal rats.

In treatment group, FSK 20 mg/kg, p.o. and 40 mg/kg, p.o. administration showed remarkable increase in GSH, SOD, and catalase activity where there was significant increase in the level of MDA and nitrite level when compared to ICH-treated rats, while the FSK 60 mg/kg, p.o., administration showed significantly modulates in biochemical activities when compared to ICH-treated rats and there was remarkably disparity found in between FSK-treated groups. As among all the doses of FSK, the dose at 60 mg/kg, p.o. found to be more effective when compared with FSK 20 and 40 mg/kg, p.o. treated rats [Tables 3 and 4].

Biochemical estimations in blood serum of rats

CBC

FSK 20 mg/kg, p.o., and FSK 30 mg/kg, p.o., remarkably increases RBC, PCV, and HB values in ICH-treated rats. MCV, MCH, and MCHC were not significantly affected. In ICH rats, WBC and lymphocytes counts were decreased. FSK 40 mg/kg, p.o., and FSK 60 mg/kg, p.o. doses significantly (P<0.05) raised the WBC and lymphocytes counts to the normal. Neutrophils and basophils were not significantly affected [Table 7]. FSK was increased the lowered platelets counts in the ICH-treated rats [Table 5].

Effect of FSK treatment against ICH-induced biochemical changes (LDH, urea, uric acid, GSH, nitrite, SOD, and catalase levels) in the rat serum

Administration of ICH significantly increases the LDH, urea, uric acid, and MDA levels and remarkably decreases the levels of SOD and catalase in blood serum of rats as compared to the normal and FSK *per se* grouped rats. FSK *per se* administration did not reveal any considerable change in these biochemical parameters when compared to normal rats.

In treatment group, FSK 20 mg/kg, p.o., and 40 mg/kg, p.o. administration showed remarkable decrease in LDH, urea, uric acid, and MDA levels and remarkably increase the levels of SOD and catalase in blood serum of rats level when compared to ICH-

Table 3: Effect of FSK treatment against ICH induced bio-chemical changes (MDA, GSH and nitrite levels) in the rat brain homogenate

Treatment	MDA (nM/mg protein)	Protein carbonyl (µM/g tissue)	Nitrite (µM/mg protein)
Normal	1.04±0.45	1.47±0.93	1.74±0.45
FSK perse	1.56±0.93	1.77±0.14	1.56±0.25
ICH control	13.37±0.46*	6.97±0.42*	10.35±0.46*
FSK 20+ICH	6.91±0.54$^{\#}$	4.36±0.15$^{\#}$	6.02±0.14$^{\#}$
FSK 40+ICH	4.45±0.16$^{\#}$	3.51±0.27$^{\#}$	3.45±0.37$^{\#}$
FSK 60+ICH	2.97±0.84$^{\#@}$	2.18±0.15$^{\#@}$	2.86±0.25$^{\#@}$

Values Are Mean±SD (N=6), *Signifies P<0.05 As Compared To Normal And FSK Perse, $^{\#}P$<0.05 Versus ICH, $^{\#@}P$<0.05 As Compared To FSK (20+ICH) And FSK (40+ICH). FSK: Forskolin, ICH: Intracerebral Hemorrhage, SD: Standard deviation, MDA: Malondialdehyde

Table 4: Effect of FSK treatment against ICH induced bio-chemical changes (SOD, catalase levels and protein carbonyl) in the brain homogenate of rat

Treatment	SOD (% control)	Catalase (% control)	GSH (µM/mg protein)
Normal	100.0±0.0	100.0±0.0	12.13±0.64
FSK perse	70.74±1.25	97.36±1.13	13.44±0.02
ICH control	20.36±4.45*	34.76±1.06*	2.17±0.45*
FSK 20+ICH	40.77±2.48$^{\#}$	55.79±1.14$^{\#}$	2.99±0.36$^{\#}$
FSK 40+ICH	54.73±3.92$^{\#}$	65.04±1.76$^{\#}$	3.94±0.32$^{\#}$
FSK 60+ICH	84.01±4.04$^{\#@}$	84.04±2.14$^{\#@}$	5.44±0.37$^{\#@}$

Values are mean±SD (n=6), * signifies P<0.05 as compared to normal and FSK perse, $^{\#}P$<0.05 versus ICH, $^{\#@}P$<0.05 as compared to FSK (20+ICH) and FSK (40+ICH). FSK: Forskolin, ICH: Intracerebral hemorrhage, SD: Standard deviation, SOD: Superoxide dismutase

Table 5: Effect of FSK treatment against ICH induced Hematological changes in rat serum

CBC parameters	Normal	ICH	FSK 40+ICH	FSK 60+ICH
RBC×1012/L	7.84	4.34	5.04	6.85
PCV %	44.20	27.64	32.31	38.47
HB g/Dl	14.2	6.23	9.12	10.83
MCV fL	63.95	60.98	50.35	55.66
MCH Pg	20.67	25.64	22.94	21.84
MCHC g/dL	29.64	40.69	35.13	32.47
Platelets×109/L	901	570	674	800
WBC×109/L	10.04	3.92	5.15	7.84
Lymphocytes %	45.15	33.47	36.19	40.04
Neutrophils %	36.34	29.16	30.47	32.58
Mid cells % (eosinophils, basophils and monocytes)	20.03	22.98	21.74	20.41

