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Research Article

ESCULETIN PROTECTS STRIATAL NEURONS FROM 3-NITROPROPIONIC ACID INDUCED NEUROTOXICITY BY MODULATING BEHAVIOURAL AND BIOCHEMICAL ALTERATIONS OF MALE WISTAR RATS

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A B S T R A C T

3-Nitropropionic acid is an inhibitor of succinate dehydrogenase known to cause blood brain barrier. It is used as a model for Huntington's disease by injecting in rodents and primates. HD is a neurodegenerative disorder characterized by chorea, dementia and cognitive decline. HD affects large number of population, but there is no cure till date. HD is caused due to expansion of polyglutamine repeats in Huntingtin gene, which leads to production of mutant huntingtin. In our study, we have used esculetin to study its protective effects on 3-NP induced neurotoxicity. Esculetin is a coumarin with well known beneficial properties. It is present in Chinese medicinal plants. We have given two doses of esculetin (25mg/kg and 50mg/kg) to rats who were administered with 3-NP (10mg/kg b.w.) the results showed that there was a significant increase in the levels of ATPases and Lactate dehydrogenase upon esculetin treatment, which had otherwise decreased due to 3-NP administration. Esculetin also increased the motor coordination of rats as shown by reduced fall-off time on rotarod apparatus. Cresyl violet staining showed an increased number of inflamed nissl positive cells in 3-NP induced rats. Thus, this study shows that Esculetin shows neuroprotective activites against 3-nitropropionic acid induced neurotoxicity.

INTRODUCTION

Huntington's disease is an autosomal dominant neurodegenerative disorder. It is caused due to expansion of CAG repeats (Polyglutamine) in the IT-15 gene of the Huntingtin (Htt) protein. Normal Htt gene encodes Htt protein of more than 350kDa molecular weight. Is a peculiar neurodegenerative disorder for which no medicine has been discovered yet. HD afflicts 30,000 people in USA and another 250,000 persons are genetically at risk (Dhadde *et al*, 2016). This disease causes atrophy of the basal ganglia, especially striatal neurons (Kumar *et al*, 2012). It is characterized by psychiatric disturbance as well as dementia and is a movement disorder. It is caused to aggregation of the mutated huntingtin protein which leads to cascade of events causing, oxidative stress, mitochondrial alterations, transcriptional dysfunction bioenergetic defects, apoptosisand subsequent excitotoxicity. HD patients often exhibit deficits in executive tasks requiring planning, cognitive flexibility and problem solving (Dhadde *et al*, 2016). Several animal models exist for HD such as stereotactic injection of kainic, quinolinic and ibotenic acids into specific region of the brain, but systemic administration of 3-nitropropionic acid (3-NP) is the recent and popularly used one (Dhadde *et al*, 2016). 3-nitropropionic acid is fungal toxin known to induce behavioural and biochemical alterations. It causes Huntington's disease like symptoms when injected into rodents and humans. It is a well-known inhibitor of succinate dehydrogenase (SDH). By inhibiting SDH it causes the behavioural, biochemical abnormalities leading to oxidative stress. 3-NP administration causes mitochondrial dysfunction which causes an imbalance in oxidant and antioxidant levels and thus leads to apoptosis of neurons. Striatal neurons are highly dependent on mitochondria because of high energy requirements. 3-NP injection causes motor impairment, memory loss and loss of muscle strength in rodents (Ahmed *et al*, 2015). Due to inhibition of SDH by 3-NP, there is a depletion of Ca intracellular Ca^{2+}, leading to activation of $Ca2^{+}$ and Na^{+}/K^{+} ATPase. This causes membrane depolarization and increased levels of intracellular sodium (Matthews *et al*, 1998). Esculetin, is a phenolic compound, a coumarin that occurs in many plants (Wang *et al*, 2015). It is also known as 6,7-dihydroxy coumarin or aesculetin. It is mainly found in *Citrus limonia* and *Artemisia capillaris.* Esculetin is an inhibitor of 5-LOX. Esculetin has anti-oxidant, anti-inflammatory and anti-cancer activities (Rzodkiewicz *et al*, 2015). It also shows neuroprotective activities against Parkisnon's disease (Subramaniam and Ellis, 2013). In the current study, we have shown the protective activity against 3-NP induced toxicity by assessing its behavioural, biochemical and histological changes.

