Research Article

To evaluate the combinational effect of Ethyl Pyruvate, Berberine and Poloxamer 188 on 3 nitropropionic acid induced Huntingtons disease

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ABSTRACT

The present study is undertaken to study the combinational effect of ethyl pyruvate, berberine, and poloxamer 188 on 3-nitropropionic acid (3NP)-induced Huntington's disease. 3NP is a well-versed experimental model to study Huntington's disease (HD)-induced motor, memory, and mitochondrial dysfunctions. Ethyl pyruvate, berberine, and poloxamer 188 are reported to exhibit a neuroprotective effect in various animal models. Moreover, berberine is reported to have a protective effect on memory dysfunction. In addition, poloxamer-188 and ethyl pyruvate reduce muscular atrophy, neuronal loss, and neuroinflammation. These evidences suggest that either single or combination of these agents may protect against 3NP model. Administration of 3NP (10 mg/kg) for 14 days in male Wistar rats significantly induced HD-like symptoms in rats as indicated by reduced locomotor activity, body weight, grip strength, oxidative defense, cognition, and mitochondrial complex enzymes activities in striatum, cortex, and hippocampus. All the behavioral and biochemical studies on control and treatment groups were done after 14 days. Marked elevation in lactate dehydrogenase (LDH), succinate dehydrogenase (SDH), and lipid peroxidation was also observed. Furthermore, a decrease in the levels of reduced glutathione was noted. However, pretreatment of combination (ethyl pyruvate [40 mg/kg], berberine [50 mg/kg], and poloxamer 188 [40 mg/kg]) significantly attenuated behavioral alterations, oxidative stress, neuronal loss, and mitochondrial enzymes complex dysfunction in 3NP-treated group and potentiates their respective protective effects.In conclusion, a combination of ethyl pyruvate, poloxamer 188, and berberine could be used to manage behavioral and biochemical alterations in HD than either of single drug therapy.

Keywords: 3-Nitropropionic acid, ethyl pyruvate, flavones: berberine, Huntington's disease, lactate dehydrogenase, neurodegeneration, oxidative stress, poloxamer 188, reactive oxygen species

Introduction

3-nitropropionic acid (3NP) is a well-known mycotoxin causing neurotoxicity in both animals and humans following neuropathological phenomenon.[1,2] Intraperitoneal administration of 3NP causes substantial neuronal damage of striatum and putamen in the brain. It produces Huntington's-like symptoms including jerky movements and Chorioform.[3,6] There are many mechanisms undergoing neuronal loss in HD-like oxidative stress, mitochondrial membrane depolarization, energy depletion, oxidative stress, and enhanced mitochondrial-dependent apoptosis.[4,5] 3NP is also associated with neuroinflammation with an increase in expression of tumor necrosis factor alpha, cyclooxygenase-2 (COX-2), and inducible nitric oxide syntheses (iNOS).[7]Thus, 3NP treatment leads to a depletion of ATP and energy failure, later resulting in altered calcium homeostasis, excitotoxic events, and neuronal death.[8,9]

Systemic administration of 3NP to rats depleted glutathione (GSH) pools and increased the production of hydroxyl radicals and nitrotyrosine, as well as oxidized proteins in the lesioned striatum.[10]

Ethyl pyruvate (EP) is a stable lipophilic ester derivative of pyruvate. It shows multiple mechanism of action such as metabolic augmentation, inflammatory response suppression, and radical scavenging which have been suggested to be involved in the protective effect of EP.[7,11]

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Under aerobic conditions, pyruvate enters into mitochondria, where it undergoes oxidative decarboxylation in a reaction catalyzed by the enzyme complex, pyruvate dehydrogenase, to form acetyl coenzyme A and carbon dioxide.[12]

Berberine is an isoquinoline alkaloid reported to exhibit anxiolytic, analgesic, anti-inflammatory, antipsychotic, antidepressant, and anti-amnesic effect.[10] Recently, berberine is reported to ameliorate spatial memory impairment by activating microglia and senile plaque clearance. Moreover, berberine is also reported to inhibit acetylcholinesterase enzyme activity and play an important role in metabolic syndrome. In addition, it showed that the anti-amnesic effect of berberine is related to increase in peripheral and central cholinergic neuronal system activity.[13] Other than this, berberine is also reported to reduce diabetic neuropathy, nephropathy, endothelial dysfunctions, and oxidative stress in diabetic animals.[13,30]

Poloxamer 188 is a copolymer having reported potent action on muscular atrophy and grip strength; it has neuroprotective action as well.[14,15]

Therefore, the present study has been designed to explore the combinational effect of ethyl pyruvate, berberine, and poloxamer 188 against 3NP-induced neurotoxicity in an animal model of Huntington's disease (HD).