FSK: Forskolin, ICH: Intracerebral hemorrhage, SD: Standard deviation, CBC: Complete blood count, RBC: Red blood cell, WBC: White blood cell, HB: Hemoglobin, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration

Table 6: Effect of FSK treatment against ICH induced bio-chemical changes such as LDH, urea and uric acid in rat serum

Treatment	LDH (IU/L)	Urea (mg/dl)	Uric acid (mg/dl)
Normal	400.23±7.32	24.2±0.32	6.24±0.45
FSK perse	401.1±6.65	23.24±0.94	4.92±0.34
ICH control	1022.3±41.78*	74.37±0.53*	14.64±0.57*
FSK 20+ICH	887.2±4.54$^{\#}$	58.23±0.47$^{\#}$	7.37±0.35$^{\#}$
FSK 40+ICH	702.4±3.94$^{\#}$	42.92±0.34$^{\#}$	6.52±0.25$^{\#}$
FSK 60+ICH	459.3±4.53$^{\#@}$	32.5±0.36$^{\#@}$	5.25±0.35$^{\#@}$

Values are mean±SD (n=6), *signifies P<0.05 as compared to normal and FSK perse, $^{\#}P$<0.05versus ICH, $^{\#@}P$<0.05 as compared to FSK (20+ICH) and FSK (40+ICH). FSK: Forskolin, ICH: Intracerebral hemorrhage, SD: Standard deviation, LDH: Lactate dehydrogenase

treated rats, while the FSK 60 mg/kg, p.o., administration showed significantly modulates these biochemical levels when compared to ICH-treated rats and there were remarkably disparity was found in between FSK-treated groups. As among all the doses of FSK, the dose at 60 mg/kg, p.o., found to be more effective when compared with FSK 20 and 40 mg/kg, p.o., treated rats [Tables 6 and 7].

Inflammatory mediators

Effect of FSK on TNF-α, IL-1β, IL-10, and IL-6 levels in rat brain homogenate

Level of TNF-α, IL-1β, and IL-6 was remarkable increased and level of IL-10 was significantly decreased in ICH-administered rats as compared to normal and FSK *per se* rats brain homogenate. There was no significant difference was found in between normal and FSK *per se* treated groups. However, these animals when treated chronically with FSK 20 mg/kg, p.o., 40 mg/kg, p.o., and 60 mg/kg, p.o., showed a dose-dependent significant decrease in the TNF-α, IL-1β, and IL-6 and increased in level of IL-10, as compared with those of ICH control group. FSK 60 mg/kg, p.o., found to be more

effective when compared with FSK 20 and 40 mg/kg, p.o. treated rats [Table 8].

Estimation of Biochemical parameters in rat urine

Effect of FSK on urine output of rats [Table 9]

Effect of FSK on urine output of rats described in Table 9.

Effect of FSK on normal urine value of rats

In rat urine in all groups, urine sample was analyzed by Combistix (reagent strips for urine analysis) with Siemen's kit. There were no significance changes found in between all grouped animals in case of urine glucose (30 s) and urine pH. There were significantly changes observed, where urine protein (mg/dl) and specific gravity (45 s) were slightly increased when compared with normal and *per se*-treated rats.

Effect of FSK treatment against ICH-induced biochemical changes such as urea and uric acid of rat urine

Level of urea and uric acid was remarkable increased in ICH-administered rats as compared to normal and *per se* in rat urine. FSK *per se* administration did not reveal any considerable change in urea and uric acid level when compared to normal rats. In treatment group, FSK 20 mg/kg, p.o. and 40 mg/kg, p.o. administration showed a remarkable decrease in urea and uric acid level when compared to ICH-treated rats, while the FSK 60 mg/kg, p.o. administration showed significantly decrease in urea and uric acid level when compared to ICH-treated rats and there were remarkably disparity was found in between FSK-treated groups. As among all the doses of FSK, the dose at 60 mg/kg, p.o. found to be more effective when compared with FSK 20 and 40 mg/kg, p.o. treated rats [Table 10].

Effect of FSK on ICH-induced histopathological changes in rat brain

Brain histological section of the normal [Figure 13] and FSK per se [Figure 14] served rats showed optimally size, not harmed the pyramidal shape neuronal cells with a certainly observable cell's nucleus and remain cell membrane. FSK *per se* treatment did not show any remarkably effect on histological changes in independent brain sections.

The disease-controlled or ICH treated rats' [Figure 15] brain histological section showed jerky impaired cells and several neuronal space and dense pyknotic nuclei follow with marked focal diffuse gliosis as compared to normal and FSK *per se*-treated rats. FSK 20, 40, and 60 mg/kg p.o. [Figure 16] administration remarkably debilate ICH-induced histological modification as correlated to ICH-treated rats.

