Progesterone induced neuroprotection in reperfusion promoted mitochondrial dysfunction following focal cerebral ischemia in rats

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Abstract

Alterations in mitochondrial permeability transition and organelle damage are key players in the development of cerebral ischemic tissue injury due to associated modifications in ATP turnover and cellular apoptosis/necrosis. Early restoration of blood flow and improvement of mitochondrial function might reverse the situation and help in recovery following an onset of stroke. Mitochondria and related bioenergetics can be effectively used as pharmacological targets. Progesterone (P4), one of the promising neurosteroids, has been found neuroprotective in various models of neurological diseases through a number of mechanisms. This influenced us to investigate the possible role of P4 via the mitochondria-mediated neuroprotective mechanism in ischemic stroke model of rat. In this study, we have shown the positive effect of P4 administration on behavioral deficits and mitochondrial health in ischemic/stroke injury model of transient middle cerebral artery occlusion (tMCAO). After induction of tMCAO, the rats received an initial intraperitoneal injection of P4 (8 mg/kg) or vehicle at 1h post-occlusion followed by subcutaneous injections at 6, 12, and 18 h. Behavioral assessment for functional deficits included grip strength, motor coordination, and gait analysis. Findings revealed a significant improvement with P4 treatment in tMCAO animals. 2, 3, 5- triphenyltetrazolium chloride staining of isolated brain slices from P4 treated rats showed a reduction in the infarct area in comparison to vehicle group indicating the presence of more viable mitochondria. P4 treatment was also able to attenuate the mitochondrial ROS as well as modulate the mitochondrial permeability transition pore (mPTP) in the tMCAO injury. In addition, it was also able to ameliorate the altered mitochondrial membrane potential and respiration ratio in the ischemic animals thereby suggesting that P4 has a positive effect on mitochondrial bioenergetics. In conclusion, these results demonstrate that P4 treatment is beneficial in preserving the altered mitochondrial functions in cerebral ischemic injury and thus can help in defining better therapies.

Keyword: Progesterone, Cerebral ischemia, Neurobehavior, Mitochondria, Apoptosis, Neuroprotection
TRANSLATIONAL IMPACT

Clinical Issue: Stroke is reported to be the third leading cause of adult disability and death globally, preceded only by cancer and cardiac disease. Among the different types of stroke, cerebral focal ischemia is the most common type that results from occlusion of an artery. The occlusion may cause irreversible tissue infarction due to cellular death in the affected brain region. Structural and functional impairment of ATP supplying mitochondria has an important role in the pathophysiology of ischemic cell death. In spite of several attempts, a completely effective treatment regime has not been found so far due to limited therapeutic possibilities/methods. We hypothesized that progesterone (P4) can be used to target mitochondrial pathways involved in neuronal damage and eventually improve the ischemic state of the brain. These preclinical data might be helpful in bringing progesterone to the clinical level in ischemic stroke as there is a critical need to fulfill the gap in this field. Due to its strong clinical connotations, P4 led to positive outcomes of phase 2 trials in traumatic brain injury (TBI) patients. Keeping in view that TBI and ischemic stroke progress through similar cascades of reactions, P4 might be a promising candidate for drug therapy for stroke patients in the future.

Results: In the current study, we have used behavioral analysis in the rat model of focal transient middle cerebral artery occlusion model to mimic ischemic conditions. Additionally, biochemical and bioenergetic assays were done along with immunohistochemical measurements corroborating the findings. This model was used to evaluate whether or not mitochondrial targeted P4 treatment improves the organelle based changes indicative of cellular damage in the frontal cortex of the rat brain which is thought to play a key role in the ischemic injury. The results clearly depict the changes in mitochondrial bioenergetics and permeability associated with the rat model of cerebral ischemia. P4 directly inhibits the mitochondrial permeability transition pore that blocks the release of cytochrome c from mitochondrial matrix. These alterations are mitigated by P4 owing to its anti-apoptotic and antioxidative potential in the context of mitochondrial dysfunction.
**Future Implications**

The new findings suggest that P4 inhibits the mitochondria-mediated neuronal death, particularly affecting the frontal cortex, which might be a part of the pathology underlying diseased state in cerebral ischemia. The experimental insights provided by this study might have implications for the development of neural-protective therapeutic agents to reduce stroke-induced brain damage. Deciphering cellular and molecular mechanisms will be an important crux in utilizing progesterone as a therapeutic agent in ischemic stroke.

**Introduction**

Stroke is the leading global cause of millions of death and disability as per reports by the World Heart Association (2015). Ischemic stroke is the most common form of stroke accounting for 87% of all types of strokes (Bennett et al., 2014). In spite of high prevalence, no 100% effective treatment is currently available that can alter the course of the disease. Tissue plasminogen factor (clot buster), is the only FDA approved drug with a narrow therapeutic time window, which is currently available for ischemic stroke patients (Peplow, 2015). Thus, there is a dire need for finding a new therapeutic strategy for stroke injury with a prolonged treatment window.

Mitochondria play a crucial role in the pathophysiology of several neurological diseases, including stroke (Andrabi et al., 2015; Rasheed et al., 2016). Mitochondria is very susceptible to any insult due to its critical role in energy metabolism, production of reactive oxygen species (ROS), and apoptotic pathways. (Waseem et al., 2016). The primary event in ischemia is disruption of electron transport chain (ETC) due to the impaired delivery of oxygen and glucose (Novgorodov et al., 2016). The mitochondrial membrane potential (MMP), generated via proton transfer across the inner mitochondrial membrane for the production of adenosine triphosphate (ATP) is derailed in cerebral ischemia (Khatri and Man, 2013). Upon reperfusion, the damage becomes severe as a consequence of excess ROS accumulation in mitochondria leading to attenuated activity of ETC components and favoring the open state of mitochondrial permeability transition pore (mPTP) (Javadov and Kuznetsov, 2013). These pathological changes eventually cause the release of various apoptotic factors such as cytochrome c and apoptosis inducing factor (AIF) culminating in initiation of apoptosis cascade and eventually leading to cell death (Manzanero et al., 2013). Neurotransmitter based excitotoxicity is another
mechanism associated with ischemic injury (Lai et al., 2014). This excitatory neurotransmitter leads to cytosolic Ca\textsuperscript{2+} overload and mitochondrial swelling (Nicholls et al., 2015). This swelling causes mitochondrial permeabilization releasing the apoptotic factors, including cytochrome c and resultant cell death in ischemia (Liu et al., 2009).

