Neuroprotective effects of combined trimetazidine and progesterone on cerebral reperfusion injury

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ABSTRACT

Cerebral ischemia-reperfusion injury induces multi-dimensional damage to neuronal cells through exacerbation of critical protective mechanisms.

Targeting more than one mechanism simultaneously namely, inflammatory responses and metabolic energy homeostasis could provide additional benefits to restrict or manage cerebral injury. Being proven neuroprotective agents both, progesterone (PG) and trimetazidine (TMZ) has the potential to add on the individual therapeutic outcomes.

We hypothesized the simultaneous administration of PG and TMZ could complement each other to synergize, or at least enhance neuroprotection in reperfusion injury. We investigated the combination of PG and TMZ on middle cerebral artery occlusion (MCAO) induced cerebral reperfusion injury in rats. Molecular docking on targets of energy homeostasis and apoptosis assessed the initial viability of PG and TMZ for neuroprotection. Animal experimentation with MCA induced ischemia-reperfusion (I/R) injury in rats was performed on five randomized groups.

Sham operated control group received vehicle (saline) while the other four I-R groups were pre-treated with vehicle (saline), PG (8 mg/kg), TMZ treated (25 mg/kg), and PG + TMZ (8 and 25 mg/kg) for 7 days by intra-peritoneal route. Neurological deficit, infarct volume, and oxidative stress were evaluated to assess the extent of injury in rats. Inflammatory reactivity and apoptotic activity were determined with alterations in myeloperoxidase (MPO) activity, blood-brain barrier (BBB) permeability, and DNA fragments. Reperfusion injury inflicted cerebral infarct, neurological deficit, and shattered BBB integrity.

The combination treatment of PG and TMZ restricted cellular damage indicated by significant ($p < 0.05$) decrease in infarct volume and improvement in free radical scavenging ability (SOD activity and GSH level). MPO activity and LPO decreased which contributed in improved BBB integrity in treated rats. We speculate that inhibition of inflammatory and optimum energy utilization would critically contribute to observed neuroprotection with combined PG and TMZ treatment. Further exploration of this neuroprotective approach for post-recovery cognitive improvement is worth investigating.

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1. Introduction

Injury to the brain due to stroke or trauma leads to severe neuronal damage resulting in death or disability of the patient. WHO reports, around half a million human individuals succumb to stroke, and another half-million are left disabled for rest of their life (Prabhakaran et al., 2015). During the last three decades (1990–2019), the incidences of stroke have increased substantially more than 70% to earlier decades. Similarly, stroke-related mortality was also increased by 43% during these decades. In 2019, stroke incidence cases were 12.2 million, while deaths from stroke mounted to 6.55 million. Stroke remained the second most relevant cause of death worldwide, and it is the third leading cause of death associated with disability. Out of all these incidences, ischemic stroke constituted 62.4%, and its associated mortality was higher (3.6 times than the high-income group) in low-income groups during 2019 (GBD 2019 Stroke Collaborators, 2021).

In India, men were slightly more prone to stroke incidences than females, while the one-month fatality rates hovered between 18 and 42% (Jones et al., 2022). The current approach in stroke management prioritizes the restoration of blood flow to the ischemic region through the use of thrombolytic agents or the execution of mechanical recanalization techniques (Goldstein, 2014). Paradoxically, this restored blood circulation exacerbates injury beyond the ischemic core into the penumbra and is termed as ischemia-reperfusion injury (Pan et al., 2007).

Ischemia-reperfusion injury destabilizes energy utilization and limits enzymatic activity that scavenges free radicals in the brain. Escalation of energy demands and generation of free radicals renders ischemic brain prone to inflammatory reactivity and deficiency of ATP (Kalogeris et al., 2014; Kawabori and Yenari, 2015). β-oxidation (utilize free fatty acid as a substrate) does provide an alternative source of energy, but it also adds on to the generation of lipid peroxides (Chomova and Zitnanova, 2016). The mitochondrial enzyme, 3-Ketoacyl coenzyme A thiolase (3-KAT, also referred to as β-keto thiolase) regulates β-oxidation, and in turn, recruits free radicals during this process which contributes to neuronal injury (Yang et al., 1987; Reichmann et al., 1988; Kantor et al., 2000; Onay--Besikci and Ozkan, 2008). Incidences of prolonged ischemia initiate cell death, both necrotic as well as apoptotic, by expressing specific proteins or up-regulating signaling pathways like apoptotic protease activating factor-1 (Apaf-1), and Rho/Rho-kinase (ROCK) signaling, respectively (Yan et al., 2015; Noshita et al., 2001). The underlying mechanisms: energy deficit, oxidative stress, and apoptosis in ischemic brain injury regulate cell death; and any modulation of enoyl-CoA hydratase enzyme, Apaf-1, or ROCK may rescue brain tissue from reperfusion injury (Yan et al., 2015; Noshita et al., 2001; Li et al., 2013).

