PI3K/AKT/mTOR Signalling Inhibitor Chrysophanol Ameliorates Neurobehavioural and Neurochemical Defects in Propionic Acid-induced Experimental Model of Autism in Adult Rats

Aarti Sharma

Indo Soviet Friendship College of Pharmacy

Sonalika Bhalla

Indo Soviet Friendship College of Pharmacy

Sidharth Mehan (✉ sidh.mehan@gmail.com)

Indo Soviet Friendship College of Pharmacy <https://orcid.org/0000-0003-0034-835X>

Research Article

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Abstract

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder marked by social and communication deficits as well as repetitive behaviour. Several studies have found that overactivation of the PI3K/AKT/mTOR signalling pathways during brain development plays a significant role in autism pathogenesis. Overexpression of the PI3K/AKT/mTOR signalling pathway causes neurological disorders by increasing cell death, neuroinflammation, and oxidative stress. Chrysophanol, also known as chrysophanic acid, is a naturally occurring chemical obtained from the plant *Rheum palmatum*. This study aimed to examine the neuroprotective effect of CPH on neurobehavioral, molecular, neurochemical, and gross pathological alterations in ICV-PPA induced experimental model of autism in adult rats. The effects of ICV-PPA on PI3K/AKT/mTOR downregulation in the brain were studied in autism-like rats. Furthermore, we investigated how CPH affected myelin basic protein (MBP) levels in rat brain homogenate and apoptotic biomarkers such as caspase-3, Bax, and Bcl-2 levels in rat brain homogenate and blood plasma samples. Rats were tested for behavioural abnormalities such as neuromuscular dysfunction using an actophotometer, motor coordination using a beam crossing task (BCT), depressive behaviour using a forced swim test (FST), cognitive deficiency, and memory consolidation using a Morris water maze (MWM) task. In PPA-treated rats, prolonged oral CPH administration from day 12 to day 44 of the experimental schedule reduces autistic-like symptoms. Furthermore, in rat brain homogenates, blood plasma, and CSF samples, cellular, molecular, and cell death markers, neuroinflammatory cytokines, neurotransmitter levels, and oxidative stress indicators were investigated. The recent findings imply that CPH also restores abnormal neurochemical levels and may prevent autism-like gross pathological alterations, such as demyelination volume, in the rat brain.

1.0 Introduction

Autism spectrum disorder (ASD) is a severe neurodevelopmental illness characterised by stereotyped or repetitive behaviour, memory and cognitive dysfunctions associated with sensory and motor function impairments (Gvozdjáková et al., 2014; Neumeyer et al., 2019). Early childhood ASD symptoms appear shortly after birth, resulting in lifelong disabilities (Jin et al., 2015). Neurochemical alterations trigger Autism-like repetitive and stereotypical behaviours in specific brain regions, including the hippocampus, cerebellum, amygdala, and cerebral cortex (Rahi et al., 2021; Lee et al., 2016; Morimoto et al., 2020; Sacai et al., 2020). Previous research has found that a subgroup of autistic children has gastrointestinal symptoms and aberrant gut microbiota (Kang et al., 2018; Finegold et al., 2012).

Autistic patients have increased numbers of the bacteria *Clostridium* and *Desulfovibrio*, which are known to produce short-chain fatty acids such as Propionic acid (PPA) (Finegold et al., 2011). Propionic acid (PPA) is a short-chain fatty acid that readily crosses the gut–blood barrier and can enter the CNS via the monocarboxylate transporter system (Mirza et al., 2019; Shams et al., 2019). PPA accumulates inside cells, causing intracellular acidification, mitochondrial malfunction (Mehan et al., 2020), increased oxidative stress markers (Rahi et al., 2021), altered neurotransmitters (Tiwari et al., 2021), and impaired synaptic transmission in autistic rat brains (Meeking et al., 2020). Sharma et colleagues found that stereotaxic PPA injection into the neocortex area promotes stereotyped behaviour and neurochemical changes in experimental models of autism in adult rats (Sharma et al., 2019).

The PI3K/AKT/mTOR signalling pathway is primarily involved in neural activities such as synaptic plasticity, neuronal development, memory consolidation, and protein synthesis (Kassai et al., 2014; Takei et al., 2014). It also affects a variety of physiological and biological processes, such as neuronal growth, axon guidance, cell proliferation, and differentiation (Rai et al., 2019; Jafari et al., 2019).

Upregulation of the PI3K/AKT/mTOR signalling pathway has also been associated with cerebral cell proliferation, axonal disruption, and megalencephaly in various brain regions (Kim et al., 2015; Subramanian et al., 2015; Hutsler et al., 2010). According to previous research, the PI3K/AKT/mTOR pathway governs translation in dendritic spines, and hence its activation increases dendritic spine malfunction, leading to autism-like symptoms (Ganesan et al., 2019; Soltani et al., 2017; Maiti et al., 2015). Overexpression of the PI3K/AKT/mTOR pathway resulted in learning and memory problems (Sharma and Mehan, 2021), social impairment (Mattioli et al., 2013), and abnormal synaptic plasticity (Li et al., 2010). The PI3K/AKT/mTOR signalling pathway has also been linked to the pathophysiology of several neurodevelopmental and neuropsychiatric disorders, including depression (Neis et al., 2020), cognitive development-associated brain malformation (Rivière et al., 2012), and epilepsy (Rivière et al., 2012). (Xiao et al., 2015; Brandt et al., 2018).

The PI3K/AKT/mTOR pathways are activated in the development of several neurodegenerative illnesses, including Huntington's disease (Abd-Elrahman et al., 2019), Alzheimer's disease (Hodges et al., 2018), and brain trauma (Xu et al., 2020). Furthermore, motor neuron illnesses such as Multiple Sclerosis (Giacoppo et al., 2017) and Parkinson's disease are caused by the overactivation of the PI3K/AKT/mTOR pathway (Chen et al., 2019).

Chrysophanol (CPH) is a 1, 8-dihydroxy-3-methyl derivative of the 9, 10-anthracenedione ring identified in *rheum rhubarbarum*, a herbaceous perennial plant in the Polygonaceae family (Singh et al., 2013). CPH has a wide range of pharmacological effects and biological activities, including anti-depressant, anti-bacterial, and anti-cancer properties (Rokaya et al., 2012). (Su et al., 2020). CPH also has antimicrobial, anti-inflammatory, and antiviral effects (Lian et al., 2017) and is used to treat a variety of neurological dysfunctions (Chae et al., 2017; Jeong et al., 2018). CPH has been shown in preclinical investigations to alleviate cognition deficits and neuronal death in streptozotocin-induced diabetic encephalopathy (Chu X et al., 2018). CPH has been demonstrated to protect against neurodegenerative diseases affecting the motor neurons, including multiple sclerosis (MS) (Lee et al., 2017) and Parkinson's disease (PD) (Chae et al., 2017). To summarise CPH's relationships with possible targets, Wang and Lv validated CPH's interaction with mTOR against malignant meningioma by blocking mTOR signalling and increasing NF2 signalling (Wang & Lv, 2021). Previous research on colorectal cancer (Deng et al., 2019) and colon cancer (Lee et al., 2011) found that CPH reduced PI3K/AKT/mTOR levels, reducing pathological conditions.

Based on the findings, we hypothesise that CPH can downregulate the abnormal PI3K/AKT/mTOR signalling mechanisms, reducing the neuropathological abnormalities in an ICV-PPA-induced experimental model of autism in adult rats. As a result, the current study looked at the

overexpression of PI3K/AKT/mTOR, which is involved in the pathogenesis of autism. We investigated the neuroprotective effect of CPH on behavioural, neurochemical, and morphological characteristics in an ICV-PPA-induced experimental model of autism in adult rats. Thus, CPH provides neuroprotection in autistic rats by downregulating the PI3K/AKT/mTOR signalling pathway, which was proven by examining neurochemical parameters in biological samples such as CSF, blood plasma, and brain homogenates.