A large set of experimental evidence strongly supports the neuroprotective role of steroid hormone progesterone (P4) in many central nervous system injury models (De Nicola et al., 2013). Additionally, pre-clinical data focused on the neuroprotective role of P4 in ischemic stroke through multiple mechanisms available (Wong et al., 2013). In addition, clinical trials involving the neuroprotective role of P4 have also been exploited in traumatic brain injury patients making it an attractive pharmacological agent for designing a treatment regime for ischemic injury (Schumacher et al., 2015; Wright et al., 2014; Xiao et al., 2008). P4 can act multi-mechanically by reducing oxidative damage, inhibiting the apoptosis, and regulating various signaling pathways in brain damage (Deutsch et al., 2013). However, the role of mitochondrial-mediated pathway in neuroprotection provided by P4 in ischemic stroke needs to be thoroughly explored before using it as a target-based therapy. Rodent models of cerebral ischemia are important tools in experimental stroke research. Such models have proven to be instrumental for the understanding of injury mechanisms in cerebral stroke as well as to identify potential new therapeutic options.

In the current study, we evaluated the mitochondria-mediated neuroprotective effects of P4, at clinically relevant concentrations, in the frontal cortex of in vivo rodent model of cerebral ischemia established in our laboratory. We examined the effects of P4 on the extent of the infarction, the neurobehavioral outcome, and neurotransmitter levels in rats subjected to transient middle cerebral artery occlusion (tMCAO), a model of focal ischemia in vivo. Then, to elucidate its mitochondrial mechanism of action, we examined whether or not P4 could act by reducing Ca\textsuperscript{2+}-induced rat brain mitochondrial swelling, an index of increased mitochondrial membrane permeability. In addition, we examined if P4 could prevent the other mitochondrial functional changes, including loss of membrane potential, alteration of and excess ROS production. To further prove our hypothesis, we analyzed the mitochondrial bioenergetics by examining the state 3, respiratory control ratio (RCR) along with some ETC. Finally, we examined the anti-apoptotic action of P4 by elucidating the translocation of cytochrome c from mitochondria to cytosol through mPTP and thereby authenticated our findings.
Results

Neurobehavioral Analysis

We have studied several behavioral parameters to analyze the effect of P4 in attenuating the neurological deficits after (tMCAO) surgery. The first test involved scoring the grip strength between the sham, tMCAO, and P4 administered groups. The mean reading of three successive trials for each rat was taken as a dependent variable. Grip strength decreased significantly (P< 0.001) in rats subjected to tMCAO when compared to the sham group. Post hoc analysis showed that repeated administration of P4 (8mg/kg) improved the grip strength (P< 0.05) at 24 h when compared to the tMCAO group (Fig. 1A). Next, we measured the time remaining on the rota rod apparatus (in seconds) and mean of three successive trials for each rat was taken (Fig. 1B). There was a significant (P< 0.001) performance reduction in the tMCAO group when compared to the sham group. The one-way ANOVA and posthoc analysis showed that P4 improved the rota rod performance significantly (P < 0.001) in the treated group. This was followed by measurement of gait analysis of animals which utilizes calculation of stride length and stride width. Ischemic injury induced severe gait impairment in the tMCAO group (P< 0.001) when compared to the sham group. This was improved significantly in P4 treated rats when stride length and stride width were evaluated after treatment (P<0.05-0.01) when compared to the tMCAO group (Fig. 1 C-D).

P4 attenuates infarct volume after tMCAO injury

In ischemic stroke models, TTC (2,3,5-triphenyltetrazolium chloride) staining is a fast and reliable visualization method for hypoxic brain tissue, dysfunctional mitochondria, and for defining the size of cerebral infarction. Brain slices of P4 treated tMCAO group after 24 h stained with TTC showed a significant reduction in the infarct volume in comparison to the tMCAO rats (Fig. 2 A&B).

P4 modulates mitochondrial complexes after tMCAO

Mitochondrial NADH dehydrogenase (complex I) plays a crucial role in ETC for energy production and is de-regulated in ischemic injury induced mitochondrial dysfunction. P4 treatment significantly (P< 0.001) elevated activity of NADH dehydrogenase in comparison to only tMCAO group where a significant (P< 0.001) decrease in complex I was seen when compared to the sham group (Fig. 3A). A similar pattern was observed while studying another ETC complex succinate dehydrogenase/complex II that transports electrons to quinone and gets depleted
due to ischemic injury. The enzyme activity of complex II was significantly (P < 0.001) reduced in the tMCAO group when compared to the sham group (Fig. 3B). P4 administration significantly (P < 0.01) elevated the succinate dehydrogenase activity in treated group when compared to the tMCAO group. Further, the MTT reduction rate (complex III) was used to assess the effect on the activity of the mitochondrial respiratory chain. P4 significantly (P<0.05) improved the cell viability, while a significant (P<0.001) decrease in cell viability in the tMCAO group was observed (Fig. 3C). Also, synthase activity (Complex V) is inhibited in mitochondrial dysfunction induced by ischemic stroke. Administration of P4 significantly (P< 0.01) restored the levels of ATPase synthase, while the same was significantly (P< 0.001) decreased in the tMCAO group (Fig. 3D).

**Effect of P4 on mitochondrial oxidative stress parameters**

Lipid peroxidation (LPO) is one of the important biomarkers of oxidative damage. The level of mitochondrial LPO was significantly (P<0.001) elevated in the tMCAO group and was significantly (P<0.001) reduced with P4 treatment (Fig. 3E). Glutathione (GSH) is one of the most crucial non-enzymatic anti-oxidant involved in rescuing the cells from oxidative damage. Mitochondrial GSH level was found to be significantly (P<0.001) reduced in tMCAO rats and was significantly (P<0.01) restored with P4 treatment (Fig. 3F).