DISCUSSION

ICH is a clinical outcome that causes a deprivation of blood supply and energy in the brain due to blockade of carotid arteries. Most affected brain area as a consequence of GCI is CA1 neurons of hippocampus, cerebral cortex, and basal ganglia.[17] Neuronal death

Table 7: Effect of FSK treatment against ICH induced bio-chemical changes such as GSH, SOD, catalase and MDA in rat serum

Treatment	GSH (μM/mg protein)	SOD (%control)	Catalase (%control)	MDA (nM/mg protein)
Normal	42.24±1.24	100±0.00	100±0.00	3.15±0.15
FSK perse	41.25±1.35	95.24±0.45	96.12±0.46	3.21±0.21
ICH control	30.24±1.25*	46.74±1.36*	39.84±1.64*	8.25±0.15*
FSK 20+ICH	33.42±0.53$^{\#}$	59.46±1.36$^{\#}$	56.93±2.47$^{\#}$	6.92±0.24$^{\#}$
FSK 40+ICH	36.34±0.66$^{\#}$	68.35±1.84$^{\#}$	79.64±1.04$^{\#}$	5.02±0.24$^{\#}$
FSK 60+ICH	38.25±0.35$^{\#@}$	85.41±1.25$^{\#@}$	85.43±2.46$^{\#@}$	4.25±0.32$^{\#@}$

Values are mean±SD (n=6), *signifies P<0.05 as compared to normal and FSK perse, $^{\#}P$<0.05 versus ICH, $^{\#@}P$<0.05 as compared to FSK (20+ICH) and FSK (40+ICH). FSK: Forskolin, ICH: Intracerebral hemorrhage, SD: Standard deviation, SOD: Superoxide dismutase, MDA: Malondialdehyde

Table 8: Effect of FSK treatment against ICH induced bio-chemical changes such as TNF-α, IL-1β, IL-10 and IL-6 of rat brain homogenate

Treatment	TNF-α (pg/mg protein)	IL-1β (pg/mg protein)	IL-10 (pg/mg protein)	IL-6 (pg/mg protein)
Normal	35.21±1.92	72.33±3.43	51.31±2.52	38.63±3.45
FSK perse	34.44±2.35	73.54±3.13	52.44±2.47	41.26±2.92
ICH control	391.3±15.73*	567.4±14.12*	32.13±1.72*	246.7±6.24*
FSK 20+ICH	275.3±11.73$^{\#}$	412.3±6.25$^{\#}$	41.11±1.66$^{\#}$	222.4±6.52$^{\#}$
FSK 40+ICH	234.4±17.53$^{\#}$	357.3±9.27$^{\#}$	48.03±2.00$^{\#}$	172.4±2.84$^{\#}$
FSK 60+ICH	171.4±12.24$^{\#@}$	255.2±7.34$^{\#@}$	52.96±1.56$^{\#@}$	132.4±5.34$^{\#@}$

Values are mean±SD (n=6), *signifies P<0.05 as compared to normal and FSK perse, $^{\#}P$<0.05versus ICH, $^{\#@}P$<0.05 as compared to FSK (20+ICH) and FSK (40+ICH). FSK: Forskolin, ICH: Intracerebral hemorrhage, SD: Standard deviation, TNF-α: Tumor necrosis factor-alpha, c

Table 9: Effect of FSK treatment against ICH change in urine volume

Parameter	Normal	FSK perse	ICH control	FSK20+ICH	FSK40+ICH	FSK60+ICH
Urine volume (ml)	1.92 ml	1.89 ml	0.7 ml	1.2 ml	1.4 ml	1.7 ml

FSK: Forskolin, ICH: Intracerebral hemorrhage, SD: Standard deviation

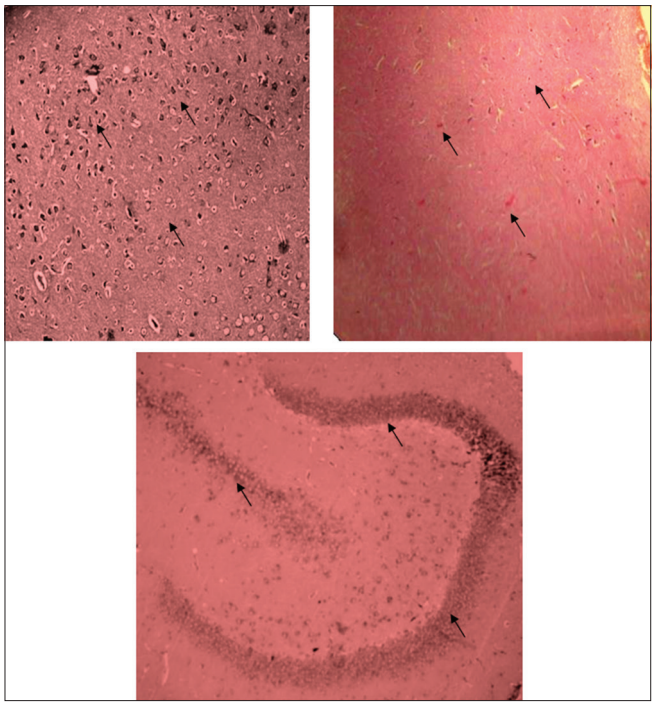

Figure 13: Panel A1 (basal ganglia), Panel A2 (cortex) and Panel A3 (hippocampus): Photomicrographs of hematoxylin and eosin stained brain sections of normal rats