2.0 Materials And Methods

Experimental animals

A total of 36 rats were used in the current study. All experiments were conducted on six-month-old adult Wistar rats' weight 250–300 g. Each group contains six rats, either sex; they were obtained from the Central Animal House, ISF College of Pharmacy, Moga, Punjab. India. Animals were housed in an acclimatized environment with a 12-hour light-dark cycle with food and water at room temperature at $23\pm 2^{\circ}\text{C}$. The Institute for Animal Ethics Committee (IAEC) approved the project as 816/PO/ReBiBt/S/04/CPCSEA as IAEC/CPCSEA/Meeting No: 27/2020/Protocol No. 454, following the guidelines provided by the government of India. The rats were randomly divided into six groups, and the sample size was based on a validated animal sampling method suggested by Charan and Kantharia, 2013. Animals were acclimatized to laboratory conditions before experimentation.

Drugs and chemicals

PPA was purchased from Sigma–Aldrich (USA). CPH was provided as an ex-gratia sample from BAPLEX, India. All other chemicals utilized in the experiments are of analytical grade. Before using the drugs and chemicals, fresh solutions were prepared. CPH was given orally by dissolving in an aqueous solution of 2% ethanol (Chu et al., 2018). The dosing of chrysophanol was determined based on previous research findings in various brain diseases, including ischemic brain injury (Zhao et al., 2016), learning and memory deficits (Dong et al., 2010), cognition deficits and neuronal loss (Chu et al., 2018), and Cerebral Ischemia (Zhang et al., 2014).

Experimental grouping of animals

The total duration of the experimentation was of 44 days. Propionic acid (PPA) was injected from day 1st to day 11th into the intracerebroventricular (ICV) region of the rat brain to induce autism. CPH was administered orally from day 12th to day 44th. Animals were randomly assigned into six groups. Group 1- vehicle control; Group 2- sham control; Group 3- Chrysophanol perse (20 mg/kg., p.o.); Group 4- PPA (10 μl /0.26M, i.c.v.); Group 5- PPA (10 μl /0.26M, i.c.v.) + Chrysophanol (10 mg/kg., p.o.); Group 6- PPA (10 μl /0.26M, i.c.v.) + Chrysophanol (20 mg/kg., p.o.). The present study was unblinded, and the experimenter was known regarding the care of animals. All behavioral parameters were conducted from day 1 to day 44th. On day 45th, after completing the protocol schedule, the blood plasma, CSF was collected from adult Wistar rats. Besides, Sodium pentobarbital 270 mg/ml, i.p., was used to anaesthetize the animals deeply. After anesthetization, the fresh brain was removed and preserved with ice-cold PBS (0.1 M) PBS for further neurochemical analysis. The experimental protocol is summarized in (Figure 1).

ICV-PPA induced experimental model of autism in adult rats

The PPA-induced experimental model of Autism in rats was established and validated by Tiwari et al., 2021. Experimental rats were treated with a PPA-ICV injection of 10 μl /0.26M for consecutive 11th days. The study by Rahi et al., 2021 suggests that PPA causes behavioral and neurochemical alteration similar to an experimental animal model of autism and is regarded as a validated experimental model for researching the pathophysiological changes identical to those seen in Autistic patients.

Rats were allowed to be habituated in a laboratory environment. Eventually, rats were anaesthetized by intraperitoneal injection of 75 mg/kg ketamine. Then, the rats were placed on the stereotaxic instrument (Stoelting Co., Wood Dale, IL, USA) in a skull-flat position. The positioning of the head was adjusted prior to the surgery to ensure that the bregma and lambda coordinates were similar and at the same level. The rats' heads were shaved, the scalp had been cleaned with 70% ethanol and incised with a blade (mid-sagittal), the skin was removed, and the skull was exposed to spot bregma and lambda that was indicated to assist in defining ICV injection coordinates. Wet cotton swabs were put on rat eyes to prevent dehydration, and cotton buds were used to stop bleeding. A hole was drilled in the skull (Stereotaxic coordinates: AP= -1.3mm; ML= -1.8mm; DV= -3.0mm), a cannula inserted in the burr hole, and the cannula was closed using a plastic ear-pin. The dental cement was filled in the hole and then sutured with an absorbable surgical suture attached to the sterile surgical needle (Gonzalez-Fraguela et al., 2013).

For post-operative care, rats were kept independently in a polyacrylic cage that contained warm cloth. Special care was needed until they restored spontaneous movement, which occurred approximately 2–3 hours after anaesthesia. The room temperature was set at $25\pm 3^{\circ}\text{C}$. For 2–3 days, milk and glucose water are provided inside the cages to avoid physical trauma after surgery. To prevent sepsis, gentamycin (35 mg/kg) was given intraperitoneally for three days, and lignocaine gel was applied to the sutured area to relieve the pain. Neosporin powder was sprinkled on them to prevent bacterial infection of the skin. After surgery, the body's overall health and clinical symptoms such as dehydration, body weight, infection, and other physical changes were closely monitored.

2.1 Parameters

Measurement of weight variations

Assessment of body weight

The body weight was measured on the 1st, 13th, 23rd, 33th, and 43rd days of the experiment protocol schedule (Sharma et al., 2019).

Measurement of relative brain-body weight ratio

The relative brain-body weight ratio was calculated on the 45th day of the experimental protocol schedule (Gopi et al., 2019).

Behaviour parameters

Morris water maze task (MWM)

The Morris water maze test was conducted to evaluate memory and cognitive impairment (Morris, 1984). Escape latency time (ELT) was measured using MWM on the protocol schedule's 40th, 41st, 42nd, and 43rd. Time (seconds) taken by rats to reach the target platform was considered as escape latency. On day 44th, rats were exposed to swim in the pool containing a hidden platform; for 120 seconds, and time spent in the target quadrant (TSTQ) was recorded. The TSTQ represents the degree of memory consolidation, which occurred after learning (Duggal et al., 2020).

Locomotor activity

The locomotor activity was performed on the 1st, 13th, 23rd, and 43rd days of the experimental protocol schedule using an actophotometer (INCO Group of Companies Dubai, United Arab Emirates). The behaviour parameter was evaluated using the method described by Mehan et al., 2018. The animal was placed in a digital actophotometer equipped with infrared photocells. They are then observed for five minutes in a square, the closed arena. The value of a digital actophotometer begins as counts per 5 minutes (Mehan et al., 2018).

Beam crossing task (BCT)

The beam crossing task was conducted on days 1st, 13th, 23rd, and 43rd of the experimental protocol schedule to evaluate motor coordination. During each trial, the number of foot slips was recorded, and additionally, the direction of an animal's fall was observed against the cut-off time of five minutes (Sharma et al., 2019).

Forced swim test (FST)

The forced swim test was used to measure the depressive-like behaviour of rats on the 1st, 13th, 23rd, and 43rd days. The first exposure of rats in the tank during the training phase is for 15 minutes, and the second is after 24 hours later for 5 minutes. A single six-minute exposure is used during the testing session. The first two minutes serve as a habituation period, with the final four minutes serving as the test itself, which determines the length of immobility (Minj et al., 2021).

Neurochemical parameters

Collection and preparation of biological samples

Blood plasma collection and separation

On day 45th of the protocol schedule, anaesthetized the rats with the chloroform before sample collection. Immediately after anesthetization, a capillary tube is placed at the medial canthus of the eye, and then the sinus is ruptured. Instantly 1-2 ml of blood was collected from the rats through retro-bulbar puncture (Kumar et al., 2017). The obtained blood samples were then cold centrifuged at 10,000×g for 15 minutes to separate the plasma. Then separated plasma was carefully stored at -80° C deep freezer for biochemical analysis.

CSF collection

Rats were deeply anaesthetized with 270 mg/ml sodium pentobarbital through i.p. injection. The rats' head was fixed using a holder to reveal the Arachnoid membrane and a skin incision was made, and a translucent dura mater was exposed. A maximum volume of 100µL CSF was obtained by direct inserting a 30-gauge needle at a 30° angle into the cisterna magna. Within 20 minutes after collection, the sample was centrifuged at 2000 g for 10 minutes at 4°C. After centrifugation, the supernatant was stored at -80°C until further analysis (Kozler et al., 2015).