The tissue plasminogen activators (tPA), the only approved anti-ischemic agent, has limitations and failed to correct multiple underlying mechanisms (Broussalis et al., 2012). Clinical reports indicate trimetazidine (TMZ) restores energy homeostasis and attenuate free radical generation to exhibit benefits in ischemia and angina pectoris (Kantor et al., 2000; Dhote and Balaraman, 2008; Iqbal et al., 2002; Nowak et al., 2006). TMZ shows its anti-ischemic activity, inhibits beta-oxidation of fatty acids by blocking long-chain 3-ketoacyl-CoA thiolase, and enhances glucose oxidation (Kantor et al., 2000). Similarly, progesterone (PG) protects brain damage, restores blood-brain-barrier, suppresses inflammation, and in turn limits apoptosis of the viable cells (Stein, 2008; Ishrat et al., 2009, 2010).

We aimed to investigate the neuroprotective potential of combination treatment of TMZ and PG that extends survival of penumbra (tissue at risk) upon exposure to reperfusion. We explored whether individual therapeutic effects of these two agents complement each other to address multiple underlying pathological mechanisms of brain injury.

The *in-silico* analysis predicted the binding of TMZ and PG to some molecular targets of β-oxidation and apoptosis. We hypothesized that positive metabolic regulation and inhibition of inflammatory reactivity by combination treatment of TMZ and PG might alleviate brain damage. The simultaneous administration may complement the therapeutic potential of individual agents to enhance neuroprotection in the middle cerebral artery occlusion (MCAO) induced ischemia-reperfusion injury in rats.

2. Materials and methods

Trimetazidine (TMZ) was gifted by US Vitamins Ltd (Mumbai, India), while progesterone (PG) and other chemicals like 2,3,5-triphenyl-tetrazolium chloride, Superoxide dismutase (SOD), Malondialdehyde (MDA), and catalase (CAT) were procured from Sigma-Aldrich (St Louis, MO, USA). Reduced glutathione (GSH), 5-,5-dithiobis 2-nitrobenzoic acid (DTNB), and thiobarbituric acid (TBA) were bought from Merck India, Ltd. (Mumbai, India). Rest of the chemicals had an analytical grade.

2.1. In silico molecular docking analysis

To predict and correlate the pharmacological activity of progesterone and TMZ, the molecular docking study was conducted, where both agents were docked against active sites of identified targets: β-ketothiolase enzymes, Enoyl reductase, Apoptotic protease-activating factor 1 (Apaf-1), and Rho Kinase.

2.1.1. Protein preparation

The three-dimensional (3D) structures of 4 targeted proteins (Table 1) were retrieved from the Protein Data Bank (http://www.rcsb.org/). The retrieved proteins were β-ketothiolase (4NZS), Enoyl reductase (2NV6), Apoptotic protease-activating factor 1 (3YGS), Rho Kinase (2H9V). Water (within 3 Å) and other nonspecific molecules were removed by using UCSF Chimera (Pettersen et al., 2004). For protein protonation to maintain cellular pH, polar hydrogens were added.

2.1.2. Molecular docking study

The structurally refined protein.pdb files of target proteins were converted to.pdbqt files after the addition of the Kollman charge using AutoDock MGL tools 1.5.6. The 3D chemical structures of PG and TMZ were retrieved from the source (http://drugbank.ca/) in sdf format and converted into pdb format using chemaxon marvin suite. After the addition of Gasteiger charges, the AutoDock tools 1.5.6 converted these pdb files.pdbqt. AutoDock vina docked and analyzed PG and TMZ (Trott and Olson, 2010) for all target proteins with the grid box parameters (Table 1). The docked structures were further visualized using Discovery Studio Visualizer 2019 to identify binding interactions.

2.2. Animals

Male Sprague-Dawley rats (16–22 weeks) procured from the central animal housing facility of the institute housed under standard light and dark cycle; the temperature and humidity were maintained throughout the study period. The rats housed had free access to standard pelleted food and water and the study protocol was approved by the Institutional Animal Ethical Committee (PH/IAEC/2k12/18).