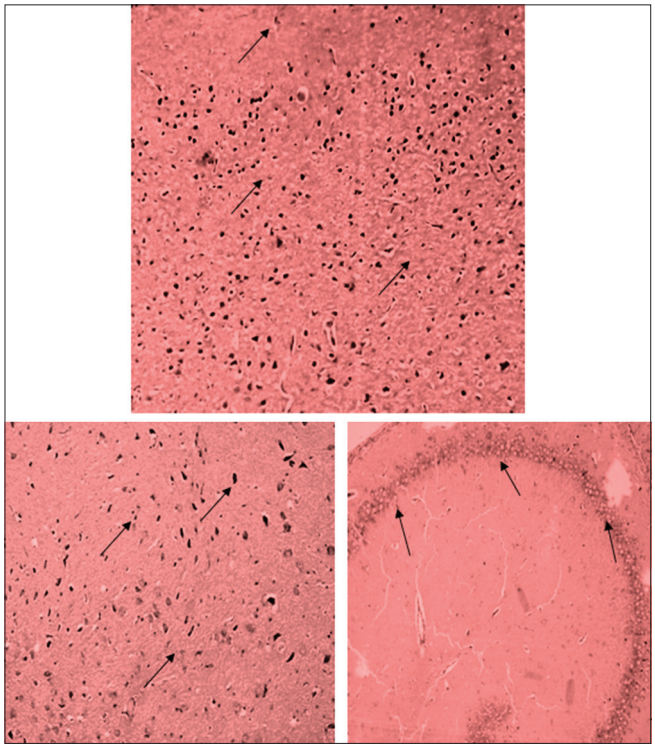

Figure 14: Panel B1 (striatum), Panel B2 (cortex) and Panel B3 (hippocampus): Photomicrographs of hematoxylin and Eosin stained brain sections of forskolin perse treated rats

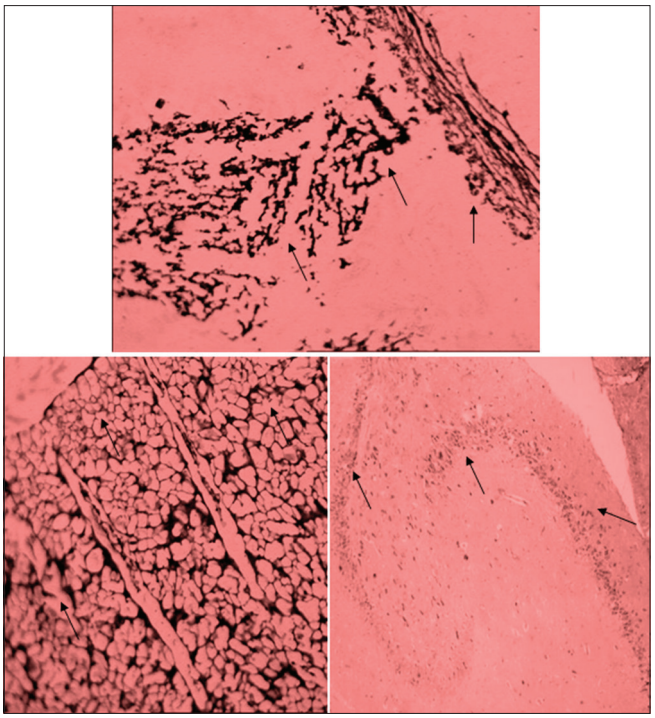

Figure 15: Panel C1 (basal ganglia), Panel C2 (cortex) and Panel C3 (hippocampus): Photomicrographs of hematoxylin and eosin stained brain sections of intracerebral hemorrhage treated rats

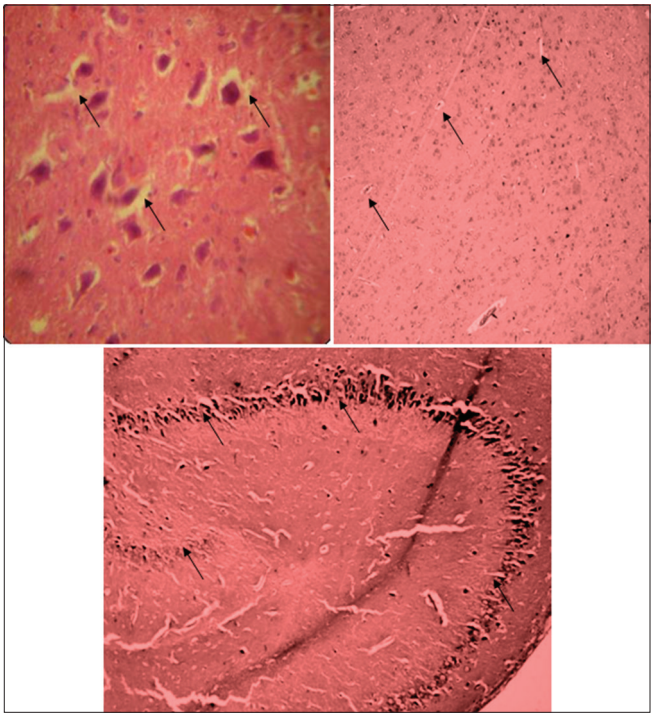

Figure 16: Panel D1 (basal ganglia), Panel D2 (cortex) and Panel D3 (hippocampus): Photomicrographs of hematoxylin and eosin stained brain sections of forskolin 60 mg/kg, p.o. treated rats

in cerebral stroke/ischemia proceeds 1–3 days after the insult, and the process is referred to as delayed neuronal death producing severe histopathological damages, cognitive deficits, and motor dysfunctions.[17]