**P4 and Mitochondrial bioenergetics**

We investigated the effect of P4 on mitochondrial oxygen consumption (state 3 respiration) and respiratory control ratio (RCR) after 24 h of tMCAO. Results show that oxygen consumption and RCR was reduced (P< 0.05-0.01) in the frontal cortex of tMCAO animals when compared to the sham group. P4 at 8mg/kg b.w. significantly (P<0.05-0.01) restored the oxygen consumption as well as the RCR in the tMCAO treated group when compared to the tMCAO alone group (Fig. 4 A and B).

**P4 attenuates mitochondrial swelling**

P4 was able to bring down the levels of the Ca^{2+} induced mitochondrial swelling. There was no significant difference in light transmission between any groups before adding the Ca^{2+}. The baseline value was taken for five minutes after addition of Ca^{2+} at the concentration of 400µm.
A significant (P<0.001) decrease in light transmission in tMCAO group occurred when compared to the sham group with addition of the Ca\(^{2+}\). This was significantly attenuated by P4 administration (P<0.05) when compared to the tMCAO group (Fig. 4C).

**P4 reduces the Mitochondrial ROS**

The mitochondrial ROS was reflected by DCF fluorescence. Measurement of ROS production in sham, tMCAO, and tMCAO + P4 was carried out as shown by changes in DCF fluorescence intensity (Fig. 5B). There was a significant (P<0.001) increase in mitochondrial ROS level in the tMCAO group when compared to the sham group. P4 treatment effectively (P<0.05) lowered the mitochondrial ROS level as shown by reduced DCF fluorescence intensity in comparison to the tMCAO group (Fig. 5C).

**Effect of P4 on MMP**

The MMP is reflected as a measure of TMRE fluorescence. Changes in MMP in sham, tMCAO, and tMCAO + P4 was reflected by changes in TMRE fluorescence (Fig. 5D). There was a significant (P<0.01) reduction in MMP as indicated by low fluorescence intensity in the tMCAO group when compared to the endogenously depolarized sham group. P4 significantly (P<0.05) elevated the MMP as shown by high TMRE fluorescence intensity when compared to the tMCAO alone group (Fig. 5E).

**Effect of P4 on Neurotransmitters**

P4 treatment significantly (P<0.001) decreased the level of serotonin (5-HT) when compared to that of the only tMCAO group. In the tMCAO group, the level of 5-HT was observed to be significantly (P<0.001) raised when compared to the sham group (Fig. 6A). Also, a significant increase in dopamine level (P<0.05) was observed after tMCAO when compared to that of the sham group. In P4 treated animals, dopamine levels were significantly (P<0.01) lowered when compared to that of tMCAO group alone (Fig. 6B).

**Effect of P4 on enzymatic neurotoxicity markers following tMCAO**

Monoamine oxidase (MAO) is an important neurotoxicity biomarker enzyme in the brain that catalyzes the breakdown of various monoamines. P4 significantly (P<0.001) attenuated the level of MAO in the treatment group when compared to the tMCAO group. There was a significant (P<0.001) elevation in only the tMCAO group (Fig. 6C) when compared to the sham...
group. Another prominent neurotoxicity marker includes the enzyme, acetylcholine esterase (AchE), which is crucial for synaptic termination of the nerve impulse by metabolism of acetylcholine. P4 administration significantly ($P< 0.01$) lowered the activity of AchE in animals in comparison to the tMCAO group. There was a significant ($P<0.001$) elevation of AchE enzyme level in the tMCAO group when compared to the sham group (Fig. 6D). Also, decreased levels of $Na^+ K^+\text{-ATPase}$ during brain injury indicated depletion of ATP. The same was lowered significantly ($P< 0.001$) in the tMCAO group alone, when compared to the sham group. This was observed to be restored in P4 treated animals at the significant rate ($P<0.01$; Fig. 6E) when compared to the tMCAO group alone.

**Histopathology**

The observed histological alterations included the presence of vacuolated spaces, pyknotic nuclei, and heavy neuronal loss in the tMCAO group compared to the sham group. In P4 treated tMCAO group, there was a reduction in vacuolation and neuronal loss as compared to the tMCAO group alone (Fig. 7 A-C). Fig. 7 D includes graphical representation of significant histological alterations in the tMCAO group ($P< 0.001$) and the P4 treated group ($P< 0.05$).

**Immunohistochemical analysis of Cytochrome c Translocation**

In the tMCAO group, the cytochrome c immunostaining was higher when compared to that in the sham group, thereby suggesting cytosolic translocation of cytochrome c. The treatment with P4 was able to reduce the translocation of cytochrome c following tMCAO (Fig. 8 A-C). Cytosolic translocation of cytochrome c was found to be significantly ($P<0.001$) increased in tMCAO rats and its translocation was significantly inhibited ($P<0.01$) with P4 treatment. Quantitative measurements of cytosolic cytochrome c release have been shown in Fig. 8 D.

**Discussion**

**Importance of the study**

In this study, we have investigated the potential mitochondrial mechanism underpinning the P4 enabled neuroprotection in cerebral ischemia model with tMCAO. Based on the results obtained from evaluating alterations in various paradigms (such as mitochondrial permeability, MMP, ROS generation, bioenergetics including ETC complexes in frontal cortex of the brain) we can infer that mitochondrial dysfunction is one of the hallmarks of ischemic/reperfusion
induced cerebral damage leading to cellular death (Kalogeris et al., 2014). Hence, targeting mitochondria could be one of the promising neuro-therapeutic tool (Jin et al., 2016). Blocking of mPTP is already being considered to be one of the prime targets for protection in various neurological diseases (Fayaz et al., 2015). The main findings are: (1) P4 inhibited the formation of mPTP that eventually blocks the translocation of cytochrome c from mitochondria to cytosol (2) P4 attenuated the tMCAO induced mitochondrial ROS that rejuvenated the mitochondrial bioenergetics. (3) P4 restored ETC components and various neurological functions.