Brain homogenate preparation

Rats were sacrificed by decapitation on the 45th day of the treatment schedule. The whole fresh brain was removed, washed with ice-cold isotonic saline solution, and homogenized with 0.1M (w/v) of chilled phosphate buffer saline (pH=7.4). The rat brain homogenate was then centrifuged at 10,000×g for 15 minutes, the supernatant was separated, and the aliquots were preserved. The samples were stored in a deep-freezer at -80°C to be used as and when the need for various neurochemical estimations (Rahi et al., 2021).

Assessment of cellular and molecular markers

Estimation of PI3K, AKT and mTOR protein level

PI3K protein level was measured in rat brain homogenate (David et al., 2015) and CSF (Tong et al., 2019) using an ELISA kit. According to the instruction given by ELISA assay kits (E-EL-H1019/PI3K; Elabsciences, Wuhan, Hubei, China). The AKT level was estimated in rat brain homogenate (Schipper et al., 2000) and CSF (Dong et al., 2018) (E-AB-21082/AKT; Elabsciences, Wuhan, Hubei, China), mTOR level was measured in rat brain homogenate (Wang et al., 2017) and CSF (Li et al., 2015) samples (E-EL-H1655/mTOR; Elabsciences, Wuhan, Hubei, China).

Estimation of MBP level

The MBP levels were assessed in rat brain homogenate using an ELISA kit (E-EL-R0642/MBP; Elabsciences, Wuhan, Hubei, China). The values were expressed in µg/mg protein (Rahi et al., 2021).

Assessment of Apoptotic Markers

Caspase-3 concentrations were assessed in brain homogenate (Rahi et al., 2021) and blood plasma (Guo et al., 2018) using an ELISA kit. Bax protein level was determined in brain homogenate (Tiwari et al., 2021) and blood plasma (Wang et al., 2019). The anti-apoptotic protein such as Bcl-2 levels was estimated in brain homogenate (Sharma et al., 2019) and blood plasma (Wang et al., 2018) using ELISA commercial kits (E-EL-R0160/Caspase-3; E-EL-R0098/Bax/Bcl2Elabsciences, Wuhan, Hubei, China). The values are expressed in ng/gm protein in brain homogenate and ng/ml in blood plasma.

Assessment of neurotransmitter levels

Measurement of Acetylcholine (Ach) level

Acetylcholine level was measured using a diagnostic kit (E-EL-008Ach; ELabSciences, Wuhan, Hubei, China). All samples and reagents were freshly prepared as per manual instruction provided by the kit's. The optical density of the reaction mixture was measured at 540 nm. The neurotransmitter in the supernatant was estimated and the value expressed as ng/mg protein (Rajdev et al., 2020).

Measurement of dopamine level

The dopamine levels in the striatal tissue sample were determined. Dopamine levels in the striatum are a sign of neural excitability, which leads to mood changes. The electrochemical detector was used to assess dopamine levels in the brain homogenate using the HPLC technique. The level of dopamine in brain homogenates is expressed as ng/mg protein (Sharma et al., 2021).

Estimation of glutamate level

Glutamate was assessed after derivatization with o-phthalaldehyde-/mercaptoethanol (OPA-/ME), and quantitative analysis in tissue samples using HPLC was carried out according to Rahi and her coworker's method. The glutamate level in rat brain homogenate is expressed as ng/mg protein (Sharma et al., 2019).

Measurement of serotonin level

Serotonin level was measured in the brain homogenate sample by HPLC using an electrochemical detector and C18 reverse-phase column. The mobile phase consisted of sodium citrate buffer (pH 4.5) – acetonitrile (87:13, v/v). The supernatant was filtered through 0.22 mm nylon filters before being injected into the sample injector. The serotonin concentration was assessed from the standard curve generated using a standard with a concentration of 10–100 mg/ml (Sharma et al., 2019).

Measurement of inflammatory cytokines levels

The level of TNF was determined in rat brain homogenate (Mehan et al., 2018) and Blood plasma (Fu et al., 2021) using a rat immunoassay kit. The activity of IL-1β was assessed in rat brain homogenate (Minj et al., 2021) and blood plasma (Nwangwu et al., 2017) (E-EL-R0019/TNF-α; E-EL-R0012/IL-1β; ELabSciences, Wuhan, Hubei, China) and value expressed as pg/mg protein (Yu et al., 2018).

Evaluation of oxidative stress markers

Estimation of acetylcholinesterase (AChE) level

Acetylcholinesterase concentration was estimated using spectrophotometrically. The assay mixture contained 0.05 ml of supernatant, 3 ml of 0.01M sodium phosphate buffer (pH 8), 0.10 ml of acetylthiocholine iodide, and 0.10 ml DTNB (Ellman reagent). The change in absorbance was recorded instantly at 412nm spectrophotometrically. The enzymatic activity in the supernatant was expressed as µM/mg protein (Deshmukh et al., 2009).

Measurement of superoxide dismutase (SOD) enzymatic activity

SOD activity was measured using spectrophotometrically by auto-oxidation of epinephrine at pH 10.4. The supernatant (0.2 ml) of the brain homogenate was mixed with 0.8 ml of 50 mM glycine buffer, pH 10.4, and the reaction was initiated with the addition of 0.02 ml epinephrine. After 5 minutes, the absorbance was spectrophotometrically measured at 480nm. SOD activity was quantified as nM/mg protein (Mehan et al., 2017).

Estimation of GSH level

The level of reduced glutathione in the brain was assessed using the method described by Ellman et al. 1959. 1ml supernatant was precipitated with 1 ml of 4% sulfosalicylic acid and cold digested at 4°C for one hour. The samples were centrifuged at 1200×g for 15 min. To 1ml 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 colour appeared immediately measured with a spectrophotometer at 412 nm. The glutathione concentration in the supernatant was expressed as µM/mg protein (Bala et al., 2015).

Estimation of nitrite level

The nitrite concentration in the supernatant, indicating the formation of nitric oxide (NO) is evaluated by a colorimetric assay using a Greiss reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid) as described by Green et al. 1982. Equal volumes of supernatant and Greiss reagent are mixed, the mixture incubated for 10 min at room temperature in the dark, and the absorbance determined spectrophotometrically at 540nm. The amount of nitrite in the supernatant is determined from a sodium nitrite standard curve and expressed as µM/mg protein (Mehan et al., 2020).

Estimation of malondialdehyde (MDA) level

The quantitative determination of malondialdehyde (MDA) was performed in brain homogenate. After its reaction with thiobarbituric acid, the concentration of MDA was measured at 532nm using a spectrophotometer and expressed as nM/mg protein (Mehan et al., 2011).

Protein estimation

The protein content was quantified by using the Coral protein estimation kit (Biuret method).

Gross pathological examination of rat brains

On the 43rd day, rats were sacrificed by decapitation; brains were removed for gross pathological analysis performed. After analyzing the whole brain, coronal sections were taken (Tiwari et al., 2021). Sectioned 2-mm thick brain pieces (coronally from the anterior pole to the posterior poles of the cerebral cortex) were placed on glass slides. A digital camera (Fujix digital camera, Fujifilm, Japan) was used to visualize all the brain regions. The demyelination region (mm) in each brain segment was measured on day 43rd after completing the procedure through MOTICAM-BA310 image plus 2.0 analysis software. The demyelination scale (mm) volume was calculated for each coronal brain segment by converting the demyelination region (mm). The demyelination size (mm³) in each brain section was measured from the dark greyish area near the striatum by image analysis on the 43rd day. The injury's size was calculated in each coronal 2-mm-thick brain section by calculating the demyelination area (l×b×h) (Rahi et al., 2021).

3.0 Statistical Analysis

Data were analyzed using two-way ANOVA followed by Post hoc test Bonferroni and one-way ANOVA repeated measures followed by Post hoc test Tukey's multi comparison test. P<0.001 was considered statistically significant. Data was found to be normalized, and the sample size was calculated by checking the normality distribution by the Kolmogorov Smirnov test. All statistical results were performed out by GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA). Statistical results were expressed as the mean±standard deviation (SD).