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MATERIALS AND METHODS

Animals

Male wistar rats weighing 200-250 gm were obtained from Central Animal House Facility (CAHF), University of Madras, Taramani campus, Chennai. All animals were placed in polypropylene cages and acclimatized to 12hrs light and 12hrs dark cycles. They were fed with standard chow pellets and had access to water *ad libitum.* All experiments were carried out according to the guidelines of Institutional Animal Ethics Committee (IAEC No.- 01/12/2016).

Chemicals and Reagents

3-Nitropropionic acid and Esculetin were purchased from Sigma Chemical Co. (USA). All other reagents used were of analytical grade.

Drug treatment

Animals were divided into 5 groups

1. Group I- Control- Rats were administered with saline.
2. Group II- Rats were administered with 3-nitropropionic acid (10mg/kg b.w.) intraperitoneally for 14 days (Kumar *et al*, 2009).
3. Group III- Rats were administered withesculetin (25mg/kg b.w) (Sulakhiya *et al*, 2016), orally, 1 hour prior to the administration of 3-nitropropionic acid (10mg/kg b.w.) intraperitoneally for 14 days.
4. Group IV- Rats were administered withesculetin (50mg/kg b.w) (Sulakhiya *et al*, 2016), orally, 1 hour prior to the administration of 3-nitropropionic acid (10mg/kg b.w.) intraperitoneally for 14 days.
5. Group V- Rats were treated with esculetin (50mg/kg b.w) orally for 14 days.

Behavioural Analysis

Rotarod analysis

Motor coordination and grip strength were assessed by usingrotarod apparatus. Animals weretrained prior aclimatize them on rotarod apparatus before making the actualassessments of drug treatments. Animals were placed on therotating rod with a diameter of 7 cm. Thecut-off time was 120 seconds. The average fall-off time was recordedand expressed as count per 2 min (Jamwal and Kumar, 2016).

Biochemical Estimations

Tissue preparation

On day 15, the animals were sacrificed and the brain was removed by decapitation. Striatum was separated from each isolated brain. A 10% (W/V) tissue homogenate were prepared in 0.1 M phosphate buffer (pH 7.4). The homogenate were centrifuged at 10,000 x g at 4º C for 15 min. Aliquots of supernatant were separated and used for biochemical estimations.

The activity of Na^+/K^+ ATPase was done by Bonting.To 1.0 ml of Tris buffer and 0.2 ml of each of the above reagents were mixed together. Thus, the assay medium in a final volume of 2.0 ml, contained 92mM tris buffer, 50mM $MgSO_4$, 60mM NaCl, 1mM EDTA and 4mM ATP. After 10 minutes, equilibrium at 37˚C in an incubator, reaction was started by the addition of 0.1 ml of homogenate. The assay medium was incubated for 15 minutes. After incubation, the reaction was arrested by the addition of 1.0 ml of 10% TCA. The enzyme activity is expressed as micromoles of Pi liberated/min/mg protein.

Assay of Mg^{2+}ATPase

The activity of Mg^{2+}ATPases was determined by the method of Ohnishi *et al* (Ohnishi *et al*, 1982). The assay was initiated by the addition of 0.1 ml of homogenate to an incubation medium containing 0.1 ml of water and 0.1 ml each of tris buffer, $MgCl_2$ and ATP having concentrations 75mM, 5mM and 2mM respectively with total incubation volume of 0.5 ml. The reaction was terminated after 15 minutes by the addition of 1.0 ml of 10 % TCA. The enzyme activity was expressed as micromoles of Pi liberated/min/mg protein.