Cyclic nucleotides, also known as second messengers, such as cAMP, are known to play an important role in memory and cognitive functioning.[24] These nucleotides lead to activation of AC/cAMP/PK_A/CREB signaling pathway that is important for regulation of

Table 10: Effect of FSK treatment against ICH induced bio-chemical changes such as urea and uric acid of rat urine

Treatment	Urea (mg/dl)	Uric acid (mg/dl)
Normal	15.25±1.35	3.92±0.21
FSK perse	16.35±1.64	3.95±0.24
ICH control	48.64±2.93*	12.34±1.32*
FSK 20+ICH	37.46±1.47$^{\#}$	8.84±0.36$^{\#}$
FSK 40+ICH	32.14±1.54$^{\#}$	7.24±0.25$^{\#}$
FSK 60+ICH	26.46±1.57$^{\#@}$	5.34±0.62$^{\#@}$

Values are mean±SD (n=6), *signifies P<0.05 as compared to normal and FSK perse, $^{\#}P$<0.05 versus ICH, $^{\#@}P$<0.05 as compared to FSK (20+ICH) and FSK (40+ICH). FSK: Forskolin, ICH: Intracerebral hemorrhage, SD: Standard deviation

neuronal growth and differentiation and several other factors related to cognition and motor functions.[34,35,53] Moreover, cAMP/PK_A/CREB signaling system has been implicated in cerebral ischemia/reperfusion-induced memory and motor dysfunction.

Modulation of cyclic nucleotides (cAMP) by direct activator of an enzyme adenylyl cyclase, i.e., FSK is found to enhance learning and memory and restore neurological functions as well as shown to enhance learning and memory and ameliorate experimentally induced amnesia or cognitive impairment in animals. Therefore, the present study was planned and executed to investigate the effects of selective adenylyl cyclase activator FSK in experimental models of ICH.

In the present study, the effect of improving mitochondrial dysfunction of FSK was evaluated using ICH-induced rats and discuss the dominant role of any played by cAMP signaling pathways in ICH-induced neurodegeneration. ICH experiment model is reported to inhibit mitochondrial complexes and also reduce the activity of complex I which leads to mitochondrial dysfunction and cellular energy deficit and capable of altering dopamine, serotonin, GABA, and glutamate systems in a manner similar to that observed in cerebral stroke and hemorrhage.

The current study, presented in this thesis, behavioral memory task was analyzed using MWM and EPM. Wherein, ICH control rats showed significant acquisition and retention deficit in MWM and EPM, compared to normal and FSK *per se*-treated animals. The ICH model has been used extensively, and after administration of autologous blood injection, it has shown to produce deficits such as memory deficits, imbalance movement will be produced in various motor tests such as EPM, Morris water maze, beam crossing, grip strength in the ICH rats. Furthermore, ICH has shown to cause a significant reduction in body and caused motor and behavioral abnormalities including bradykinesia, muscle weakness, and rigidity in animals.

In the present findings, a variety of neurobehavioral abnormalities observed including cognitive impairment, psychiatric complications, mood disturbances, and motor deficit in rats following ICH-treated rats. FSK dose dependently decreased the TL, increased time in target quadrant zone, immobility period; improve grip strength and motor movements. ICH-treated rodents pronounced biochemical damages of serotonergic, glutamatergic, GABAergic, cholinergic, and dopaminergic system in a similar manner to observe in ICH patients. ICH rats produce same behavioral, biochemical, electrophysiological, and neuropathological effects as seen in the cerebral stroke/ischemia. ICH affects diverse physiological processes such as cell signaling, neurotransmitter synthesis and release, mitochondrial function, lipid metabolism, immune functions, gap junctional gating, modulation of gene expression through DNA methylation, and histone acetylation. The neuropathological findings of ICH patients often show loss of purkinje cells (PCs) in cerebellum. A defect of mitochondrial respiratory chain activity results in impaired oxidative phosphorylation and an increase in free radical generation and thus will affect level of ATP.[53]

Mitochondrial dysfunctioning is associated with loss of ATP in the cell, further leads to decrease in the level of cAMP. In response to binding of a number of neurotransmitters to G-protein coupled receptors (GPCRs) and subsequent activation of AChE, cAMP is generated. Cyclic nucleotides by activating cAMP response element-binding protein (CREB) through PKA have been reported to play an important role in cognitive, motor functions and performs neuroprotective functioning associated with mitochondrial dysfunctioning. As increase in the ATP levels in the present study was found to be effective in attenuating ICH-induced neurotoxicity in the different brain regions. In the present findings, FSK, efficiently increases the ATP levels, is the sign of cAMP/CREB activation in the individual samples of brain homogenates.

During ICH, the most severely affected is the cholinergic system where learning and memory capacity of brain is governed by cholinergic system, especially the hippocampus, amygdala, and cortical regions are involved in cholinergic transmission. In the present study, there was found a significant increase in AChE activity, an enzyme responsible for degradation of Ach. This increase AChE activity was partially restored dose dependently by FSK. By restoring the AChE, the availability of Ach was prolonged and enhanced cholinergic function.