Materials and Methods

Animals

Male Wistar rats (250–300 g) were procured from the National Institute Of Bioscience, Pune. Animals were acclimatized to laboratory conditions before experimentation. The animals were kept under standard conditions of light and dark cycle with food and water *ad libitum*. All the experiments were carried out between 09:00 am and 4.00 pm. The experimental protocol was approved by the Institutional Animal Ethics Committee and carried out in accordance with the Indian National Science Academy Guidelines for the use and care of animals. The protocol was approved by the Institutional Animal Ethics Committee and was carried out in accordance with the CPCSEA Guidelines for the use and care of animals.

Drugs and treatment schedule

The drugs used in the present study are 3NP and procured from Sigma Chemicals, St. Louis, MO, USA. 3NP was diluted with saline (adjust pH 7.4) and administered intraperitoneally to animal, whereas ethyl pyruvate, berberine, and poloxamer 188 were also procured from Sigma Chemicals, St. Louis, MO, USA. Animals were randomly divided into six groups of 12 animals in each. All the drugs were diluted in saline (adjusted to pH 7.4), and ethyl pyruvate and berberine were administered intraperitoneally to animals. Poloxamer 188 was suspended in a saline and administered by oral route through an oral cannula in a constant volume of 0.5 ml/100 g of body weight. Rats were administered 3NP for 14 days to induce Huntington's-like symptoms. The study was conducted as follows:

Group 1 is vehicle-treated group, Group 2 received 3NP (10 mg/kg, i.p.) for 14 days, Group 3 received ethyl pyruvate (40 mg/kg i.p.) + poloxamer 188 +3NP (10 mg/kg, i.p.) for 14 days, Group 4 received berberine (50 mg/kg) + P-188 + 3NP (10 mg/kg, i.p.) for 14 days, Group 5 received ethyl pyruvate (40 mg/kg) + berberine (50 mg kg) + 3NP (10 mg/kg, i.p.) for 14 days, and Group 6 received ethyl pyruvate+berberine+poloxamer 188+3NP for 14 days.[31]

Behavioral assessment

Measurement of body weight

Animal body weight was noted on the 1st and last day of the experimentation. Percent change in body weight was calculated in comparison to the initial body weight on the 1st day of experimentation.

$$\frac{\text{Body weight}\left[1^{st}\ \text{day} - 15^{th}\ \text{day}\right]}{1^{st}\ \text{day body weight}} \times 100$$

Muscle grip strength (rotarod)

All animals were evaluated for motor ability and balance using the Rotarod apparatus (Techno, India). The rats were given a prior training session before initialization of therapy to acclimate them to rotarod apparatus. Rats were placed on the rotating rod with a diameter of 7 cm (speed 25 rpm). The cutoff time as 180 s, and each rat performed three separate trials. The average results were recorded as fall of time.[14]

Assessment of gross behavioral activity (locomotor activity)

The locomotor activity was monitored using Actophotometer (IMCORP, India). The horizontal motor activity was detected by infrared beams located 2.5 cm above the floor of the testing area. Each interruption of a beam on the X- or Y-axis generated an electric impulse, which was presented on a digital counter. The apparatus was placed in a darkened, light, and sound attenuated and ventilated the testing room. Each animal was observed over 5 min, and values expressed as counts per 5 min.[13]

Dissection and homogenization

After completion of the behavioral evaluation, animals were randomized into two groups, one group was used for the biochemical evaluation and second group was used for mitochondrial dysfunction analysis. For the biochemical analysis, animals were scarified by cervical dislocation, immediately after behavioral assessment. Brains were put on ice and the cortex and striatum, and hippocampus were separated and weighted. A 10% (WV −1) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000× g for 15 min, and aliquots of supernatant were separated and used for biochemical estimation.