Assay of Ca^{2+}ATPase

The activity of Ca^{2+}ATPase was estimated by the method of Ohnishi *et al* (Ohnishi *et al*, 1982). 0.1ml of homogenate was added to an incubation medium containing 0.1ml of water and 0.1ml of each Tris buffer (75mM), $CaCl_2$ (5mM) and ATP (2mM), with total incubation volume of 0.5ml. The reaction was terminated by the addition of 1.0ml of 10% TCA after 15 minutes. The enzyme activity was expressed as micromoles of Pi liberate /min/mg protein. Inorganic phosphorus was estimated by the method of Fiske and Subbarow (Fiske and Subbarow, 1925).

Activity of Lactate dehydrogenase (LDH)

The Activity of Lactate dehydrogenase was done according to the method of King *et al*. (King *et al*, 1965) the reaction mixture contained 1.0ml of buffered substrate, 0.1 ml of homogenate, 0.2ml of NAD+ and was incubated for 15 mins at 37^0C. Then, 1.0ml of DNPH and 0.4N NaOH were added and read at 420 nm using a UV-VIS Spectrophotometry. The activity of LDH is expressed as Units/ mg protein.

Histopathological observation of striatum by Cresyl Violet staining

The animals were sacrificed on day 15 by cervical decapitation. The striatum was removed and was fixed in formalin for 24hrs and then dehydrated. The specimens were cleared with xylene and were embedded in paraffin block for sectioning at 4µm. tissue sections were stained with Nissl staining liquid (Medox Biotech, Chennai, India). Sections were then washed with distilled water, dehydrated and mounted.

Statistical Analysis

All the values were expressed asmean ± S.D. of six animals each group. Data was analyzed by using one-way analysis of variance (ANOVA) followed by Tukey's test using SPSS 20 software. Values with $P < 0.05$ were considered as statistically significant

RESULTS

Effect of Esculetin on 3-NP induced Motor coordinationin Rotarod test in control and experimental rats

Systemic administration of 3-NP for 14 days showed a significant ($P < 0.01$) impairment in the grip strength performances on day 5, 10 and 14 when compared to control animals. Treatment with esculetin (25mg/kg and 50 mg/kg) delayed the mean fall off time significantly ($P<0.01$ and $P<0.05$ respectively) as compared to 3-NP induced group. Esculetin alone treated animals were ableto balance on rods similar to control animals (Fig.1).

Effect of Esculetinon 3-NP induced alterations in the activity of Na^+/K^+ ATPase in the striatum of control and experimental rats

The activity of Na^+/K^+ATPase in 3-NP intoxicated rats decreased significantly as compared with normal rats (P<0.01). Esculetintreatment 25mg/kg b.w (P<0.01) and 50mg/kg b.w (P<0.05) has protectedNa^+/K^+ATPase when compared to 3-NP induced group. There were no significant changes observed in Esculetin (50 mg/kg, b.w.) alone treated rats (Fig.2).

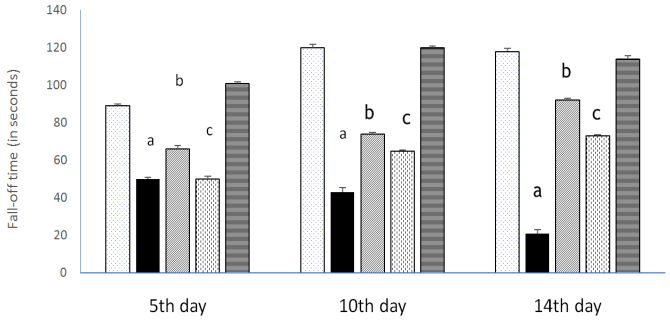

Fig 1 Effect of Esculetin on 3-NP induced Motor coordination in Rotarod test in control and experimental rats

Data represents mean ± SD (n=6). a*P*< 0.01 versus control group, b*P*< 0.01; cP<0.05 versus 3-NP induced group (One way ANOVA followed by Tukey's *Post-hoc* test).