**Behavioural Outcomes**

We have performed a number of behavioral assays in rats to support the existence of neurological deficits/anomalies associated with the cerebral ischemic condition. Muscle weakness or motor impairment is a common complaint after stroke in humans. Our results have demonstrated that tMCAO leads to severe impairment in motor coordination which was improved with P4 treatment. Also, abnormal changes occurred in grip strength and gait patterns of tMCAO animals which were further ameliorated by repeated P4 administration at the dose of 8 mg/kg b.w. These observations are in agreement with previous findings of other research groups showing that P4 is able to improve motor coordination and various other neurological deficits (Yousuf et al., 2014). P4 treatment was also able to reduce the infarct size in animals which can be associated with improvement of ischemia-induced neurological deficits.

**Ischemia induced neuronal death**

Infarct volume is a crucial indicator of how severe the ischemic damage is; therefore to confirm this, TTC staining was performed that reliably identifies the infarct core. Only tMCAO operated animals showed a significant cortically lesioned areas, thereby indicating loss of dehydrogenase activity accompanied with increased presence of ROS in the region and probable secondary excitotoxicity (Drose et al., 2016). Another supporting cause might be the inhibition of blood supply to the injured area. This was attenuated by P4 administration post-occlusion which can be attributed to its antioxidant, anti-apoptotic, and free radical scavenging properties (Cai et al., 2015). It can also cross blood brain barrier that might have helped it in reaching the injured brain region easily (Wang et al., 2009).
Oxidative stress causes mitochondrial dysfunction

This ischemia-induced ROS damages the various macromolecules and eventually may lead to cell death by apoptosis and necrosis. LPO, one of the oxidative stress markers, could lead to loss of mitochondrial membrane integrity and uncoupling of mitochondrial respiration as was observed in our study and in a prior study by Rood and Hall (2000). A loss of membrane integrity was observed after 24 h of ischemia/ perfusion. It was accompanied by significant loss of endogenous anti-oxidant GSH, due to increased mitochondrial ROS as well as the compromised activity of ETC components. The change in DCF fluorescence intensity thus correlates linearly with the amount of intracellular ROS formed. Post–occlusion treatment of P4 significantly decreased the mitochondrial ROS as shown by reduction in fluorescence when compared to that of only tMCAO rats. We assume that antioxidant property of P4 might have helped in increasing the mitochondrial GSH content and providing protection against excess ROS (Gaignard et al., 2016). From these data, the pharmacological intervention targeting mitochondrial health seems to be a promising option for treating ischemic damage. Due to mitochondrial LPO and oxidative stress, there was a loss of integrity of respirosomes (complexes I-V) that alters the efficacy of electron transport along the ETC (Lenaz et al., 2010).

In ischemia/reperfusion, the primary event is the lack of oxygen and glucose as a result of a loss of blood supply compromising the mitochondrial ETC complexes I-V/respirosomes (Sims and Muyderman, 2010; Sanderson et al., 2013). We made similar observations in the animals with tMCAO injury alone. P4 treatment after ischemic injury showed the elevated level of these complexes suggesting the role of P4 in stabilizing the mitochondrial membrane.

Modulation of mPTP a possible neuroprotective mechanism of P4

Ischemia/reperfusion induced mitochondrial ROS reduces the electron transfer kinetics which eventually reduces the MMP. MMP and synthesis of ATP can get collapsed in ischemic stroke due to altered respirosomes and disturbed transport of protons across the inner mitochondrial membrane (Kalogeris et al., 2012). Flow cytometric monitoring of TMRE fluorescence demonstrated that ischemic insult resulted in a quick drop in MMP, visible as a strong decline in
fluorescence intensity, and as a result of less MMP and decreased sequestration of this potentiometric-sensitive dye. This was significantly improved in P4 treated group. Moreover, mitochondrial respiration also decreased due to ischemic injury as shown by less state 3 respiration and RCR. P4 significantly attenuated this deficit. P4 due to its antioxidative property helps in preserving the respirosomes assembly by protecting the mitochondrial membrane from LPO (Aggarwal et al., 2008).

Mitochondrial swelling is one of the initial post-ischemic changes (Liang et al., 2013). In the present study, we measured mitochondrial swelling as an index of mitochondrial membrane permeabilization. After an ischemic injury, there was a significant increase in mitochondrial swelling that modulates PTP which leads the passage to the cytochrome c from mitochondria to the cytosol where it forms the apoptosome. This finding led to this presumption that P4 might have blocked the mPTP by binding to cyclosporine D. However, additional molecular studies need to be done to prove this finding. We further presume that if P4 blocks mPTP then translocation of cytochrome c from mitochondria to the cytosol must have been inhibited in P4 treated rats. To prove our hypothesis, we performed immunohistochemical analysis of translocation of cytochrome c from mitochondria to cytosol. The translocation was lowered after P4 administration and thus this finding strengthened our hypothesis that P4 might have blocked the mPTP. The novel finding for the sheds light on mitochondrial protective and the anti-apoptotic role of P4 that can be utilized therapeutically in stroke injury. A previous study on isolated mitochondria has also shown that P4 and its metabolite Allopregnanolone inhibits the release of cytochrome c from mitochondria to cytosol (Sayeed et al., 2009).