Measurement of oxidative stress parameters

Measurement of lipid peroxidation

The amount of malondialdehyde, a measure of lipid peroxidation, was measured by reaction with thiobarbituric acid at 532 nm using

Perkin Elmer Lambda 20 spectrophotometer.[16] The values were calculated using molar extinction coefficient of chromophore (1.56 × 105/M/cm) and expressed as a percentage of control.

Estimation of nitrite

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO), was determined with a colorimetric assay with Griess reagent [0.1% N-[1-naphthyl] ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid]. Equal volumes of supernatant and Griess reagent were mixed, the mixture was incubated for 10 min at room temperature in the dark, and the absorbance at 540 nm was determined with the Perkin Elmer Lambda 20 spectrophotometer. The concentration of nitrite in the supernatant was determined from a sodium nitrite standard curve and expressed as a percentage of control.[17]

Reduced GSH

GSH estimation was done according to the method described earlier (Ellman, 1959). Briefly, 160 µl of supernatant was added to 2 ml of Ellman's reagent [5′5 dithiobis [2-nitrobenzoic acid] 10 mM, $NaHCO_3$ 15 mM], and the mixture was incubated at room temperature for 5 min and absorbance was read at 412 nm. The values are expressed as nmol/g of wet tissue.[18]

Superoxide dismutase activity (SOD)

SOD activity was assayed according to the method of Konno,[19] wherein the reduction of nitroblue tetrazolium (NBT) was inhibited by the SOD which is measured at 560 nm using spectrophotometer. Briefly, the reaction was initiated by the addition of the hydroxylamine hydrochloride to the mixture containing NBT and sample. The results were expressed as unit/mg protein, where one unit of enzyme is defined as the amount of enzyme inhibiting the rate of reaction by 100%.

Protein estimation

The protein content was measured by biuret method using bovine serum albumin as the standard of Gornall.[21]

Estimation of acetylcholinesterase levels

The quantitative measurement of acetylcholinesterase levels in the brain was performed according to the method of Elleman.[20] 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 412 nm using Perkin Ellman Lambda 20 spectrophotometer. Results were calculated using molar extinction coefficient of chromophore (1.36 × 104/M/cm) and expressed as percentage of vehicle-treated group.

Mitochondrial enzyme estimation (complex-II- SDH activity)

It was measured spectrophotometrically according to Kumar.[23] The method involves oxidation of succinate by an artificial electron acceptor, potassium ferricyanide. The reaction mixture contained 0.2 M phosphate buffer pH 7.8, 1% BSA, 0.6M 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.[24]

Mitochondrial enzyme estimation (LDH estimation)

The LDH enzyme activity was estimated by the method of King.[22] The reaction mixture contained 0.5 ml of buffered substrate made in 0.1 M glycine buffer (pH 7.9) and 0.1 ml of tissue homogenate. The tubes were allowed to be incubated for 30 min at 37°C. Finally, the color was intensified by the addition of 5.0 ml of 0.4 N NaOH. The absorbance was read at 440 nm. The activity was expressed as pyruvate formed/min/mg protein.

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. All the values are expressed as mean ± SEM. In all the tests, the criterion for statistical significance was $P < 0.05$.

Results

Effect of ethyl pyruvate, berberine, and poloxamer 188 on body weight and brain weight in 3NP-treated rats

3NP treatment caused a significant decrease in body weight and brain weight on the day 15th as compared to vehicle-treated group. Further, combination treatment of ethyl pyruvate and berberine, berberine and poloxamer 188, and ethyl pyruvate, berberine, and poloxamer 188 attenuated the body weight of 3NP-treated rats ($P < 0.05$) [Figures 1 and 2]. The combination dose of ethyl pyruvate and poloxamer 188 did not influence body weight significantly.