Fig.2

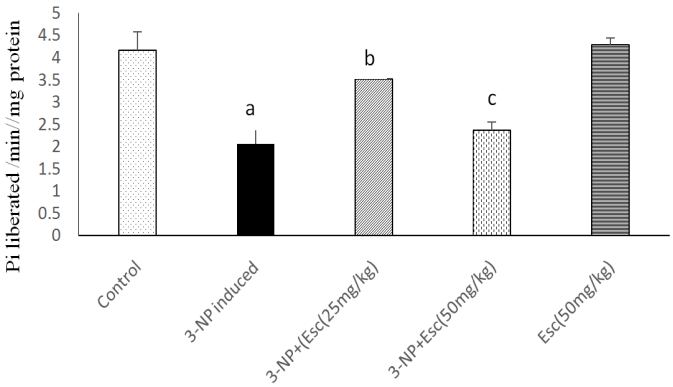

Fig 2 Effect of Esculetin on 3-NP induced alterations in the activity of Na^+/K^+ ATPase in the striatum of control and experimental rats

Data represents mean ± SD (n=6). a*P*< 0.01 versus control group, b*P*< 0.01; cP<0.05 versus 3-NP induced group (One way ANOVA followed by Tukey's *Post-hoc* test).

Effect of Esculetin on 3-NP induced alterations in the activity of Mg^{2+} ATPase in the striatum of control and experimental rats

3-NP administered rats showed significant decrease (p<0.01) in the activity of Mg^{2+} ATPase as compared to control animals. Esculetin treatment (25mg/kg and 50mg/kg) significantly increased (p<0.01 and p<0.05 respectively) the activity of Mg^{2+} ATPase. Esculetin alone treated rats showed no significant difference as compared to control group (Fig.3).

Fig.3

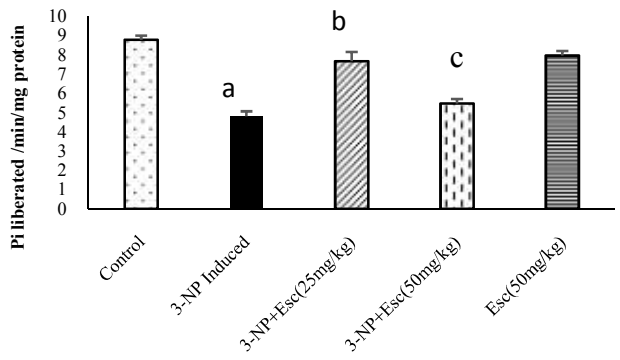

Fig 3 Effect of Esculetin on 3-NP induced alterations in the activity of Mg^{2+} ATPase in the striatum of control and experimental rats

Data represents mean ± SD (n=6). a*P*< 0.01 versus control group, b*P*< 0.01; cP<0.05 versus 3-NP induced group (One way ANOVA followed by Tukey's *Post-hoc* test).

Effect of Esculetin on 3-NP induced alterations in the activity of Ca^{2+} ATPase in the striatum of control and experimental rats

Systemic 3-NP administration caused a significant decrease (p<0.01) in activity of Ca^{2+} ATPase. When 3-NP administered rats were orally given esculetin (25mg/kg and 50mg/kg), there was a significant increase (p<0.01 and p<0.05 respectively) in the activity of Ca^{2+} ATPase. Esculetin alone treated rats showed no significant difference as compared to control group (Fig.4).