4.0 Results

4.1 Effect of chrysophanol on weight variations in ICV-PPA induced experimental model of autism in adult rats

Restoration of body weight after chronic administration with chrysophanol

Body weight was measured on the 1st, 13th, 23rd, 33th, and 43rd days of the protocol schedule. There was no significant difference in body weight between any treatment groups prior to treatment beginning. Rats who received daily PPA injections for 11 consecutive days had lower body weight on the 13th day as compared to the vehicle, sham, and CPH 20 per se treated groups. Prolonged oral administration of CPH 10 mg/kg and CPH 20 mg/kg restored body weight significantly on the 23rd, 33rd, and 43rd days when compared to PPA-treated autistic rats [two-way ANOVA: F(20,120)=157.72, p<0.001]. On the 43rd day, CPH 20 mg/kg was found to be more effective than CPH 10 mg/kg in successfully restoring body weight. (Figure 2a).

Restoration of relative brain-body weight ratio after chronic administration with chrysophanol

On the final day of the procedure, the relative brain-body weight ratio was assessed. On the 43rd day, there was no significant difference in the relative brain-body weight ratio among all groups. When compared to the vehicle, sham, and CPH 20 per se treatment groups, chronic ICV injection of PPA for 11 days resulted in a substantial reduction in the relative brain-body weight ratio. Long-term CPH 20 mg/kg and CPH 10 mg/kg administration led to an increase in relative brain-body weight ratio on the 43rd day when compared to PPA-treated autistic rats [one-way ANOVA: F(5,25)=1.218, p<0.001]. CPH 20 mg/kg, on the other hand, significantly restored the relative brain-body weight ratio as compared to the CPH 10 mg/kg treated group (Figure 2b).

4.2 Effect of chrysophanol in the amelioration of neurobehavioral alterations in ICV-PPA induced experimental model of autism in adult rats

Improvement in memory and cognition after chronic administration with chrysophanol

To assess memory and cognitive impairment, the Morris water maze test was used. The escape latency was measured on the 40th, 41st, 42nd, and 43rd days of the experiment protocol schedule. PPA-treated rats have a progressive increase in escape latency time (ELT) as compared to vehicle, sham, and CPH 20 perse treatment rats. Long-term oral CPH 10 mg/kg and CPH 20 mg/kg administration significantly reduced ELT in a dose-dependent manner when compared to the PPA-injected group [two-way ANOVA: $F(15,90)=19.48$, $p<0.001$]. Furthermore, CPH 20 mg/kg administered to rats reduced ELT more effectively than CPH 10 mg/kg administered to animals (Figure 2c). On the 44th day of the protocol schedule, the time spent in the target quadrant (TSTQ) was measured. TSTQ in chronic PPA-infused rats is considerably lower than in a vehicle, sham, and CPH 20 perse treated rats. Long-term CPH 10 mg/kg and 20 mg/kg administration enhances TSTQ in a dose-dependent manner compared to PPA-treated autistic rats [one-way ANOVA: $F(5,25)=25$, $p<0.001$]. CPH 20 mg/kg-treated rats improved TSTQ and memory consolidation more than CPH 10 mg/kg-treated rats (Figure 2d).

Improvement in locomotion after chronic administration with chrysophanol

Rat locomotion was observed using locomotor activity. The actophotometer device was used to perform the test on the 1st, 13th, 23rd, and 43rd days. On the first day of the protocol schedule, there were no significant differences between the treatment groups. PPA-injected rats had significantly lower locomotion on the 13th day compared to the vehicle, sham, and CPH 20 perse treated groups. In comparison to PPA-treated autistic rats, persistent oral treatment of CPH 10 mg/kg and CPH 20 mg/kg significantly improved locomotion on the 23rd and 43rd days [two-way ANOVA: $F(15,90)=644.72$, $p<0.001$]. CPH 20 mg/kg was more effective than CPH 10 mg/kg in improving locomotor activity on the 43rd day than CPH 10 mg/kg (Figure 2e).

Improved motor coordination after chronic administration with chrysophanol

The beam crossing task was used to assess rats' motor coordination abilities. The task was completed on the 1st, 13th, 23rd, and 43rd days. There were no significant changes between treatment groups on the first day. On the 13th day, chronic PPA-treated rats experienced considerably more slips than the vehicle, sham, and CPH 20 perse treated groups. Prolonged oral treatment with CPH 10 mg/kg and CPH 20 mg/kg on days 23 and 43 significantly reduced the number of slips in a dose-dependent manner compared to the PPA treatment group [two-way ANOVA: $F(15,90)=35.18$, $p<0.001$]. CPH 20 mg/kg was substantially more effective than CPH 10 mg/kg in reducing slip count and enhancing beam efficiency on the 43rd day (Figure 2f).

Reduced depression-like behavior after chronic administration with chrysophanol

The forced swim test was used to assess rats' depressive-like behaviour. The immobility time was recorded on the 1st, 13th, 23rd, and 43rd days. There was no significant difference between treatment groups on the first day. PPA-injected rats show longer immobility time significantly on the 13th day of the protocol schedule compared to the vehicle, sham, and CPH 20 perse treatment groups. Long-term oral administration of CPH 10 mg/kg and CPH 20 mg/kg to rats on the 23rd and 43rd days significantly reduces immobility time in a dose-dependent manner when compared to PPA-treated rats [two-way ANOVA: $F(15,90)=910.07$, $p<0.001$]. On the other hand, CPH 20 mg/kg was found to be more effective than CPH 10 mg/kg in considerably reducing immobility time and restoring depressive-like behaviour on the 43rd day (Figure 2g).

4.3 Effect of chrysophanol on neurochemical alterations in ICV-PPA induced experimental model of autism in adult rats

Decreased PI3K level after chronic administration with chrysophanol

The PI3K protein level was evaluated in rat brain homogenate and CSF samples at the end of the experimental protocol. When compared to the vehicle, sham, and CPH20 perse treated groups, PPA-treated rats have a significant increase in PI3K protein levels in rat brain homogenate and CSF samples. Long-term oral treatment of CPH at doses of 10 mg/kg and 20 mg/kg for 44 days consistently lower PI3K levels as compared to PPA-treated rats. CPH 20 mg/kg treatment group was shown to be more efficient than CPH 10 mg/kg treatment group in reducing PI3K levels in rat brain homogenate [one-way ANOVA: $F(5,25)=1.136$, $p<0.001$] and CSF samples [one-way ANOVA: $F(5,25)=0.256$, $p<0.001$]. (Table 1a.)

Table 1

Effect of chrysophanol on PI3K, AKT, mTOR and myelin basic protein level in ICV-PPA induced experimental model of autism in adult rats

S. no.	Groups	PI3K		AKT		mTOR		Myelin basic protein
		Brain homogenate (pg/g protein)	CSF (ng/ml)	Brain homogenate (pg/g protein)	CSF (ng/ml)	Brain homogenate (ng/g protein)	CSF (ng/ml)	Brain homogenate (µg/mg protein)
1.	Vehicle control	7.133±0.142	2.312±0.092	3.682±0.103	0.470±0.031	1.332±0.081	7.485±0.083	110.3±1.972
2.	Sham control	7.123±0.116	2.277±0.105	3.743±0.095	0.465±0.029	1.308±0.077	7.415±0.129	110.1±2.133
3.	CPH20 <i>perse</i>	7.070±0.130	2.298±0.097	3.822±0.076	0.460±0.036	1.303±0.050	7.422±0.084	110.7±1.526
4.	PPA	28.04±0.158*	4.485±0.091*	19.06±0.197*	1.560±0.020*	7.435±0.101*	11.01±0.069*	50.58±1.040*
5.	PPA+CPH10	21.77±0.146@	3.802±0.076@	14.71±0.260@	1.178±0.046@	5.865±0.078@	9.818±0.0875@	66.67±1.413@
6.	PPA+ CPH20	14.16±0.209@#	2.795±0.094@#	9.202±0.209@#	0.858±0.051@#	3.532±0.094@#	8.792±0.129@#	76.91±1.634@#
(Values expressed as mean±SD (n=6 rats per group). *p<0.001 v/s vehicle control, sham control and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (one-way ANOVA followed by Tukey's multiple comparison test)								

Decreased AKT level after chronic administration with chrysophanol

The AKT level was determined using an ELISA kit in rat brain homogenate and CSF samples. Chronic PPA injection significantly elevated AKT levels in rat brain homogenate and CSF when compared to the vehicle, sham, and CPH20 treatment groups. Prolonged oral CPH 10 and 20 mg/kg therapy significantly lowered AKT levels in brain homogenate and CSF compared to PPA-injected groups. Furthermore, CPH 20 mg/kg is shown to be more efficient than CPH 10 mg/kg in reducing AKT levels in brain homogenate [one-way ANOVA: $F(5,25)=1.209$, $p<0.001$] and CSF samples [one-way ANOVA: $F(5,25)=0.151$, $p<0.001$] (Table 1b).