**Attenuation of neurotoxicity in ischemia by P4**

We also studied the levels of brain specific enzymes as a marker for neurotoxic damage due to ischemic injury. Alterations in these brain specific enzymes are one of the mechanisms behind neuronal cell death in ischemic stroke. Altered AchE activity and disrupted cholinergic system play an important role in disturbed synaptic transmission and neuronal health in ischemic stroke (Kim and Kim, 2013). There was a significant elevation in AchE levels observed in only tMCAO animals that were lowered with P4 administration. Studies have reported that AchE activity is increased by oxygen and nitrogen reactive species (Affonso et al., 2013). Considering that increased AchE activity has been related to progressive neurological decline and neurodegenerative diseases such as Alzheimer and Parkinson’s disease, it is presumed that a disruption in the cholinergic system may be involved in the neurological deficit associated with the tMCAO model of stroke.
We also evaluated the effect on levels of MAO that leads to oxidation of monoamines such as dopamine, 5-HT, and adrenaline (Wasik et al., 2014). In aging and other neurodegenerative process, an increase in the activity of the mitochondrial enzyme MAO has been seen. MAO catalyses the oxidative deamination of various biogenic and xenobiotic amines, including monoamine neurotransmitters 5-HT and dopamine, along with generating aldehydes and H$_2$O$_2$ (Weinreb et al., 2016). MAO inhibitors and anti-depressants are most commonly used as a treatment for post-stroke depression in stroke patients (McCann et al., 2014). Ischemia-induced injury leads to excessive release of MAO that can lead to depression and anxiety. P4 treatment was able to reduce the excess MAO levels in the tMCAO group. Another enzyme, Na$^+$ K$^+$-ATPase, is an ATP dependent pump for nerve conduction maintaining the resting potential of cell membranes (de Lores Arnaiz and Ordieres, 2014). Due to altered mitochondrial bioenergetics in ischemia, physiological processes such as ATP-dependent ion channels can be derailed leading to alteration of membrane potential as observed in tMCAO injury. P4 was able to substantially elevate the activity of Na$^+$ K$^+$-ATPase by protecting the mitochondrial damage and thus providing the consistent support to the previous findings that P4 has a positive effect on mitochondrial bioenergetics.
Changes in Neurotransmitter affecting the mitochondrial functions

This study also incorporated the analysis of neurotransmitters that play a crucial role in proper functioning of nervous system. After the onset of ischemic stroke, cessation or reduction of blood flow to the brain induces neuronal damage. Monoamine neurotransmitters such as dopamine and 5-HT are important biogenic amines vital for transmitting nerve impulses for cognitive functions of the body. Over-release of above mentioned neurotransmitters triggered by many types of neurotoxic insult such as stroke, hazardous chemicals, tumors, and neurodegenerative diseases affect their respective receptors in the brain which can further induce cytotoxic responses including altered calcium influx, free radical damage, oxidative stress, inflammatory responses, and apoptosis, potentially leading to neuronal cell death, and neurodegeneration. (Chen et al., 2014).

Dopamine not only works as a neurotransmitter but acts as a good metal chelator and electron donor in in vivo conditions generating toxic free radicals through the redox reaction. It has a high tendency to generate H$_2$O$_2$ through Fenton’s chemistry. This, in turn, causes mitochondrial respiratory chain breakdown and oxidative stress leading to cardiomyopathy and neurodegeneration (Uttara et al., 2009). 5-HT has an inhibitory effect on mitochondrial respiration causing brain ATP depletion. It causes an excitotoxic death of nerve cells which involves both limitations of energy production and increased cellular activation (Chen et al., 2014). Their levels were found to be elevated in tMCAO group and were attenuated by P4 treatment. P4 is known to possess specific anti-serotonin actions (Morton et al., 2015). P4 can attenuate neuronal excitotoxicity by blocking calcium channels or by upregulating inhibitory neurotransmitter GABA which in turn can block the release of other excitatory neurotransmitters in the CNS (Wei and Xiao, 2013). Also, we have shown in the present study, P4 inhibited the activity of enzyme MAO which breaks down dopamine to generate toxic products such as H$_2$O$_2$, oxygen-derived radicals, semiquinones, and quinones leading to mitochondrial LPO and exerts neurotoxic effects.

P4 improves anatomical damage in ischemic tissue

tMCAO operated animals showed a series of histological alterations, including marked neurodegeneration with pyknotic neurons. P4, successfully ameliorated these histological alterations as observed by the reduced number of pyknotic neurons.
Conclusion
The current study adds further evidence that P4 is neuroprotective in cerebral ischemia. Our results indicate that P4 mediates its neuroprotection through mitochondrial pathways in male rats after cerebral ischemia. In conclusion, the results demonstrate that P4 was able to modulate the functional and cellular deficits associated with ischemic injury via mitochondrial pathways. It is important to assess the potential neuroprotective candidate, such as P4, in the group which is at higher risk of stroke. As this study focused on mitochondrial pathways in male rats, further studies are warranted to study the effects on female rats.
Material and Methods

Animals and treatment regimen
Male Wistar rats (250-300 g) were obtained from the Central Animal House Facility of Jamia Hamdard, New Delhi, India. The animals were kept under a 12-hour light-dark cycle with free access to food and water. Use of animals and all experimental procedures were conducted according to the procedures approved by Animal Ethics Committee, Jamia Hamdard.

Experimental groups
The rats were divided randomly into three groups: (1) Sham operated group (2) tMCAO group (3) tMCAO + P4 (8 mg/kg b. w). The experimenter was blinded to the treatment group. All parameters were done in the frontal cortex of brain and n = 6 were taken for each set of parameters in each group respectively.

Surgical procedure
Focal tMCAO was performed according to the method of Vaibhav et al. (2013). Prior to tMCAO surgery, animals were anesthetized with choral hydrate (400 mg/kg). A midline incision was made on the ventral surface of neck to expose the right common carotid artery. External carotid artery (ECA) was ligated and internal carotid artery (ICA) was isolated near to bifurcation. An intraluminal monofilament of filament size 4.0, length 30 mm, and diameter 0.19 mm having a silicon rubber coated tip was introduced into ECA and advanced through ICA up to the origin of middle cerebral artery (MCA). The suture was withdrawn slowly after 1 h occlusion of MCA, and rats were returned to their cages for the period of 23 h for reperfusion. In the sham group, ECA was surgically prepared but the filament was not inserted. Animals were returned to their normal environment in air conditioned room at an ambient temperature (25 ± 2 °C) and relative humidity (45-50%) with 12 h light/dark cycles and allowed free access to the pellet diet and purified drinking water.
**P4 treatment**

P4 (P-0130; Sigma-Aldrich Co) at a dose of 8 mg/kg b.w. was dissolved in 50% dimethyl sulphoxide (DMSO) and 50% saline, which was administrated by intraperitoneal (i.p.) injection 1 h post-occlusion followed subcutaneously (s.c) at 6, 12, and 18 h post-occlusion. This dose is considered to be the optimal dose for producing neuroprotection (Wali et al., 2014)

**Behavioral tests**

All naïve animals were given random training for testing motor coordination, grip strength, and gait pattern before experiment for five days in order to acclimatization and get a maximum possible score.