2.3. Induction of reperfusion injury by middle cerebral artery occlusion

The rats were randomly divided into 5 groups and each group comprises 30 rats (in total 150 rats), and exposed to focal cerebral reperfusion (I/R) injury with middle cerebral artery occlusion by a 3–0 nylon monofilament (Ethilon; Johnson and Johnson, Mumbai, India) as described by Longa et al., with few modifications (Longa et al., 1989).

The ischemia of 2 h was followed by a 24-h reperfusion period in the brain region. The group I (sham operated, SO, no occlusion) and group 2 (I/R, MCA occlusion), the rats were treated (*i.p.*) with 5% DMSO and saline 7 days before I/R injury; in group 3, the rats received 7-day pretreatment of PG (8 mg/kg) while in group 4 the rats were pretreated with TMZ (25 mg/kg). The rats in group 5 were treated with the combination

Table 1
Docking interactions of PG and TMZ with various targets.

Target	PDB ID	Resolution	Binding Energy (Kcal/mol)		Grid Box			
			PG	TMZ	Dimensions	Coordinates		
						X	Y	Z
β-ketothiolase	4NZS	2.29 A	−6.5	−5.7	76*76*76	−20.43	20.53	−28.46
Enoyl reductase	2NV6	1.90 A	−8.3	−5.8	56*56*56	−3.48	37.03	15.08
Apoptotic protease-activating factor-1	3YGS	2.05 A	−6.3	−4.6	50*50*50	61.96	29.24	62.99
Rho-Kinase	2H9V	3.10 A	−9.1	−5.8	76*76*76	9.70	−17.55	−23.96

Table legend text: Progesterone PG (PG; 8 mg/kg) trimetazidine (TMZ; 25 mg/kg).

of PG and TMZ for 7 days before the I/R injury. Each group was further divided into five sets of 6 animals (n = 6) to evaluate different parameters. Details of set are as follow.

Set I: For estimation of neurological score and infract analysis.
Set II: For estimation of oxidative stress markers.
Set III: For estimation of DNA fragmentation.
Set IV: For estimation of MPO.
Set V: For estimation of histology.

The body temperature of the rats was maintained with a heating blanket throughout the experiment; the rats were sacrificed after 24-h reperfusion and the brain was isolated. PG and TMZ dissolved in 5% dimethyl sulfoxide (DMSO) and saline for *ip* administration to rats.

2.4. Quantification of neurological damage

The neurological outcomes were measured after 24 h of MCAO in rats using a five-point scale, based on the motor coordination of a rat, which was followed to access the neurological score. The scoring of the neurological deficit was as follows: *0*, no neurological deficit; *1*, failure to extend the right paw fully; *2*, circle movement to the right; *3*, the fall to the right; *4*, an inability to walk spontaneously, and the loss of consciousness (Longa et al., 1989).

2.5. Infarct analysis

The rat was sacrificed and the isolated brain was immediately frozen and coronal sections (2 mm thick slice) to stain with 2% 2,3,5-triphenyl tetrazolium chloride (TTC) for 30 min at room temperature. Brain sections were fixed overnight with a 10% formalin solution; the infarct area was measured in each brain section with Image j software 1.30 V (http://www.rsb.into.nih/ij). The infarct volume (mm^3) was calculated after multiplying the total area of infarct from all slices with its thickness (Bederson et al., 1986, Thiyagarajan and Sharma, 2004). Similarly, brain swelling was determined using the following equation (Maier et al., 1998).

Brain swelling (%) = (volume of the ipsilateral hemisphere – the volume of the contralateral hemisphere)/volume of contralateral hemisphere x 100.

2.6. Estimation of oxidative stress biomarkers

We measured oxidative stress in homogenized brain tissue (1 gm) using ice-cold 10% trichloroacetic acid (TCA). Lipid peroxidation (LPO) is expressed as malondialdehyde (MDA) contents (mmol MDA /mg of tissue protein) and estimated with the formation of thiobarbituric acid-reactive substances (Draper and Hadley, 1990). Superoxide dismutase (SOD) activity was determined with pyrogallol autoxidation using a kinetic reaction at 420 nm for 5 min (Marklund and Marklund, 1974). One unit SOD activity expressed as the amount of enzyme that inhibited oxidation of pyrogallol by 50%. The activity of catalase at 240 nm was estimated by the method described by Aebi et al., and expressed as mmol H_2O_2 consumed/min mg protein (Aebi, 1984). Similarly, reduced glutathione (GSH) was determined using DTNB reagent at 412 nm and expressed as µg of GSH/mg of protein (Moron et al., 1979).