Effect of ethyl pyruvate, berberine, and poloxamer 188 on muscular grip strength (Rotarod apparatus) in 3NP-treated rats

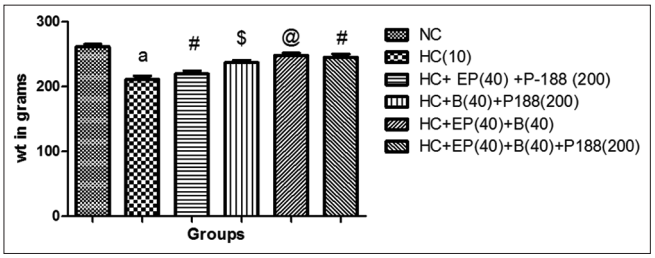

Figure 1: Effect of combinations treatment of EP, B, and P 188 on body weight. Each value represents mean ± SEM. $n = 6$. $^{a}P < 0.001$ compared with NC rats, $^{\#}P < 0.001$, $^{@}P < 0.01$, $^{\$}P < 0.05$ compared with Huntington control rats. NC: Normal control, HC: Huntington control, EP: Ethyl pyruvate, B: Berberine, P 188: Poloxamer 188, SEM: Standard error of the mean

3NP treatment decreased grip strength as assessed by rotarod test on the 10th day and 15th day compared to naive. Further, ethyl pyruvate, berberine, and poloxamer 188 treatment significantly improved muscle grip strength of 3NP-treated rats on the 15th day (Pb < 0.05) [Figure 3].

Effect of ethyl pyruvate, berberine, and poloxamer 188 on locomotor activity

In the present series of experiments, mean scores for locomotor activity on the day 1 for each rat were relatively stable and showed no significant variation. 3NP (10 mg/kg) treatment caused a significant decrease in locomotor activity as compared to vehicle-treated group. Further, ethyl pyruvate (40 mg/kg), berberine (50 mg/kg), and P 188 (200 mg/kg/oral) treatment significantly improved the locomotor activity in 3NP-treated rats [Figure 4]. However, a combination of two drugs, i.e., berberine + poloxamer-188 did not show a significant improvement in the locomotor activity [Figure 4].

Effect of ethyl pyruvate, berberine, and poloxamer 188 on lipid peroxidation, nitrite, SOD, and GSH levels in 3NP-treated rats

Systemic administration of 3NP caused a significant increase in lipid peroxidation, nitrite level, depleted SOD, and catalase enzyme activity as compared to vehicle-treated group in striatum areas of rat brain. Different combinations of ethyl pyruvate, berberine, and poloxamer 188 administration attenuated lipid peroxidation and nitrite levels and restored the decrease levels of SOD and GSH activities in striatum areas of 3NP-treated rats as compared to vehicle-treated rats ($P < 0.05$) [Table 1].

Effect of ethyl pyruvate, berberine, and poloxamer 188 on acetylcholinesterase levels in 3NP-treated rats

Further, chronic 3NP treatment significantly increased acetylcholinesterase enzyme level in striatum, cortex, and hippocampus regions of rats' brain as compared to the vehicle-treated group ($P < 0.05$). Different combinations of ethyl pyruvate, berberine, and poloxamer 188 treatments significantly attenuated acetylcholinesterase enzyme activity in striatum regions as compared to 3NP-treated rat [Figure 5].

Effect of ethyl pyruvate, berberine, and poloxamer 188 on the SDH activity in 3NP-treated rats

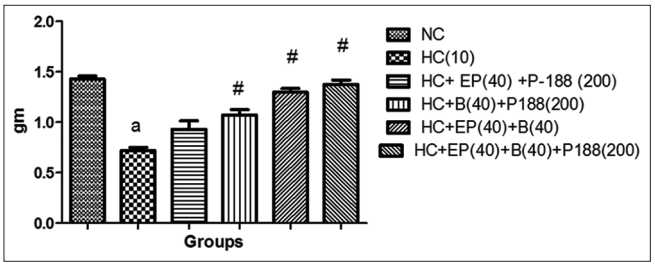

Figure 2: Effect of combinations treatment of EP, B, and poloxamer 188 on brain weight and relative brain weight. Each value represents mean ± SEM. n=6. $^{a}P < 0.001$ compared with NC rats, $^{\#}P < 0.001$, $^{@}P < 0.01$, $^{\$}P < 0.05$ compared with Huntington control rats. NC: Normal control, HC: Huntington control, EP: Ethyl pyruvate, B: Berberine, P 188: Poloxamer 188, SEM: Standard error of the mean