1. **Assessment of motor impairment**

To assess the sensorimotor coordination, the rats were evaluated in the rota rod task before sacrifice. In this study, motor function was assessed by using Rota rod unit (Omni Rotor, Omnitech Electronics, Inc., Columbus, OH, USA) which consists of rotating rod of diameter 75 mm which is divided into four compartments to test four animals at a time after 23 h post-occlusion (Ashafaq et al., 2012b). The time for each animal remaining at rotating rod was recorded for three trials at a min interval of 5 min with a maximum trial length of 180 sec for each trial. The apparatus automatically recorded the time in 0.1 sec till the rat falls on the floor. The speed was set at 10 rotations per min and cut off time was 180 sec. The score was presented as mean of three trials to which rat remains on the rotating rod.

2. **Assessment of Grip strength**

Grip test was performed by using the method of (Ashafaq et al., 2012a). The apparatus consists of a string measuring about 50 cm in length, pulled tight between two vertical supports and elevated 40 cm from the flat surface. The rats were put on the string at a midway and scoring was done according to the following scoring scale, 0 = fall of, 1 = hangs onto string by two forepaws, 2 = hangs on string by two forepaws and also trying to climb on string, 3 = hangs onto string by two forepaws along with one or both hind paws, 4 = hangs onto string by all forepaws along with tail wrapped around the string, 5 = escape.
3. Gait analysis
The gait patterns were evaluated to find the gait-related anomalies after 24 h of tMCAO were performed according to the previously published method (Ashafaq et al., 2012a). Stride length and stride width was measured by using enclosed wooden walkway of width 12 cm. The fore paws were stained with green non-poisonous coloring agent and hind paws were stained with red color. Stride length was measured as the distance between ipsilateral fore paw and hind paw. Stride width was taken as the distance between two fore paws and hind paws respectively.

Measurement of Infarction volume
The animals were sacrificed after 1 h of occlusion followed by 23 h of reperfusion (Ashafaq et al., 2016). The brains were dissected out and kept in a brain matrix. 1.5 mm coronal sections of the brains were cut down with the help of sharp blades and stained with 0.1 % 2,3,5-triphenyl-tetrazolium chloride (TTC) prepared into normal saline at 37 °C for 15 min. For imaging, the sections were scanned by a high-resolution scanner. The infarct volume was measured with software ImageJ.

Tissue collection and mitochondrial preparation
Mitochondria of brain frontal cortex of rat were isolated by differential centrifugation method (Waseem and Parvez, 2016). Rats were decapitated and frontal cortex were dissected and homogenized by using a mechanically driven Teflon-fitted Potter-Elvehjem type homogenizer in ice cold buffer A. Frontal cortex mitochondria were isolated in three buffers A, B and C. Buffer A containing 250 mM sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM EGTA, and 0.1 % fat-free BSA adjusted by Tris to pH 7.4 and centrifuged at 1000g for 8 min at 4 °C. The supernatant was collected and centrifuged at 10,000g for 10 min at 4 °C. Thereafter, the obtained pellet was resuspended and washed twice with washing medium (B) containing 250 mM sucrose, 10 mM HEPES, and 0.1 mM EGTA adjusted by Tris to pH 7.4 and centrifuged at 12,300g for 10 min. Finally, the pellet was resuspended again in an isolation medium (C) containing 250 mM sucrose, 10 mM HEPES, and 0.1 % fat-free BSA adjusted by Tris to pH 7.4 and centrifuged at 12,300g for 10 min.
Mitochondrial Complexes Measurement

NADH dehydrogenase activity (Complex I)
NADH dehydrogenase activity was assayed by spectrophotometrically according to (Waseem and Parvez, 2016). The enzyme activity was expressed as micromoles of NADH oxidized per minute per milligram protein using molar extinction coefficient of 21,000 M$^{-1}$cm$^{-1}$.

Succinate dehydrogenase activity (Complex II)
The activity of succinate dehydrogenase was assayed according to the method described by (Waseem and Parvez, 2016) by using the spectrophotometer. The enzyme activity was expressed as micromoles of succinate oxidized per minute per milligram protein using molar extinction coefficient of 1000 M$^{-1}$cm$^{-1}$.

Cytochrome c reductase (Complex-III)
Cytochrome c reductase activity was measured by a described by (Waseem and Parvez, 2016). The results were expressed as micromoles of formazan formed per minute per milligram protein with the molar excitation coefficient of 51,000 M$^{-1}$cm$^{-1}$.

F1 –F0 synthase activity (Complex V)
ATP synthase is also referred to as mitochondrial complex V. Its activity was assayed as a hydrolysis of ATP into ADP plus inorganic phosphate (P$_i$) as described by (Waseem and Parvez, 2016). The enzyme activity was expressed as microgram of P$_i$ liberated per minute per milligram protein.

Assessment of oxidative stress damage
Mitochondrial LPO was assayed according to the procedure of (Chaudhary and Parvez, 2012) with certain modifications. The results were expressed as nanomoles of thiobarbituric acid reactive substances (TBARS) formed per hour per gram tissue using molar extinction coefficient of $1.56 \times 10^5$ M$^{-1}$ cm$^{-1}$. GSH concentration in brain mitochondria was measured according to the method of (Tabassum et al., 2007). The GSH content was calculated as nanomoles of GSH reduced per gram tissue.
Mitochondrial Respiration measurement

Mitochondrial oxygen consumption was measured by using Clark-type oxygen electrode (Hansatech Instrument) (Waseem and Parvez, 2016) at 37 °C pH 7.4 in a KCl medium containing 0.1 mM EDTA, MgCl₂, sucrose, and KH₂PO₄. Rats were sacrificed 24 h after tMCAO and frontal cortex was isolated for mitochondrial preparations. Mitochondrial respiratory energy coupling was evaluated by determining respiratory control ratio (RCR) calculated as the rate of ADP–induced state 3 respiration to the state 4 rate without ADP. The rate of mitochondrial respiration was measured as nanomoles of oxygen (O₂)/min/mg of protein.