Recently, it has been reported that the experimentally ICH leads to neurotoxicity and impaired mitochondrial function through inhibition of oxidative phosphorylation and mitochondrial complexes I, II, IV, and V leading to respiratory chain dysfunction causing ATP depletion and increases oxidative stress. Moreover, there is disequilibrium is occurred between the ROS production, free radical scavenging, and antioxidant capacity of the cells, i.e. increase in the free radicals and decrease the activity of GSH, SOD, GPx, and catalase has been reported in the ICH brains, which might have led to improper removal of H_2O_2 and an increase in the production of hydroxyl radicals which are highly reactive. SOD is a potent scavenging enzyme that can selectively scavenge superoxide anion radical, which is an important source of hyperperoxides and first reduction product of molecular oxygen, by converting into H_2O_2 and O_2. H_2O_2 is the substrate of catalase and GP_X that catalyze the H_2O_2 conversion to water. ICH also potentiates the lipid peroxidation, indicated by formation of MDA, another oxidative stress marker. FSK treatment significantly enhanced the levels of SOD, catalase, total glutathione, reduced glutathione, MDA, and efficiently reduced the level of oxidized free radical, i.e., H_2O_2 and restored the activities of complex I, II, IV, and V.[54,55]

In ICH-treated rats, there was activation of microglia and elevates the levels of reactive astrocytes; these are known to produce nitric oxide and pro-inflammatory cytokines which is in tune with earlier

reports. In case of ICH increased levels of inflammatory cytokines were found. The inflammatory action of ICH was reported through the induction of pro-cytokines such as interferon-γ, IL-6, IL-10, and tumor necrosis factor-α (TNF-α). In this experiment, it was found that FSK treatment significantly reduced the level of inflammatory cytokines such as TNF-α, IL-6, and IL-1β and increases the level of IL-10 in brain tissue homogenate. Moreover, the protein oxidation (PC) and LDH were findings increased in the ICH-mediated rats. Chronic FSK administration regulates the level of protein and significantly reduced the levels of urea, uric acid, LDH, and PC following ICH induction in rats. It was also find that, in rat's urine, there was no significant changes were found in between all grouped animals in case of urine glucose and urine pH. However, there were some changes observed in urine protein and specific gravity, which are slightly increased in ICH rats.

The results of the present study suggest that chronic administration of FSK *per se* did not have any significant effect on behavioral and biochemical parameters in normal animals. However, FSK treatment groups at the dose of 20, 40, and 60 mg/kg, p.o., showed marked improvement in marked improvement in both behavioral and biochemical parameters, when compared to ICH-treated rats. The presented data in this study also suggest that FSK possesses potent antioxidant activity by scavenging ROS and exerting a neuroprotective effect against oxidative damage induced by ICH. FSK at the dose of 60 mg/kg, p.o., was found to be more neuroprotective in all behavioral and biochemical evaluations.

Moreover, a rodent ICH-mediated model system that shows impairment in motor and cognitive functions. We obtained results showing neuroprotective effects of FSK against ICH-associated neurotoxicity. Further, the AC activation found to be more effective in attenuating ICH-induced neurotoxicity. The cAMP/PKA/CREB pathway has been suggested to play a major role in the neuroprotective and restorative effects of AC activation. Moreover, the expression of CREB was shown to be markedly reduced during the progression of ICH as well as following ICH administration in experimental animals. Therefore, in current observations, neuroprotective, and mitochondrial complexes restorative effects of cAMP-mediated CREB initiated through direct AC activator, i.e., FSK. The possible mechanism of said effects of the compounds investigated may be due to their adenylyl cyclase activation, resulting in enhanced levels of second messengers which may be consisting to the neuroprotective mechanism of FSK.

CONCLUSION

The results presented in the present thesis show convincing that the autologous blood injection ICH rats exhibit brain lesions that were similar to the behavioral, histological, morphological, biochemical, neurochemical, and pathological features of ICH. ICH-mediated rats for continuous 8 days significantly induced cerebral hemorrhage in the rats as proven by behavior and biochemical parameters. Treatment with FSK (20, 40, and 60 mg/kg, i.p.) for 8–21 days effectively restore and remodulates the motor dysfunctions, biochemical, and neurochemical alternation induced by ICH rats. The findings observed in the current study reveal that adenylyl cyclase activator, i.e., FSK-mediated cAMP/PK_A/CREB activation might be a unique platform for the prevention of neurodegenerative diseases**.** Thus, in conclusion, we observed neuroprotective and neurorestoration effects of FSK may be due to favorable modulation of CREB-mediated signaling. The involvement of AC/cAMP/PK_A/CREB pathway, anti-oxidant, anti-inflammatory, and neuroprotective effect of test drug FSK may be the possible mechanisms at least in part underlying the observed effects. Furthermore, with AC/cAMP/PK_A/CREB signaling in regulation of neuronal functioning, the present study was designed to investigate the protective and therapeutic potency of FSK in ICH-mediated rat and to find out if cAMP-mediated CREB pathway is equally implicated in the disease pathogenesis or progression. Hence, now, we can finally conclude the magical mitochondrial restorative effects of the FSK may be due to showing its improved motor and cognitive functions as well as to restore the energy levels and antioxidant and anti-inflammatory defense system in experimental model of ICH.