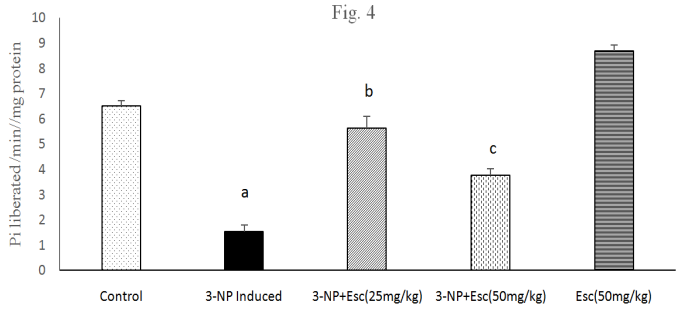

Fig 4 Effect of Esculetin on 3-NP induced alterations in the activity of Ca^{2+} ATPase in the striatum of control and experimental rats

Data represents mean ± SD (n=6). a*P*< 0.01 versus control group, b*P*< 0.01; cP<0.05 versus 3-NP induced group (One way ANOVA followed by Tukey's *Post-hoc* test).

Effect of Esculetin on 3-NP induced alterations in the activity of Lactate dehydrogenase (LDH) in the striatum of control and experimental rats

3-NP administration caused a significant decrease (p<0.01) in activity of LDH. When 3-NP administered rats were orally given esculetin (25mg/kg and 50mg/kg), there was a significant increase (p<0.01 and p<0.05 respectively) in the activity of LDH. Esculetin alone treated rats showed no significant difference as compared to control group (Fig.5)

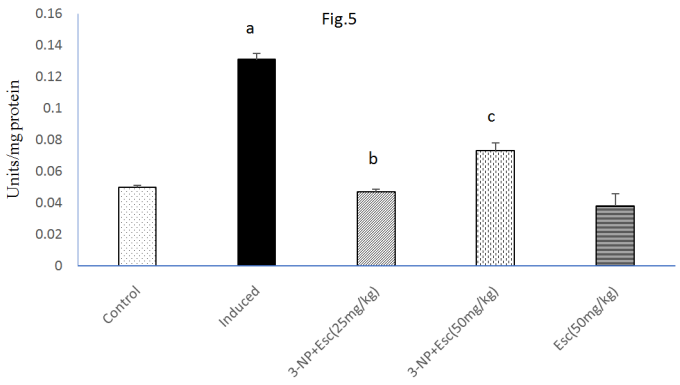

Fig 5 Effect of Esculetin on 3-NP induced alterations in the activity of Lactate dehydrogenase (LDH) in the striatum of control and experimental rats
Data represents mean ± SD (n=6). a*P*< 0.01 versus control group, b*P*< 0.01; cP<0.05 versus 3-NP induced group (One way ANOVA followed by Tukey's *Post-hoc* test).

Effect of esculetin on 3-NP induced histological alterations in striatum of control and experimental rats

Tissue sections were stained with Cresyl violet and visualized under light microscope at an original magnification of 400x. Striatum of control rats had intact viable neurons. 3-NP-induced rats showed increased number of darkly stained nonviable neurons and pyknotic nuclei in the striatum. Esculetin 25mg/kg and 50 mg/kg treated rats has lesser nonviable neurons in the striatum. Esculetin 50 mg/kg alone treated rats showed similar pattern as that of control (Fig.6).

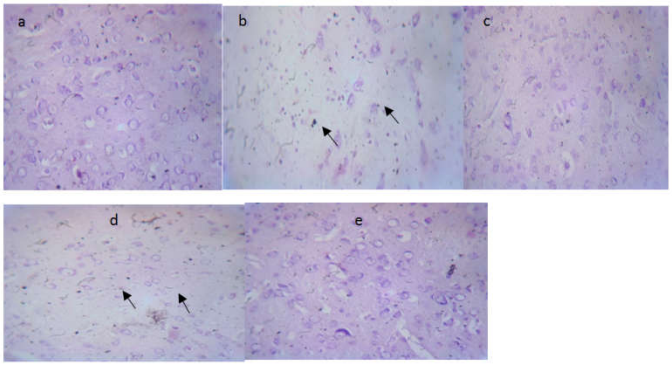

Fig. 6 Effect of esculetin on 3-NP induced histological alterations in striatum of control and experimental rats