Decreased mTOR level after chronic administration with chrysophanol

The level of mTOR was determined in rat brain homogenate and CSF samples. Long-term PPA-treated rats have higher mTOR levels in rat brain homogenate and CSF samples than the vehicle, sham, and CPH20 groups. Continuous oral administration of CPH 10 and 20 mg/kg significantly reduces mTOR levels in rat brain homogenate [one-way ANOVA: $F(5,25)=1.212$, $p<0.001$] and CSF [one-way ANOVA: $F(5,25)=0.551$, $p<0.001$] compared to PPA treatment groups. CPH 20 mg/kg was found to be more efficient in lowering mTOR levels in brain homogenate and CSF samples (Table 1c).

Restored myelin basic protein level after chronic administration with chrysophanol

The level of myelin basic protein (MBP) was determined in rat brain homogenates using an ELISA kit. PPA-injected rats have significantly lower MBP levels when compared to vehicle, sham, and CPH20 *perse* treated groups. Long-term oral treatment of CPH 10 and 20 mg/kg causes a significant increase in MBP levels as compared to PPA-injected rats [one-way ANOVA: $F(5,25)=1.687$, $p<0.001$]. CPH 20 mg/kg was more efficient than CPH 10 mg/kg in restoring MBP levels in rat brain homogenate (Table 1d).

Reduction in caspase-3, Bax, and increased Bcl-2 levels after chronic administration with chrysophanol

Neuronal apoptotic markers such as Caspase-3, Bax, and Bcl-2 were measured in rat brain homogenate and blood plasma. Prolonged PPA exposure resulted in significant elevations in Caspase-3 and Bax protein levels in rat brain homogenate and blood plasma. Furthermore, when compared to the vehicle, sham, and CPH20 *perse* groups, ICV-PPA treated rats have a significant drop in anti-apoptotic Bcl-2 levels in brain homogenate and blood plasma. Caspase-3 levels in brain homogenate [one-way ANOVA: $F(5,25)=0.210$, $p<0.001$] and blood plasma [one-way ANOVA: $F(5,25)=1.052$, $p<0.001$] are significantly reduced by persistent oral CPH therapy at 10 and 20 mg/kg.

Similarly, chronic oral CPH treatment at dosages of 10 mg/kg and 20 mg/kg results in a significant reduction in Bax levels in rat brain homogenate [one-way ANOVA: $F(5,25)=1.213$, $p<0.001$] and blood plasma samples [one-way ANOVA: $F(5,25)=1.246$, $p<0.001$]. Continuous oral treatment with CPH 10 and 20 mg/kg for 44 days resulted in significantly higher Bcl-2 levels in rat brain homogenate [one-way ANOVA: $F(5,25)=2.193$, $p<0.001$] and blood plasma [one-way ANOVA: $F(5,25)=3.179$, $p<0.001$]. In comparison, CPH 20 mg/kg was more effective than CPH 10 mg/kg in lowering apoptotic indicators and restoring anti-apoptotic markers in PPA-induced autistic rats (Table 2).

Table 2
Effect of chrysophanol on apoptotic markers level in ICV-PPA induced experimental model of autism in adult rats.

S. no.	Groups	Caspase-3		Bax		Bcl-2	
		Brain homogenate	Blood plasma	Brain homogenate	Blood plasma	Brain homogenate	Blood plasma
		(nM/mg protein)	(ng/ml)	(ng/mg protein)	(ng/ml)	(ng/mg protein)	(ng/ml)
1.	Vehicle control	117.4±0.858	1.840±0.049	9.167±0.269	0.833±0.035	34.69±0.553	9.208±0.036
2.	Sham control	118.5±0.622	1.830±0.043	9.098±0.595	0.846±0.033	35.22±0.437	9.143±0.043
3.	CPH20 <i>perse</i>	117.7±1.156	1.858±0.046	8.690±0.310	0.840±0.035	34.78±0.919	9.153±0.054
4.	PPA	178.4±1.313*	5.798±0.132	15.21±0.359*	5.223±0.092*	23.34±0.530*	1.815±0.056*
5.	PPA+CPH10	161.0±1.461@	3.248±0.136@	13.16±0.387@	4.315±0.152@	25.80±0.725@	4.425±0.203@
6.	PPA+CPH20	147.3±1.022@#	2.493±0.350@#	10.92±0.275@#	2.562±0.301@#	29.27±1.022@#	7.173±0.103@#
(Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (one-way ANOVA followed by Tukey's multiple comparison test)							

Restoration of neurotransmitters level after chronic administration with chrysophanol

Neurotransmitters such as serotonin, dopamine, acetylcholine, and glutamate were assessed in rat brain homogenate at the end of the treatment schedule. ICV-PPA injections resulted in a significant decrease in dopamine, serotonin, and acetylcholine levels, as well as an increase in glutamate levels, in rat brain homogenate, as compared to vehicle, sham, and CPH20 *perse*-treated rats. Long-term oral CPH 10 and 20 mg/kg treatment significantly increases dopamine [one-way ANOVA: $F(5,25)=2.546$, $p<0.001$], serotonin [one-way ANOVA: $F(5,25)=0.228$, $p<0.001$], and acetylcholine levels while decreasing glutamate levels in comparison to PPA-infused autistic rats [one-way ANOVA: $F(5,25)=0.807$, $p<0.001$]. Among these, CPH 20 mg/kg was more effective than CPH 10 mg/kg in restoring neurotransmitter levels in rat brain homogenate (Table 3).

Table 3
Effect of chrysophanol on neurotransmitters level in ICV-PPA induced experimental model of autism in adult rats.

S. no.	Groups	Neurotransmitters (Brain homogenate)			
		Serotonin	Glutamate	Dopamine	Ach
		(ng/mg protein)	(ng/mg protein)	(ng/mg protein)	(ng/mg protein)
1.	Vehicle control	46.99±0.416	119.9±0.945	102.2±1.867	9.047±0.215
2.	Sham control	47.22±0.519	120.2±0.518	101.8±2.186	8.955±0.279
3.	CPH20 <i>perse</i>	48.09±0.517	119.5±0.809	102.9±1.852	8.855±0.189
4.	PPA	17.34±0.648*	328.4±1.781*	38.15±1.265*	2.392±0.104*
5.	PPA+CPH10	24.05±0.457@	234.4±1.791@	54.84±0.842@	4.278±0.057@
6.	PPA+CPH20	31.06±0.407@#	198.6±1.690@#	61.91±1.163@#	5.872±0.191@#
(Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (one-way ANOVA followed by Tukey's multiple comparison test)					

Decreased inflammatory cytokines level after chronic administration with chrysophanol

Using an ELISA kit, inflammatory cytokines such as TNF and IL-1 β were quantified in rat brain homogenate and blood plasma samples. Chronic ICV-PPA treatment results in significant increases in proinflammatory mediators such as TNF and IL-1 β in rat brain homogenate and blood plasma as compared to the vehicle, sham, and CPH20 treated groups. TNF levels in rat brain homogenate [one-way ANOVA: $F(5,25)=0.357$, $p<0.001$] and blood plasma [one-way ANOVA: $F(5,25)=1.180$, $p<0.001$] are significantly reduced by continuous oral administration with CPH 10 and 20 mg/kg.