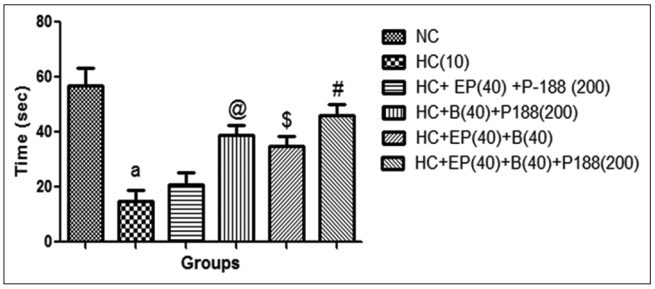

Figure 3: Effect of combinations treatment of EP, B, and poloxamer 188 on muscle grip strength. Each value represents mean ± SEM. n=6. $^{a}P < 0.001$ compared with NC rats, $^{\#}P < 0.001$, $^{@}P < 0.01$, $^{\$}P < 0.05$ compared with Huntington control rats. NC: Normal control, HC: Huntington control, EP: Ethyl pyruvate, B: Berberine, P 188: Poloxamer 188, SEM: Standard error of the mean

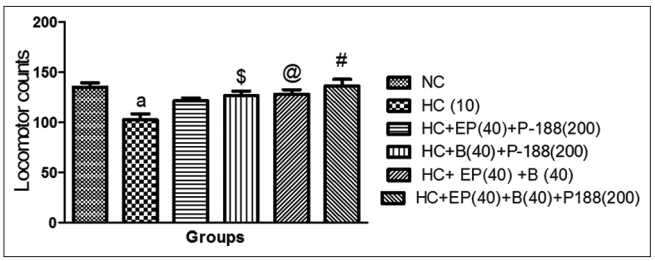

Figure 4: Effect of combination treatments of EP, B, and P 188 on locomotor activity. Each value represents mean ± SEM n=6. $^{a}P < 0.001$ compared with NC rats, $^{\#}P < 0.001$, $^{@}P < 0.01$, $^{\$}P < 0.05$ compared with Huntington control rats. NC: Normal control, HC: Huntington control, EP: Ethyl pyruvate, B: Berberine, P 188: Poloxamer-188, SEM: Standard error of the mean

Table 1: Effect of combination treatments of EP, B, P 188 on MDA, GSH, NO levels, and SOD activity in the brain striatum

Group	MDA (nmol/mg protein)	GSH (μmol/mg of protein)	NO (mmol/mg of protein)	SOD unit/mg of protein
NC	119.00±4.8	112.01±4.1	115.10±2.2	112.36±4.0
HC (10)	170.14±11.0^{a}	63.97±4.5^{a}	315.21±3.7^{a}	56.26±2.46^{a}
HC+EP (40)+P 188 (200)	142±8.6	70.14±1.4	274.69±3.6	65.61±1.63
HC+B (40)+P 188 (200)	131±7.4$^{\$}$	77.28±1.6	236.26±2.5$^{\#}$	71.50±2.46$^{@}$
HC+EP (40)+B (40)	122±6.4$^{@}$	86.85±4.6$^{\#}$	240.80±1.8$^{\#}$	78.36±2.27$^{\#}$
HC+P (40)+B (40)+P 188 (200)	110±3.3$^{\#}$	91.42±3.2$^{\#}$	268.37±1.5$^{\#}$	89.76±1.31$^{\#}$

Each value represents mean±SEM. n=6. $^{a}P<0.001$ compared with NC rats, $^{\#}P<0.001$, $^{@}P<0.01$, $^{\$}P<0.05$ compared with Huntington's control rats. NC: Normal control, HC: Huntington control, EP: Ethyl pyruvate, B: Berberine, MDA: Malondialdehyde, SEM: Standard error of the mean, SOD: Superoxide dismutase, NO: Nitric oxide, GSH: Glutathione, EP: Ethyl pyruvate, B: Berberine, P 188: Poloxamer 188

SDH is important for the regulation of TCA cycle in mitochondria of brain cells. 3NP treatment significantly decreased SDH activity as compared to the vehicle-treated group ($P < 0.05$). Different combinations of ethyl pyruvate, berberine, and poloxamer 188 treatment significantly increased SDH activity in striatum regions of 3NP-treated rats [Figure 6] ($P < 0.05$).