Fig. 6 shows cresyl violet stained images of striatum of control and experimental rats.

a-Control rats showing normal architecture of healthy neurons.
b-3-NP induced showing reduced number of striatal neurons, condensed dark nuclei.
c-3NP+Esc(25mg/kg) showing presence of normal cells and increased loss of inflammed Nissl positive cells.
d-3NP+Esc(50mg/kg) showing less apoptotic nuclei with condensed nucleus and less Nissl positive cells.
e- Esculetin (50mg/kg) striata showing normal architecture as control group.

DISCUSSION

HD is disorder which affects mental ability as well as body movements. One of the important symptom of HD is bradykinesia and chorea. HD patients show a marked increase in depression and anxiety. The abnormal motor coordination is caused due to increased dopamine levels in striatum (Chakraborty *et al*, 2014). The number of CAG repeats in people suffering from HD is indirectly proportional to their age. People with more than 35 CAG repeats develop juvenile HD.

In the current study we used 3-NP- induced- Huntington's Disease model because it is well established that it induced behavioural and biochemical abnormalities and mimics- HD phenotype. 3-NP administration for 14 days produced behavioral alterations which were observed in the form of motor incoordination as assessed by rotarod on 5th, 10th and 14th day respectively. Intraperitoneal administration of esculetin 1 hour prior to 3-NP administration, reduced the behavioural abnormality in rats (Menze *et al*, 2012).3-NP causes degeneration of GABAergic neurons in striatum (Kumar *et al*, 2009 b). Esculetin shows neuroprotective effect even in Parkinson's disease. Basal ganglia control the coordination of body movements. Thus, the decreased motor coordination ability in rats exemplifies that there is striatal neurodegeneration.

3-NP administration causes energy deficits by depleting ATP levels due to mitochondrial dysfunction as a result of SDH inhibition. There results the condition of increased oxidative stress on 3-NP administration.Alterations in the activities of ATPases affects electrophysiological energetics and normal homeostasis (Suganya and Sumathi, 2016). Intoxication of animals with 3-NP causes a decrease in levels of Na^+/K^+ ATPase. This enzyme is responsible for maintaining normal cellular electrochemical gradient. It is also important for normal cell cycle and differentiation of nervous system. Decreased levels of Na^+/K^+ ATPase results in impairment of learning and memory (Ramalho *et al*, 2017).

Nervous system controls an important function of communication of signals in any organism. Calcium plays an important role in signal transmission at pre- and post-synaptic sites. Along with playing a crucial role in neuronal development and differentiation, calcium is also controls neuronal death. An imbalance in calcium regulation results in neuronal death in neurodegenerative disorders. Calcium ATPases present in plasma membrane are mainly responsible for maintaining Ca^{2+} homeostasis. Decrease in Calcium ATPase levels or activity causes an increase in cytosolic Calcium concentration.

The conversion of lactate to pyruvate is catalysed by lactate dehydrogenase. LDH levels are used a marker for tissue injury. Chronic administration of 3-NP causes an increase in the levels of LDH. Treatment with esculetin reduces the LDH levels in 3-NP intoxicated rats.

In our study, as presented by cresyl violet staining of striata, there is a marked degeneration of neurons upon 3-NP intoxication, whereas healthy neurons are visible when 3-NP adminsteredanimals are also given esculetin. Our study shows that Esculetin has neuroprotective effect on 3-NP induced neurotoxicity.

CONCLUSION

We have presented a 3-NP induced model of Huntington's disease using esculetin as a treatment option. Esculetin has shown protective effect against 3-NP induced oxidative stress by improving locomotory behaviour and altering biochemical parameters. Thus, we can conclude thats some studies be carried out further to validate the role of esculetin providing neuroprotection against 3-NP induced neurotoxicity.

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