Similarly, long-term treatment with CPH10 and 20 mg/kg significantly reduced IL-1 β levels in rat brain homogenate [one-way ANOVA: $F(5,25)=4.582$, $p<0.001$] and blood plasma [one-way ANOVA: $F(5,25)=0.782$, $p<0.001$] compared to PPA treated rats. CPH 20 mg/kg treatment, on the other hand, was found to be more effective in lowering inflammatory cytokines when compared to CPH 10 mg/kg treated rats (Table 4).

Table 4
Effect of chrysophanol on inflammatory cytokines level in ICV-PPA induced experimental model of autism in adult rats.

S. no.	Groups	Inflammatory cytokines			
		TNF- α		IL-1 β	
		Brain homogenate (pg/mg protein)	Blood plasma (pg/ml)	Brain homogenate (pg/mg protein)	Blood plasma (pg/ml)
1.	Vehicle control	37.84 $\pm$ 0.838	30.04 $\pm$ 0.543	18.69 $\pm$ 0.621	13.30 $\pm$ 0.664
2.	Sham control	37.05 $\pm$ 0.548	30.07 $\pm$ 0.363	18.75 $\pm$ 0.627	13.53 $\pm$ 0.336
3.	CPH20 <i>perse</i>	36.90 $\pm$ 0.267	30.22 $\pm$ 0.382	18.72 $\pm$ 0.702	13.22 $\pm$ 0.100
4.	PPA	80.45 $\pm$ 1.342*	96.78 $\pm$ 1.545*	34.89 $\pm$ 0.478*	83.91 $\pm$ 0.363*
5.	PPA+CPH10	71.40 $\pm$ 1.370@	80.82 $\pm$ 1.955@	28.84 $\pm$ 0.668@	69.05 $\pm$ 0.610@
6.	PPA+CPH20	57.61 $\pm$ 1.850@#	59.98 $\pm$ 0.419@#	23.64 $\pm$ 0.440@#	46.68 $\pm$ 1.080@#

(Values expressed as mean $\pm$ SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (one-way ANOVA followed by Tukey's multiple comparison test)

Amelioration of oxidative stress markers level after chronic administration with chrysophanol

In rat brain homogenate, the levels of oxidative stress markers AchE, LDH, SOD, GSH, nitrite, and MDA were evaluated. Chronic PPA administration rats showed a significant increase in AchE, LDH, nitrite, and MDA levels in rat brain homogenate compared to the vehicle, sham, and CPH 20 *perse* treatment groups. ICV-PPA injected groups showed a significant reduction in anti-oxidant enzyme levels such as SOD [one-way ANOVA: F(5,25)=0.968, p0.001] and GSH [one-way ANOVA: F(5,25)

Prolonged oral treatment with CPH 10 and 20 mg/kg for 44 days resulted in significantly lower levels of AchE [one-way ANOVA: F(5,25)=1.266, p0.001], LDH [one-way ANOVA: F(5,25)=1.584, p0.001], nitrite [one-way ANOVA: F(5,25)=0.801, p0.001], and MDA [one-way ANOVA: F(5,25)=4.699, p<0.001] levels in brain homogenate. Similarly, continuous oral CPH 10 and 20 mg/kg treatment results in a significant rise in SOD and GSH levels in rat brain homogenate. Likewise, CPH 20 mg/kg is more effective than CPH 10 mg/kg in restoring anti-oxidant enzyme levels in rat brain homogenate (Table 5).

Table 5
Effect of chrysophanol on oxidative stress markers level in ICV-PPA induced experimental model of autism in adult rats.

S.no.	Groups	Oxidative stress markers (Brain homogenate)					
		AchE	LDH	SOD	GSH	Nitrite	MDA
		(μ M/mg protein)	(Unit/mg protein)	(μ M/mg protein)	(μ M/mg protein)	(μ M/mg protein)	(nM/mg protein)
1.	Vehicle control	23.86 $\pm$ 0.971	130.1 $\pm$ 1.206	492.3 $\pm$ 2.583	38.27 $\pm$ 0.790	6.89 $\pm$ 0.261	36.91 $\pm$ 0.315
2.	Sham control	24.24 $\pm$ 0.790	125.4 $\pm$ 0.545	491.9 $\pm$ 3.425	38.63 $\pm$ 0.886	7.11 $\pm$ 0.220	37.67 $\pm$ 0.622
3.	CPH20 <i>perse</i>	23.90 $\pm$ 0.886	126.6 $\pm$ 0.628	493.1 $\pm$ 3.004	39.10 $\pm$ 0.421	6.91 $\pm$ 0.181	37.11 $\pm$ 1.079
4.	PPA	60.25 $\pm$ 1.214*	428.7 $\pm$ 1.835*	347.8 $\pm$ 1.806	10.95 $\pm$ 0.422*	13.41 $\pm$ 0.458*	80.00 $\pm$ 0.880*
5.	PPA+CPH10	45.41 $\pm$ 0.874@	318.1 $\pm$ 1.057@	359.8 $\pm$ 0.914@	16.88 $\pm$ 0.732@	10.82 $\pm$ 0.450@	71.19 $\pm$ 0.824@
6.	PPA+CPH20	36.97 $\pm$ 1.346@#	289.8 $\pm$ 2.058@#	393.2 $\pm$ 2.183@#	24.76 $\pm$ 0.769@#	8.85 $\pm$ 0.234@#	63.37 $\pm$ 0.881@#

(Values expressed as mean $\pm$ SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (one-way ANOVA followed by Tukey's multiple comparison test)

4.4 Effect of chrysophanol in the restoration of gross pathological alterations in ICV-PPA induced experimental model of autism in adult rats

Restoration of whole-brain alterations after chronic administration with chrysophanol

The normal, vehicle, and CPH 20 *perse* treated groups all showed normal brain size and morphology. In comparison to the vehicle, sham, and CPH 20 *perse* treatment groups, the ICV-PPA-treated rat brains had a disrupted clotted outermost layer with rupture meninges. Prolonged oral administration of CPH at 10 mg/kg and 20 mg/kg doses repaired the morphological changes and aided the rat brain's recovery from subsequent injury. Similarly, animals given CPH 20 mg/kg showed considerable recovery in the damaged area of the brain as well as recovery of brain injury when compared to rats given CPH 10 mg/kg (Figure 3a).

Brain sections of rats treated with vehicle, sham, and CPH 20 mg/kg perse were structurally intact and undamaged, with clearly visible basal ganglia, cortex, and hippocampal tissue. The brain sections of the ICV-PPA treated rats showed cortical and hippocampus shrinkage, as well as atrophy in subcortical areas such as the medial thalamus, putamen, caudate nucleus, and internal medullary lamina, as compared to the vehicle, sham, and CPH 20 mg/kg, treated rats. The pathological abnormalities in rat brain slices were reversed by long-term oral treatment of CPH 10 and 20 mg/kg (Figure 3b).

Reduction in demyelination volume after chronic administration with chrysophanol

The normal, vehicle, and CPH20 mg/kg treated groups all demonstrated no significant change in demyelination volume. However, when compared to the normal, vehicle, and CPH20 mg/kg treatment groups, long-term administration of the neurotoxin PPA for 11 days significantly increased the area of demyelination. Long-term oral CPH administration at dosages of 10 mg/kg and 20 mg/kg significantly reduced demyelination volume in autistic-like rats compared to PPA-treated autistic-like rats. As a result, CPH 20 mg/kg had a dose-dependent effect on demyelination volume reduction when compared to CPH 10 mg/kg treated rats [one-way ANOVA: $F(5,25)=0.241$, $p=0.001$] (Figure 4)

5.0 Discussion

The current study used a PPA-induced experimental adult rat model to evaluate neurobehavioral and neurochemical abnormalities in autistic-like animals. Several investigations have found that ICV-PPA injection plays a significant role in developing autistic-like behaviour in adult rats (Rahi et al., 2021; Tiwari et al., 2021; Sharma et al., 2019). PPA-exposed rats exhibit behavioural and neuropathological abnormalities that are comparable to those seen in ASD patients, including hyperactivity, poor social interaction, stereotypic and repetitive movements, and have been recognized as a viable adult ASD model in rodents (Nemechek and Kathryn Moore, 2020; Chow et al., 2012).