2.7. Estimation of myeloperoxidase activity

The myeloperoxidase (MPO) enzyme extracted from brain tissue its activity was estimated by the method described by Bradley et al. Tissue homogenized using phosphate buffer and hexadecyl trimethyl ammonium bromide; the homogenate was freeze-thawed three times to extract enzyme and centrifuged at high speed. Ortho-dianisidine dihydrochloride and hydrogen peroxide dissolved in phosphate buffer was used to dilute supernatant; and a kinetic reaction, for 2 min, recorded the absorbance at 460 nm. The consumption of 1 nmol peroxide/min at 22 °C was reported as a unit of MPO activity (unit/mg of tissue protein) (Bradley et al., 1982).

2.8. Determination of blood brain barrier (BBB) permeability

The integrity of BBB was evaluated by Evans blue (EB) extravasation due to leakage in the brain by spectrophotometric method at 610 nm. BBB permeability was quantified as µg of the EB/hemisphere (Gursoy-ozdemir et al., 2000).

2.9. Estimation of tissue protein

Tissue protein content was estimated in aliquots of diluted membrane fractions using a colorimetric reaction with Folin's phenol reagent. Change in colour of the reaction mixture at 640 nm was reported as mg of protein/gm of wet tissue (Lowery, 1951).

2.10. Analysis of DNA fragmentation by gel electrophoresis

We isolated the brain and harvested DNA from the cortex of the hemisphere (ipsilateral) where blood flow was occluded. The tissue was digested immediately with RNAase enzyme in Tris-HCL and DNA extracted using phenol-chloroform. Each agarose gel (0.8%) lane had 8 µg DNA, which was stained with ethidium bromide (0.5 µg/mL) to separate the DNA in multiple bands (Simonian et al., 1996) using electrophoresis; strands were compared with 1 K DNA ladder (Gene ruler 1 K Fermentas, St Leon, Germany). DNA fragmentation and subsequent fragmented bands provide a qualitative measure of the apoptotic process.

2.11. Observation of histological changes

Deeply anesthetized rats were transcardially perfused with normal saline, the brain was isolated, and washed immediately to fix in ice-cold 10% buffered formalin. After 48 h, tissue was dehydrated with ethanol and embedded in paraffin to section (40 µm) with microtome. Haematoxylin and eosin (H and E) stained sections were placed on glass slides, fixed, and observed under the microscope (BX4O; Olympus, Tokyo, Japan) to identify histopathological alterations. The changes in the frontal cortex were graded as: *0* (no change); *1* (partial loss with intact neurons); *2* (vacuolated spaces), *3* (pyknotic nuclei); *4* (vacuolization and pyknotic nuclei); *5* (severe neuronal injury).

2.12. Statistical analysis

All the data were summarized as mean $\pm$ SEM, analyzed with One Way Analysis of Variance (ANOVA) followed by Tukey's test. Neurological deficit and histological changes were analyzed with the Kruskal Wallis test and reported as median values with the quartile range (25–75%). Statistical difference was considered significant with the values $P < 0.05$ using GraphPad Prism 5 (GraphPad Software, USA).

3. Results

3.1. In-silico docking analysis

Docking study analyzed the binding energies of PG and TMZ with all four targets; the protein-ligand binding free energy was measured to express drug-target interaction. We found PG had maximum bind energy with Rho-kinase (−9.1 kcal/mol) and enoyl reductase (−8.3 kcal/mol) indicating marked inhibition of these enzymes. Similarly, TMZ demonstrated comparable binding energies with all targets (Table 1).

The macromolecular target interactions of these agents showed multiple bond formations with amino acids (Figs. 1–4). PG developed multiple alkyl interactions (VAL-25, ALA-32, LEU-33) and TMZ involved in pi-sigma (ALA-179) as well as pi-cation (ARG-186) interaction at A chain of β-ketothiolase. With enoyl reductase, PG again formed multiple

alkyl bonds (ALA-191, ILE-194, MET-199) at A chain, while TMZ developed H-bonding (GLY-, ILE-194) and pi-sigma (ILE-21) bonds. Further with Apaf-1, PG repeated the same pattern by forming multiple alkyl interactions (PHE-34, VAL-69, VAL-93, VAL-94) at C-chain, while TMZ ensured pi-pi stacking (PHE-34) and H-Bonding (HIS 77). Rho kinase interactions with PG resulted in multiple alkyl reactions (VAL-106, LYS-121, MET-169, LEU- 221, ALA-231) & pi-sigma bond (PHE-103) at A-chain while TMZ participated by developing H-bonding (GLU-110) (Figs. 1–4).