ACKNOWLEDGMENT

The authors express their gratitude to Chairman, Mr. Parveen Garg and Director, Dr. G.D.Gupta, ISF College of Pharmacy, Moga (Punjab), India for their great vision and support.

REFERENCES

1. Swerdlow RH. Mitochondrial DNA - Related mitochondrial dysfunction in neurodegenerative diseases. Arch Pathol Lab Med 2002;126:271-80.
2. Kermer P, Jochen HJ. Neuronal apoptosis in neurodegenerative diseases: From basic research to clinical application. Neurodegener Dis 2004;1:9-19.
3. Alavi A, Clark C, Fazekas F. Cerebral Ischemia and Alzheimer's disease: Critical role of PET and implications for therapeutic intervention. J Nucl Med 1998;39:8.
4. Lipton P. Ischemic cell death in brain neurons. Physiol Rev 1999;79:1431-568.
5. Smith WS. Pathophysiology of focal cerebral ischemia: A therapeutic perspective. J Vasc Interv Radiol 2004;15:S3-12.
6. Sharma SS. Emerging neuroprotective approaches in stroke treatment. CRIPS 2003;4:8-12.
7. Yun YJ, Bombi LE, Dae-Hyun H. Neuroprotective effect of palmul-chongmyeong-tang on ischemia - Induced learning and memory deficits in the rat. Biol Pharm Bull 2007;30:337-42.
8. Minino AM, Arias E, Kochanek KD, Murphy SL, Smith BL. Deaths: Final data for 2000. Natl Vital Stat Rep 2002;51:119.
9. Steven JJ, Reinhart P, Menelas NP. Current concepts in therapeutic strategies targeting cognitive decline and disease modification in Alzheimer's disease. J Am Soc Exp Neuro Thers 2005;2:612-26.
10. Miyamoto E. Molecular mechanism of neuronal plasticity: Induction and maintenance of long term potentiation in the hippocampus. J Pharmacol Sci 2006;100:433-42.
11. Cai D, Qiu J, Cao Z. Neuronal cyclic AMP controls the developmental loss in ability of axons to regenerate. J Neurosci 2001;21:4731-9.
12. Frey U, Huang YY, Kandel ER. Effects of cAMP simulates a late stage of LTP in hippocampal CA1 neurons. Science 1993;260:1661-4.
13. Cammarota M, Bevilaqua LR, Rossato JI, Ramirez M, Medina JH, Izquierdo I. Relationship between short- and long-term memory and short- and long-term extinction. Neurobiol Learn Mem 2005;84(1):25-32.
14. Yao WD, Rusch J, Poo MM, Wu CF. Spontaneous acetylcholine secretion from developing growth cones of Drosophila central neurons in culture: Effects of cAMP-pathway mutations. J Neuroscience 2000;20:2626-2637.