Mitochondrial swelling

Mitochondrial swelling caused by the influx of solutes through open PT pores results in an increase in light transmission (i.e., a reduced turbidity). This turbidity change offers a convenient and frequently used assay of the MPT by measurement of absorbance in mitochondrial suspensions. Mitochondrial permeability was assayed by Ca²⁺ induced mitochondrial swelling and was assayed by spectrophotometrically (Li et al., 2012). The mitochondrial pellet was re-suspended in ice cold BSA free and EDTA free sucrose buffer after the last step of washing (300 mmol sucrose and 10 mmol/L Tris-Base, pH 7.4). The aliquot of 100µg of mitochondria was added to 1 ml of BSA free and EDTA free buffer and 400µm Ca²⁺ was added after five minutes and reading was taken for 5 minutes at 540 nm.

Flow cytometric analysis of MMP and mitochondrial ROS

Flow cytometry analysis was performed using a FACS Calibur equipped with a 488 nm argon laser and a 635 nm red diode laser according to (Li et al., 2012). Data from the experiments were analyzed using the Cell Quest software (BD Bioscience). To exclude debris, samples were gated based on light scattering properties in the side scattering (SSC) and forward scattering (FSC) modes, and 10000 events per sample within the R1 gate were collected. The mitochondrial sample was suspended in analysis buffer containing 250 mmol/L sucrose, 20 mmol/L MOPS, 10 mmol/L Tris-Base, 100 µmol/L Pi(K), and 0.5 mmol/L Mg²⁺ and 5 mmol/L succinate at pH 7.4. The mitochondria were then stained with TMRE (100 nmol/L, excitation at 488 nm and emission at 590 nm) and H₂DCFDA (10 mmol/L, excitation at 488 nm and emission at 525 nm), which were used to measure the mitochondrial membrane potential and the production of ROS respectively.
Neurotransmitter detection using HPLC

Estimation of 5-HT and dopamine in the brain FC was done using HPLC-ECD by the method of Chaudhary and Parvez, (2012). Chromatographic analyses were performed at room temperature. Data were acquired and processed in Empower Pro Operating System. 5-HT peaks were identified by comparing their retention time in the sample and its concentration was estimated according to the area under the curve using straight line equation \( y=mx+c \). Dopamine concentration was represented as ng/mg protein.

Neuronal insult markers

MAO was measured according to the method reported by Vishnoi et al., (2015). The enzyme activity was assayed as nanomoles of BAHC hydrolyzed per minute per milligram protein using a molar extinction coefficient 7.6925 M\(^{-1}\)cm\(^{-1}\). AchE was estimated according to the method by (Vishnoi et al., 2015). The enzyme activity was measured as nmoles of ATC hydrolyzed/min/mg protein using a molar extinction coefficient of 1.36 \(\times10^{4}\) M\(^{-1}\) cm\(^{-1}\). Na\(^+\), K\(^+\), ATPase activity was measured according to the method developed by (Chaudhary and Parvez, 2012). The activity was measured as µg of pi liberated/min/mg protein.

Histology

After performing neurobehavioral tests, animals were deeply anesthetized intraperitoneally with chloral hydrate (400 mg/kg b. wt) after 24 h of ischemia/reperfusion and were transcardially perfused through ascending aorta with 0.9% saline according to the method of Ashfaq et al. (2012). This was followed by ice-cold buffered 4% paraformaldehyde and then the brain was removed and put in paraformaldehyde for 48 hr. Brain removal was followed by dehydration with ethanol. It was then embedded in paraffin then coronal sections (40 µm thick) were taken for hematoxylin and eosin staining. Histopathological scoring was done by taking the help of pathologist of our animal house. Scoring was done on the basis of morphological changes in the frontal cortex. According to the morphological changes, sections were given scores as follows: 0 (no change), 1 (minor changes), 2 (vacuolated spaces), 3 (pyknotic nuclei), 4 (vacuolated spaces and pyknotic nuclei), 5 (heavy neuronal loss).
Cytochrome c

Immunohistochemical analysis was performed according to the method of (Vishnoi et al., 2015), with some modifications. The brain was removed and fixed in 4% paraformaldehyde. The brain sections were cut from paraformaldehyde-fixed, paraffin embedded and mounted on poly-L-lysine coated microscopic slides. Sections were de-paraffinized three times (5 min) in xylene followed by dehydration in graded ethanol and finally rehydrated in running tap water. For antigen retrieval, sections were boiled in 10 mM citrate buffer (pH 6.0) for 10–15 min. Sections were incubated with hydrogen peroxide for 15 min to minimize non-specific staining and then rinsed three times (5 min each) with PBST (0.05% Tween-20). Blocking solution was applied for 10 min, then sections were incubated with the anti-Cytochrome c antibody mouse monoclonal (dilution 1:200, Calbiochem®, cat #QIA87) overnight at 4°C in a humid chamber. The anti-cytochrome C antibody is a mouse monoclonal (isotype IgG2b) that reacts with human, mouse and rat cytochrome C. Next day, the slides were washed with PBST three times and slides were incubated with secondary anti body goat anti-mouse IgG for two hours. The peroxide complex was visualized with 3, 3-Diaminobenzidine (DAB). Next, the slides were counter stained with hematoxylin and dried. Finally, the sections were mounted with DPX and covered with cover slips. The slides were ready to be observed under the microscope. The intensity of the cytosolic immunostaining protocol was used for quantitative evaluation of immunostaining. Measurements were carried out using an inverted light microscope using objectives with 40x magnifications. Quantification of IHC slides has been done by image j 1.49V (Wayne Rasband National Institute of Health, USA).