Effect of ethyl pyruvate, berberine, and poloxamer 188 on the LDH activity in 3NP-treated rats

3NP treatment significantly increased LDH activity due to the anaerobic condition in the brain as compared to the vehicle-treated group ($P < 0.05$). Different combinations of ethyl pyruvate, berberine, and poloxamer 188 treatment significantly decrease LDH activity in striatum regions of 3NP-treated rats [Figure 7] ($P < 0.05$).

Discussion

Chronic administration of 3NP, a suicide inhibitor of SDH, causes prolonged energy impairments and replicates most of the pathophysiological features of HD, which causes prolonged energy impairment and replicates most of the clinical and pathophysiological hallmarks of HD, including spontaneous choreiform and dystonic movements, as well as selective degeneration of striatum.[2] The symptoms developed by chronic administration of 3NP are analogs to juvenile onset and late hypokinetic stages of HD.[27] This is the extensive report that highlights the effect of ethyl pyruvate, berberine, and poloxamer 188 in 3NP-induced HD-like symptoms in rats. Ethyl pyruvate is a well-known anti-inflammatory drug supporting the STAT signaling pathway modulation, currently reported as neuroprotective in neonatal brain injury and brain ischemia.[29] Moreover, berberine has potent antioxidant property and is reported to protect against memory dysfunction,[31] whereas poloxamer 188 provides protection against muscular atrophy and neuronal loss.

Mitochondria play a key role in maintaining cellular energy balance, cell apoptosis process. Most established function of the cell organelle is to synthesize ATP through oxidative phosphorylation, which is associated with ROS production, including primarily superoxide (O2 •−) formation by the respiratory chain complexes I and III. Increasing evidences suggest that mitochondrial dysfunction is linked with oxidative damage that plays a crucial role in oxidative neurodegenerative pathologies, and therefore, mitochondrial scavenging of ROS can be a promising therapeutic approach. Supporting to above, 3NP significantly altered mitochondrial enzyme complex activities that could be due to the generation of free radicals in the striatum.[7]These alterations in enzyme mitochondrial complex activities were significantly restored by ethyl pyruvate and berberine pretreatment. It is also reported that 3NP inhibits complex-II enzyme of the respiratory chain, mitochondrial calcium release, increased ROS, and apoptosis.[8,11] Antioxidants have also been proved to have metal chelating and free radical scavenging properties such as neutralization of superoxide and singlet oxygen and inhibit the hydrogen peroxide-induced lipid peroxidation.[32] Our study results show that berberine significantly modulates oxidative stress in HD rats.

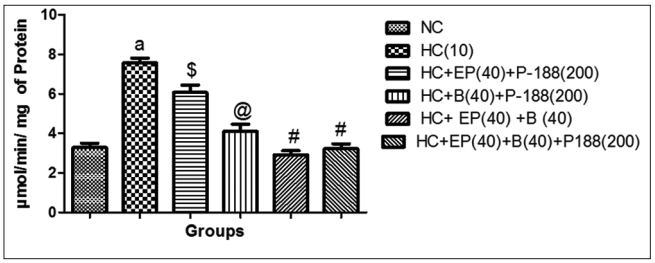

Figure 5: Effect of combination treatments of EP, B, and poloxamer 188 on cholinesterase activity. Each value represents mean ± SEM n=6. $^{a}P < 0.001$ compared with NC rats, $^{\#}P < 0.001$, $^{@}P < 0.01$, $^{\$}P < 0.05$ compared with Huntington control rats. NC: Normal control, HC: Huntington control, EP: Ethyl pyruvate, B: Berberine, P 188: Poloxamer-188, SEM: Standard error of the mean