PPA, as a weak organic acid, can passively accumulate within CNS cells, resulting in a fall in intracellular pH, which has several physiological implications (Thomas et al., 2010). PPA exerts a range of physiological effects on the brain, corresponding to the increased locomotion found in experimental rats (Lobzhanidze et al., 2020). As a result, it can alter neurotransmission in brain regions related to locomotor behaviour, such as the hippocampus and prefrontal cortex (Meeking et al., 2020; MacFabe et al., 2007). PPA-treated rat brains had higher amounts of cytokines and oxidative stress markers, as well as other abnormalities associated with ASD (MacFabe et al., 2011; Bhandari et al., 2017).

CPH was investigated to see whether it may protect against the behavioural changes caused by neurotoxin PPA in an experimental model of autism in adult rats. PPA infusion causes ASD-like behavioural and neuroinflammatory responses in adult rats. It is a neurotoxin that alters rat behaviour by impairing learning and memory, disrupting social interactions, and causing anxiety (Shultz et al., 2008; Ku et al., 2016; Wu et al., 2017). Additionally, we measured the protein levels of various cellular and molecular markers, apoptotic markers, neurotransmitters, inflammatory cytokines, and oxidative stress parameters in rat brain homogenate, blood plasma, and cerebrospinal fluid (CSF) samples. Long-term oral treatment of CPH at two different doses showed a neuroprotective effect against neurobehavioral and neurochemical changes in the ICV-PPA induced experimental model of autism in adult rats.

Bodyweight measurements taken on several days revealed that rats significantly decreased bodyweight after 11 days of ICV-PPA infusions. Prior research has shown that PPA, a short-chain fatty acid, influences weight loss in both animal and human populations due to altered fatty acid metabolism and increased gluconeogenesis after entering the citric acid cycle (Nankova et al., 2014). (Choi et al., 2018). In the current investigation, this weight loss increased in a dose-dependent manner after CPH administration. The brain-body weight ratio was also calculated by dividing the fresh brain weight by the bodyweight at the end of the experimental treatment schedule. The brain-body weight ratio was significantly lower in ICV-PPA-treated autistic rats, although it increased dose-dependently with CPH therapy. During the forced swim test, the immobility time was used to assess depressive-like behaviour in ICV-PPA induced experimental model of autism in adult rats. A previous study found that PPA therapy had an increased depressed effect (Bhandari et al., 2017). The current study's findings revealed an increase in immobility time after PPA injection that was mitigated by continuous CPH treatment.

Previous research has linked the PI3K/AKT/GSK3/mTOR/BDNF pathway to the development of mood-related disorders and has also been linked to the adaptive stress response (Lu et al., 2015). Numerous research has revealed an association between PI3K/AKT/mTOR and depression and anxiety (Leibrock et al., 2013; Moretti et al., 2014). In a chronic stress model of depression in mice, CPH treatment promotes antidepressant efficacy by blocking the mTOR signalling pathway (Zhang et al., 2016). All of these data confirmed our study's findings that CPH reduced depression symptoms in the PPA-induced experimental model of autism in adult rats.

Additionally, the hyperactivity and repetitive behaviours associated with autistic individuals have been highlighted as primary symptoms (Kong et al., 2021). As a result, PPA and other short-chain fatty acids increase glial and intracellular neuronal acidification and calcium levels, affecting neurotransmitter release, including serotonin, dopamine, glutamate, and norepinephrine (Daghestani et al., 2017; Thomas et al., 2012).

PPA has also been shown to enhance glutamatergic transmission, which causes excitability in brain areas related with locomotor activity. Our locomotor activity results showed increased locomotion after ICV-PPA injection, which is consistent with the findings of our previously reported studies (Tiwari et al., 2021; Sharma et al., 2019). There was a significant and dose-dependent improvement in hyperactive and repetitive behaviour after CPH treatment at doses of 10 mg/kg and 20 mg/kg. The beam crossing task assessed balance and motor coordination by counting the number of slips while moving across a wooden beam. Our results show that PPA autistic rats had a higher number of slips, indicating poor motor coordination, which was reduced in a dose-dependent way after CPH treatment.

Autistic children are reported to have poor spatial memory, cognitive impairments, and intellectual deficiencies (Zhang et al., 2020). The Morris water maze was used to test rats' long-term memory and spatial learning abilities. Mephem et al. found that intracerebroventricular PPA injection decreased spatial cognition in adult rats (Mephem et al., 2019). Our results demonstrated that rats treated with PPA had severe memory loss as a result of increased ELT and decreased TSTQ. Additionally, CPH treatment reduces the escape latency time (ELT), even though increased TSTQ in MWM indicates enhanced spatial memory.

In order to investigate a cellular signalling mechanism, we also examined the effect of CPH on the PI3K/AKT/mTOR protein levels. Upregulation of PI3K/AKT/mTOR has been linked to the onset and progression of neurological dysfunctions (Bozdagi et al., 2013; Chen et al., 2014). Recently, this signaling pathway overactivation has been linked to pathological processes that may be responsible for autism (Kwon et al., 2006). In comparison, inhibiting the PI3K/AKT/mTOR signalling pathway resulted in a neuroprotective effect that was employed as a diagnostic marker in autistic patients (Yeung et al., 2017). As a result, we found elevated PI3K/AKT/mTOR levels in the CSF and brain homogenate of ICV-PPA induced experimental model of autism. However, CPH therapy decreases the PI3K/AKT/mTOR protein levels, which protects against autism by lowering apoptosis, neuroinflammation, oxidative stress in CSF and rat brain homogenate.

Researchers used new techniques to detect abnormalities in the white matter region of the brain that is connected with autistic dysfunction. A previous study revealed that MBP levels were lower in the brains of autistic patients (Gonzalez-Gronow et al., 2015). The current study found decreased MBP levels in ICV-PPA rats' brain homogenate. Furthermore, CPH administration restored MBP levels in autistic rats' brain homogenates. Apoptotic cell death impairs brain maturation and is thought to be a risk factor for the development of autism (Eftekharian et al., 2019). PPA exposure elevated the levels of apoptotic indicators such as Bax and Caspase 3, while decreasing the levels of anti-apoptotic marker Bcl-2. Long-term CPH therapy has been demonstrated to protect cells against cell death by decreasing Bax and Caspase-3 levels while increasing Bcl-2 levels.

The neurochemical analysis in our study provides a clear indication of CPH neuroprotective potential. Neurotransmitters, which regulate memory, emotion, and behaviour, must balance normal function and neuronal development. Neurotransmitter disruption was identified to be one of the primary causes of the onset of behavioural traits. One of the most prominent aspects of autism is neurotransmitter imbalance (Kuo et al., 2018). The most investigated neurotransmitters in autism include serotonin, dopamine, glutamate, and Ach. As a result of serotonin's role in the brain's development, autistic individuals exhibit socially impaired, repetitive, and depressive behaviour (Kane et al., 2012; Amodeo et al., 2021).

Furthermore, a decrease in dopamine, Ach, and an increase in glutamate were linked to autistic behaviour (Drenthen et al., 2016; Karvat et al., 2014; DiCarlo et al., 2019). Increased glutamate levels activate microglia and promote neuroinflammation, whereas decreasing dopamine and acetylcholine levels influence neuronal excitability and cause mood abnormalities (Acharjee et al., 2018). Our results show that repeated ICV-PPA injections considerably affect the amount of neurotransmitters in rat brain homogenates. Dopamine, serotonin, and acetylcholine levels in ICV-PPA-treated rats decreased, whereas glutamate levels significantly increased, indicating neuronal excitotoxicity. CPH treatment restores neurotransmitter levels in a dose-dependent manner and improves autistic-like behaviour.