15. Nagakura A, Niimura M, Takeo S. Effects of a Phosphodiesterase IV inhibitor rolipram on microsphere embolism-induced defects in memory function and cerebral cyclic AMP signal transduction system in rats. Br J Pharmacol 2002;135:1783-94.
16. Zhou X, Xiao-Wei D, Crona J. Vinpocetine is a potent blocker of rat $Na_V1.8$ TTX-resistant sodium channels. J Pharm Exp Ther 2003;306:498-504.
17. Euler MV, Bendel O, Bueters T. Profound but transient deficits in learning and memory after global ischemia using novel water maze test. Behav Brain Res 2006;166:204-10.
18. Francois M, Le CV, Dupont MA. Induction of necrosis in human neutrophils by Shigella flexneri requires Type III secretion, IpaB and IpaC invasins, and actin polymerization. Infect Immun 2000;68:1289-96.
19. Finkbeiner S. CREB couples neurotrophin signals to survival messages. Neuron 2000;25:11-4.
20. Fujita M, Zoghbi TS, Crescenzo MS. Quantification of brain phosphodiesterase 4 in rat with (R)-[11C]Rolipram-PET. Neuro Image 2005;26:1201-10.
21. Chong YH, Shin YJ, Suh YH. Cyclic AMP inhibition of tumor necrosis factor production induced by amuloidigenic c-Terminal peptide of alzheimer's Amyloid precursor protein in macrophages: Involvement of multiple pathways and cyclic AMP response element binding protein. Mol Pharmacol 2003;63:690-8.
22. Nakamura Y. Regulating factors for microglial activation. Biol Pharm Bull 2002;25:945-53.
23. Bishop JE, Joshi G, Mueller GP. Localization of putative calcium-response regions in the rat BDNF gene. Mol Brain Res 1997;50:154-60.
24. Rutten K, Lieben C, Smits L. The PDE4 inhibitor rolipram reverses object memory impairment induced by acute tryptophan depletion in the rat. Psychopharmacol Press 2007;192:275-82.
25. Imanishi T, Sawa A, Ichimaru Y. Ameliorating effects of rolipram on experimentally induced impairments of learning and memory in rodents. Eur J Pharmacol 1997;321:273-8.
26. Rutten K, Prickaerts J, Hendrix M. Time-dependent involvement of cAMP and cGMP in consolidation of object memory: Studies using selective phosphodiesterase Type 2, 4 and 5 inhibitors. Eur J Pharmacol 2007;558:107-12.
27. Prickaert J, Sik A, van Staveren WC, Koopmans G, Steinbusch HW, van der Staay FJ. Phosphodiesterase Type 5 inhibition improves early memory consolidation of object information. Neurochem Int 2004;45:915-28.
28. Rutten K, Prickaerts J, Blokland A. Rolipram reverses scopolamine-induced and time-dependent memory deficits in object recognition by different mechanisms of action. Neurobiol Learn Mem 2006;85:132-8.
29. Rydel RE, Greenet LA. cAMP analogs promote survival and neurite outgrowth in cultures of rat sympathetic and sensory neurons independently of nerve growth factor (neurotrophic agents/neuronal regeneration/neuronal differentiaion/8-(4-chlorophenylthio)-cAMP/8-bromo-cAMP). Proc Natl Acad Sci 1998;85:1257-61.
30. Bodison SC. Developmental dyspraxia and the play skills of children with autism. Am J Occup Ther 2015;69:1-10.
31. Mark HL, Bodfish JW. Repetitive behavior disorders in autism. Mental retardation and developmental. Disabil Res Rev 1998;4:80-9.
32. Persico AM, Napolioni V. Autism genetics. Behav Brain Res 2013;251:95-112.
33. Lamar M, Cutter WJ, Rubia K, Brammer M, Daly EM, Craig MC. 5-HT, prefrontal function and aging: Functional MRI of inhibition and acute tryptophan depletion. Neurobiol Aging 2009;30:1135-46.
34. Zafeiriou DI, Ververi A, Vargiami E. The serotonergic system: Its role in pathogenesis and early developmental treatment of autism. Curr Neuropharmacol 2009;7:150-7.
35. Chugani DC, Muzik O, Behen M, Rothermel R, Janisse JJ, Lee J. Developmental changes in brain serotonin synthesis capacity in autistic and nonautistic children. Ann Neurol 1999;45:287-95.
36. Anderson GM, Hertzig ME, McBride PA. Brief report: Platelet-poor plasma serotonin in autism. J Autism Dev Disord 2012;42:1510-4.
37. Burgess NK, Sweeten TL, McMahon WM, Fujinami RS. Hyperserotoninemia and altered immunity in autism. J Autism Dev Disord 2006;36:697-704.
38. Kumar P, Kumar A. Protective effect of rivastigmine against 3-nitropropionic acid-induced Huntington's disease like symptoms: Possible behavioural, biochemical and cellular alterations. Eur J Pharmacol 2009;615:91-101.
39. Singh S, Jamwal S, Kumar P. Piperine enhances the protective effect of curcumin against 3-NP induced neurotoxicity: Possible neurotransmitters modulation mechanism. Neurochem Res 2015;40:1758-66.
40. Olsson M, Nikkhah G, Bentlage C, Bjorklund A. Forelimb akinesia in the rat Parkinson model: Differential effects of dopamine agonists and nigral transplants as assessed by a new stepping test. J Neurosci 1995;15:3863-75.
41. Kumar P, Padi SS, Naidu PS, Kumar A. Cyclooxygenase inhibition attenuates 3-nitropropionic acid-induced neurotoxicity in rats: Possible antioxidant mechanisms. Fundam Clin Pharmacol 2007;21:297-306.
42. Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJ. cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. Cell 1997;89:73-82.
43. Wills ED. Mechanism of lipid peroxide formation in animal tissue. Biochem J 1966;99:667-76.
44. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70-4.
45. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972;247:3170-5.
46. Imaki J. A developmental study of cyclic AMP response element binding protein (CREB) by *in situ* hybridization histochemistry and immunocytochemistry in the rat neocortex. Brain Res 1994;651:269-74.
47. Silva AJ. CREB and memory. Annu Rev Neurosci 1998;21:127-48.
48. Sottocasa GL, Kuylenstierna B, Ernster L, Berqstrand A. An electron-transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. J Cell Biol 1967;32:415-38.
49. Ellman GL, Courtney KD, Anders V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88-94.
50. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 1990;186:464-78.
51. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal Biochem 1982;126:131-8.
52. Sassone-Corsi P. The cyclic AMP pathway. Cold Spring Harb Perspect Biol 2012;4:1-19.
53. Molinari G. Pathogenesis of secondary brain hemorrhage after ischemia: Lessons from animal models and a few from man, too! Thrombolytic Therapy in Acute Ischemic Stroke. Berlin: Springer-Verlag; 1993. p. 29-36.
54. Chauhan A, Gu F, Essa MM, Wegiel J, Kaur K, Brown WT. Brain region-specific deficit in mitochondrial electron transport chain complexes in children with autism. J Neurochem 2011;117:209-20.
55. MacFabe DF, Rodriguez-Capote K, Hoffman JE. A novel rodent model of autism: Intraventricular infusions of propionic acid increase locomotor activity and induce neuroinflammation and oxidative stress in discrete regions of adult rat brain. Am J Biochem Biotechnol 2008;4:146-66.