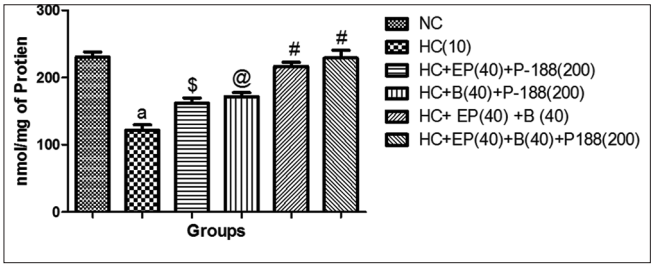

Figure 6: Effect of combination treatments of EP, B, and poloxamer 188 on succinate dehydrogenase activity. Each value represents mean ± SEM n=6. a$P < 0.001$ compared with NC rats, $^{\#}P < 0.001$, $^{@}P < 0.01$, $\$P < 0.05$ compared with Huntington control rats. NC: Normal control, HC: Huntington control, EP: Ethyl pyruvate, B: Berberine, P 188: Poloxamer 188, SEM: Standard error of the mean

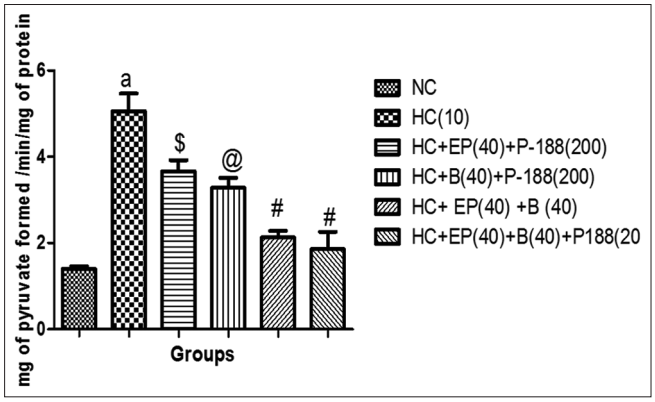

Figure 7: Effect of combination treatments of EP, B, and P 188 on lactate dehydrogenase activity. Each value represents mean ± SEM of n=6. $^{a}P < 0.001$ compared with NC rats, $^{\#}P < 0.001$, $^{@}P < 0.01$, $^{\$}P < 0.05$ compared with Huntington control rats. NC: Normal control, HC: Huntington control, EP: Ethyl pyruvate, B: Berberine, P 188: Poloxamer 188, SEM: Standard error of the mean

The JAK-STAT signaling pathway is a chain of interactions between proteins in a cell and is involved in processes such as inflammation, cell division, cell death, andtumor formation. Disrupted JAK-STAT signaling may lead to a variety of diseases, such as inflammation, skin conditions, cancers, and disorders affecting the immune system. Furtehrmore, it has been proven that ethyl pyruvate significantly alters neuroinflammation by the inhibition of STAT 1 and STAT3 signaling pathways. They prevent their translocation to the nucleus and consequently inhibited expression of iNOS and COX-2 by inhibiting STAT1- and STAT3-mediated

transcriptional activity. It also modulates GSH level in astrocytes as it is essential for cellular defense against ROS.[20,25,26] The result of our study increases the possibility of modulation of GSH in astrocytes by ethyl pyruvate. Hence, pretreatment with ethyl pyruvate and berberine significantly attenuated their protective effect on mitochondrial complex enzyme activities by restoring mitochondrial enzyme complexes. However, poloxamer 188 pretreatment with these drugs potentiated their neuroprotective effect by membrane healing mechanisms and recovering muscular atrophy. Furthermore, Poloxamer 188 is a polymer widely used in many pharmaceutical formulations; it can give further extension to this research to process a formulation using nanotechnology. These observations suggest that ethyl pyruvate, berberine, and poloxamer 188 treatment might have its protective effects on HD.

In conclusion, these results suggest that (i) 3NP induces striatal oxidative stress that remarks for all HD symptoms; (ii) ethyl pyruvate could protect against 3NP-induced neurotoxicity and neuroinflammation, (iii) neuroprotective effect of berberine could be due to its antioxidant mechanism, and (iv) polymer (poloxamer 188) could be effective against muscular atrophy.

All the two authors have equally contributed to the design and conduct of the study.

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