TNF and IL-1 are significant mediators of oxidative stress, and neuroinflammation are implicated in neurodegenerative diseases (Saghazadeh et al., 2019; Mirza and Sharma, 2019). Clinical studies on autistic children clearly showed higher inflammatory cytokine levels in CSF, resulting in immunological response and brain damage (Chez et al., 2007). according to our findings, PPA infusion elevated inflammatory cytokines such as TNF and IL-1 β in blood plasma and brain homogenate. CPH treatment significantly decreases inflammatory cytokine levels in blood plasma and brain homogenate, resulting in anti-inflammatory actions. Previous research has identified elevated oxidative stress as one of the pathogenic characteristics of autism (Morimoto et al., 2020; Abruzzo et al., 2019; El-Ansary et al., 2012). We measured oxidative stress markers in the brain homogenates of PPA and CPH-treated rats to characterize the severity of the disease and the preventive effects of CPH against oxidative stress. Our data clearly reveal an increase in AchE, LDH, nitrite, and MDA, as well as a decrease in antioxidants, particularly SOD and GSH, in an ICV-PPA induced experimental model of autism in adults rats. PPA-induced autistic rats treated with CPH showed significantly reduced levels of oxidative stress markers, and showing antioxidant properties.

This study looks at the morphological structure of the brain, whole-brain sections, and demyelination volume. Previous research has indicated that the hippocampus is relatively sensitive to PPA exposure. The gross pathological and morphological findings show that PPA-treated rats' brains differ in size and shape (Rahi et al., 2021). Prolonged CPH administration improved morphological abnormalities in ICV-PPA-treated autistic rats, such as damaged meninges and constricted prefrontal cortex. Coronal sections of ICV-PPA-treated rats exhibited malformed basal ganglia, a defragmented hippocampal area, and degraded white matter. Furthermore, the measurement of demyelination volume in rat brains revealed a significant decrease in white matter volume following PPA injections (Rahi et al., 2021). Continuous CPH therapy was found to lessen the severity of pathological and morphological changes. Long - term treatment of CPH10 mg/kg and CPH20 mg/kg to autistic rats recovered abnormalities in brain sections and enabled remyelination of damaged areas as compared to ICV-PPA treated autistic rats.

Our research primarily focuses on CPH's neuroprotective potential by decreasing the PI3K/AKT/mTOR protein levels, alleviating behavioural, neurochemical, morphological, and gross pathological abnormalities in the ICV-PPA induced experimental model of autism in adult rats. Concurrent studies, such as Western Blot and immunohistochemistry, are also required to provide molecular support for this hypothesis. Despite these limitations, CPH's neuroprotective potential in resolving or downregulating aberrant PI3K/AKT/mTOR signalling pathways in the CNS appears promising.

6.0 Conclusion

Based on the findings, we conclude that PPA injection causes considerable changes, including irregular neural cell structure, autism-like neurobehaviors, and neurochemical abnormalities. To date, there have been no pre-clinical investigations on the neuroprotective effect of CPH via PI3K/AKT/mTOR signalling downregulation in an ICV-PPA generated experimental model of autism in adult rats. It has been observed that PPA can cause autistic-like behaviour in experimental rats. Our findings suggest that CPH substantially improves social interaction, learning, and memory deficiencies in PPA-exposed rats. CPH significantly lowered oxidative stress, neuroinflammation, and apoptotic cell death in autistic rats by reducing the PI3K/AKT/mTOR protein levels in brain homogenate, blood plasma and CSF samples. Furthermore, the recovery of gross pathological defects in the whole brain and brain sections demonstrates CPH's potential to protect against PPA-induced neurological impairments. However, additional genetic study and immunohistochemical investigation are necessary to explain the underlying pathways governing such interactions. Indeed, PI3K/AKT/mTOR can be used as a therapeutic target in combination with other standard pharmacological interventions.

7.0 Abbreviations

CNS : Central nervous system

mTOR : Mammalian target of rapamycin

PI3K : Phosphoinositide 3-kinase

CPH : Chrysophanol

AKT : Protein kinase B (PKB)

ASD : Autism spectrum disorder

AD : Alzheimer's disease

ASD : Autism spectrum disorder

BBB : Blood brain barrier

DA : Dopamine

ICV :Intracerebroventricular

PD : Parkinson's disease

PPA : Propionic acid

SOD : Superoxide dismutase

MDA : Malondialdehyde

TNF : Tumor necrosis factor

GSH : Glutathione

LDH : Lactate dehydrogenase

IL-1 β : Interleukin-1 β

HPLC : High performance liquid chromatography

AchE : Acetylcholinesterase

Ach : Acetylcholine

TSTQ : Time spent in target quadrant

ELT : Escape latency time

MWM : Morris water maze

BCT : Beam crossing task

FST : Forced swim test

MBP : Myelin basic protein

Declarations

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Data availability statement

All data generated or analyzed during this study are included in this article. There are no separate or additional files.

Compliance with ethical standards

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Disclosure of potential conflicts of interest

The authors declare that they have no competing interests.

Ethical approval

All applicable institutional guidelines for the care and use of animals were followed.

Contribution of Authors

Ms. Aarti Sharma (First Author)

M. Pharm, Neuropharmacology Division, Department of Pharmacology

ISF College of Pharmacy, Moga, Punjab, India

Contribution: Thesis research work, Performed experimental animal studies

E-mail: sharmaarti3399@gmail.com

Ms. Sonalika Bhalla (Fourth author)

M. Pharm, Neuropharmacology Division, Department of Pharmacology

ISF College of Pharmacy, Moga, Punjab, India

Contribution: Revision of entire research manuscript, editing.

E-mail: sonalikabhalla97@gmail.com

Dr. Sidharth Mehan (Corresponding Author* and Third Author)

PhD, M. Pharm, Neuropharmacology Division, Department of Pharmacology

ISF College of Pharmacy, Moga, Punjab, India

Contribution: Original research hypothesis, guide, and compilation of all manuscript data

E-mail: sidh.mehan@gmail.com; sidharthmehan@isfcp.org;

Contact: +91-8059889909, +91-9461322911

Note: All authors approved the final version of this study.

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Figures

Figure 1

Experimental protocol schedule (Behavioral and biochemical estimations)

Figure 2

2 (a): Effect of chrysophanol on body weight in ICV-PPA induced experimental model of autism in adult rats (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control, and CPH20Perse; @p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (two-way ANOVA followed by post hoc multiple comparison test Bonferroni) 2 (b): Effect of chrysophanol on relative brain-body weight in ICV-PPA induced experimental model of autism in adult rats (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (one-way ANOVA followed by Tukey's multiple comparison test) 2 (c): Effect of chrysophanol on escape latency time in ICV-PPA induced experimental model of autism in adult rats. (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control, and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (two-way ANOVA followed by post hoc multiple comparison test Bonferroni) 2 (d): Effect of chrysophanol on TSTQ in ICV-PPA induced experimental model of autism in adult rats (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (one-way ANOVA followed by Tukey's multiple comparison test) 2 (e): Effect of chrysophanol on locomotor activity in ICV-PPA induced experimental model of autism in adult rats (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control, and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (two-way ANOVA followed by post hoc multiple comparison test Bonferroni) 2 (f): Effect of chrysophanol on neuromuscular coordination in ICV-PPA induced experimental model of autism in adult rats (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control, and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (two-way ANOVA followed by post hoc multiple comparison test Bonferroni) 2 (g): Effect of chrysophanol on immobility time in ICV-PPA induced experimental model of autism in adult rats (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control, and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (two-way ANOVA followed by post hoc multiple comparison test Bonferroni)

Figure 3a

Figure 3b

Figure 3

: Effect of chrysophanol in the restoration of gross pathological alterations of whole rat brain and brain sections in ICV-PPA induced experimental model of autism in adult rats (a) Whole rat brain (a) Vehicle control (b) Sham control (c) CPH20 perse (d) PPA (e) PPA + CPH10 (f) PPA + CPH20 (Scale bar = 2 mm) Note: Yellow circles are pointing to the site of the brain injury (b) Brain sections (a) Vehicle control (i) Cerebral cortex (ii) Hippocampus (iii) basal Ganglia (b) Sham control (c) CPH20 perse (d) PPA (e) PPA + CPH10 (f) PPA + CPH20 (Scale bar = 5 mm) Note: Yellow circles are pointing to the injured site.

Figure 4

Effect of chrysophanol on demyelination volume in ICV-PPA induced experimental model of autism in adult rats (Values expressed as mean±SD (n=6 rats per group). *p<0.001 v/s vehicle control, sham control and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (one-way ANOVA followed by Tukey's multiple comparison test)