Determination of protein

Protein contents in different fractionations of brain frontal cortex, viz., supernatant, homogenate, and mitochondria were assayed by Bradford method using BSA as a standard.

Statistical Analysis

All data were analyzed by taking as mean ± standard error of mean (SEM). All data were analyzed by using analysis of variance (ANOVA) followed by Tukey’s test. All data were analyzed by using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Values P < 0.05 were considered significant.
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References:


Fig. 1. Behavioral test parameters. All animals were subjected to various neurological tests (n=6). Fig. 1A depicts the grip strength test done to assess the muscular strength ***P<0.001 versus sham, *P<0.05 versus tMCAO. Fig. 1B shows the time remaining on the rota rod ***P<0.001 versus sham, ###P<0.001 versus tMCAO. Fig. 1C represents stride length, ***P<0.001 versus sham, ##P<0.01 versus tMCAO to assess the gait impairment. Fig. 1D measures stride width ***P<0.001 versus sham, *P<0.05 versus tMCAO, (n=6 in each group).
**Fig. 2. Effect of P4 on Infarct volume.** Fig. 2A shows the images of TTC staining. In the tMCAO group, there was severe infarction as compared to that of the P4 group. Infarcts are shown as white (unstained) regions involving cortex. Fig. 2B shows infarct volume $#P<0.05$ versus tMCAO.
Fig. 3. Effect of P4 on mitochondrial complexes and oxidative parameters. Analysis of mitochondrial complexes of isolated mitochondria from frontal cortex of the brain (n=6 in each group). Effect of Progesterone on NADH dehydrogenase (complex I) ***P<0.001 versus sham, ###P<0.001 versus tMCAO (Fig.3A). Effect of progesterone on succinate dehydrogenase (Complex II) ***P<0.001 versus sham, ##P<0.01 versus tMCAO (Fig.3B). MTT assay was done to assess the metabolic activity of cells in the frontal cortex of the brain (n = 6), ***P<0.001 versus sham, #P<0.05 versus tMCAO (Fig.3C). Effect of progesterone on F1–FO synthase activity (Complex V) ***P<0.001 versus sham, ##P<0.01 versus tMCAO (Fig.3D), (n=6 in each group). Effect of P4 on mitochondrial lipid peroxidation ***P<0.001 versus sham, ###P<0.001 versus tMCAO (Fig. 3E) (n= 6 in each group). Effect of P4 on mitochondrial GSH ***P<0.001 versus sham, ##P<0.01 versus tMCAO (Fig.3F; n= 6 in each group).
Fig. 4. Effect of progesterone on mitochondrial bioenergetics. Effect of P4 on mitochondrial oxygen consumption (state 3 respiration rate) *P<0.05 versus sham, #P<0.05 versus tMCAO (Fig.4A). Effect of P4 on respiratory control ratio in sham, tMCAO and tMCAO+P4 (n=6 in each group) **P<0.01 versus sham, ##P<0.01 versus tMCAO (Fig.4B). Effect of P4 on mitochondrial swelling ***P<0.001 versus sham, #P<0.05 versus tMCAO (Fig.4C), (n=6 in each group).
Fig. 5. Measurement of mitochondrial ROS and Mitochondrial membrane potential

In the FSC/SSC plot of the isolated mitochondria, 10000 events were collected within gate R1 (Fig. 5A). Measurement in ROS production in sham, tMCAO and tMCAO + P4 as shown by changes in DCF fluorescence. The relative changes in DCF fluorescence intensity (Fig. 5B), (n=6 in each group). The relative changes in DCF fluorescence intensity (n=6 in each group). **P<0.001 versus sham, #P<0.05 versus tMCAO (Fig. 5C). (n=6 in each group). Changes in mitochondrial membrane potential in sham, tMCAO and tMCAO + P4 as reflected by changes in TMRE fluorescence (Fig. 5D). The relative changes in TMRE fluorescence intensity are shown (n=6 in each group). **P<0.001 versus sham, #P<0.05 versus tMCAO (Fig. 5E). (n=6 in each group).
Fig. 6. Effect of P4 on 5-HT, Dopamine and neurotoxicity parameters.

Effect of P4 on 5-HT ***P<0.001 versus sham, ###P<0.001 versus tMCAO (Fig.6A). Effect of P4 on dopamine *P<0.05 versus sham, ##P<0.01 versus tMCAO (Fig.6B; n= 6 in each group).

Effect of P4 on monoamine oxidase activity ***P<0.001 versus sham, ###P<0.001 versus tMCAO (Fig.6C).

Effect of Progesterone on Acetylcholine esterase activity ***P<0.001 versus sham, ##P<0.01 versus tMCAO (Fig.6D). Effect of Progesterone on Na⁺ K⁺-ATPase ***P<0.001 versus sham, ##P<0.01 versus tMCAO (Fig.6E; n= 6 in each group)
**Fig. 7. Effect of P4 on histopathology**

Representative histopathological photomicrograph of frontoparietal layers of the sham, tMCAO and tMCAO + P4 group. In the sham group, there was no vacoulation or any neuronal loss. In the tMCAO group, there was vacoulation and heavy neuronal loss. In P4 treated group there was the partial neuronal loss (Fig. 7 A-C). Magnification at 40x. Histological alterations are represented graphically in Fig. 7 D. shows significant (P<0.001) in tMCAO and in P4 treated group there was the significant improvement (P<0.05).
Fig. 8. Effect of P4 on cytochrome c Translocation.
Representative images of frontoparietal layers of the brain were taken for analyzing the translocation of cytochrome c from mitochondria to cytosol. In the tMCAO group, the cytochrome c immunostaining is higher as compared to that in the sham group, which suggests cytosolic translocation of cytochrome c. Treatment with P4 is able to reduce the translocation of cytochrome c following tMCAO (Fig. 8 A-C). Cytosolic translocation of cytochrome c was found to be significant (P<0.001) in tMCAO rats and its translocation was significantly inhibited (P<0.01) with P4 treatment. Quantitative measurements of cytosolic cytochrome c release have been shown in Fig. 8 D.