3.2. Neurological outcome

Cerebral reperfusion injury-induced neurological deficit is an indicator of muscle coordination of the rats. The I/R control rats had severe impact on motor activity; the gait got altered. Treatment with PG and TMZ reduced this motor in-coordination and circling of the rats, but this difference did not reach statistical significance (Table 2).

3.3. Infarct volume

Induction of cerebral reperfusion injury in rats increased infarct volume in the ipsilateral hemisphere of the I/R control rats (212.4 $\pm$ 13.65 mm^3; Figs. 5 and 6). The treatment with PG and TMZ reduced the infarct volume in treated rats; the combination of both drugs decreased
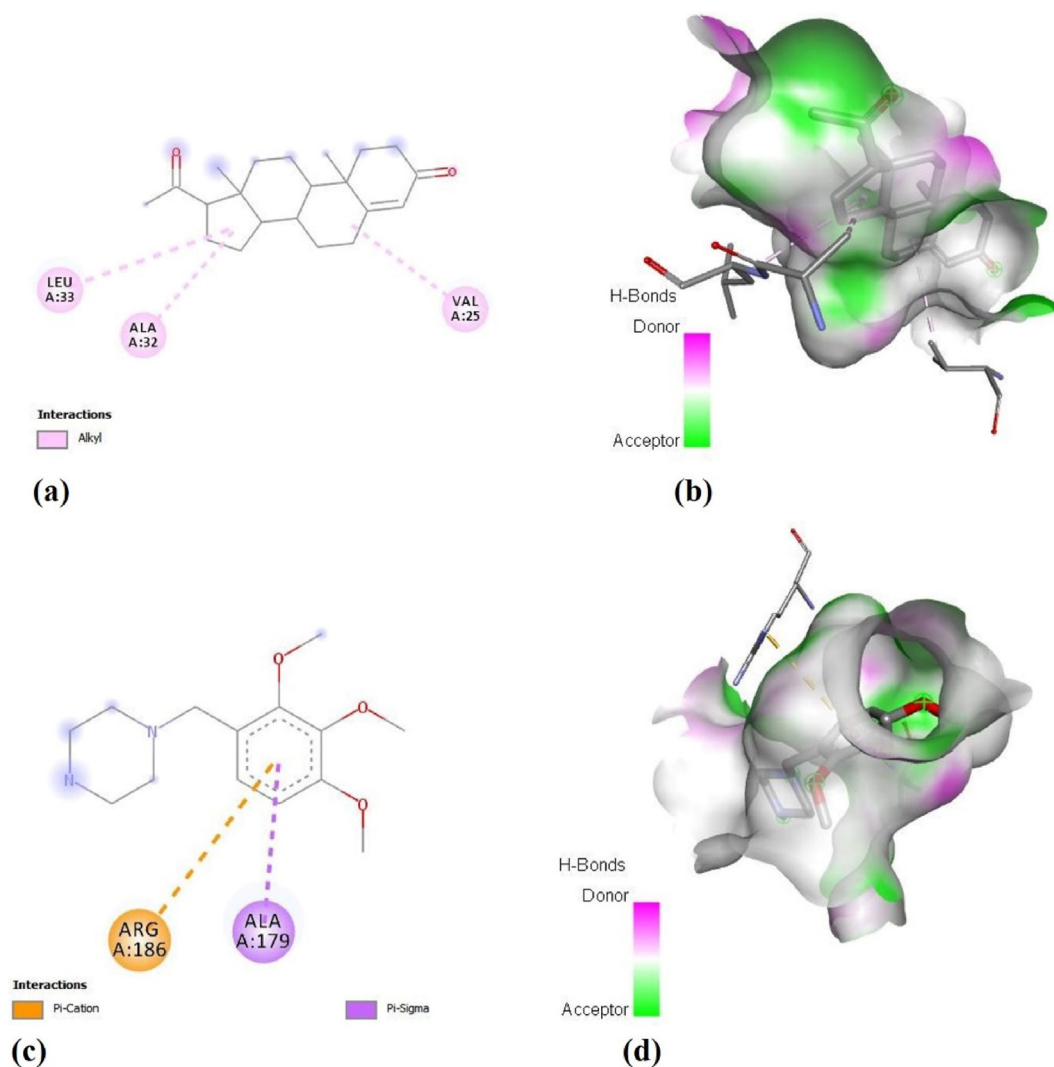

Fig. 1. Docking details of Progesterone [(a) 2D-interactions (b) 3D-interactions (H-bonding surface)] and Trimetazidine [(c) 2D-interactions (d) 3D-interactions (H-bonding surface)] against β-ketothiolase.
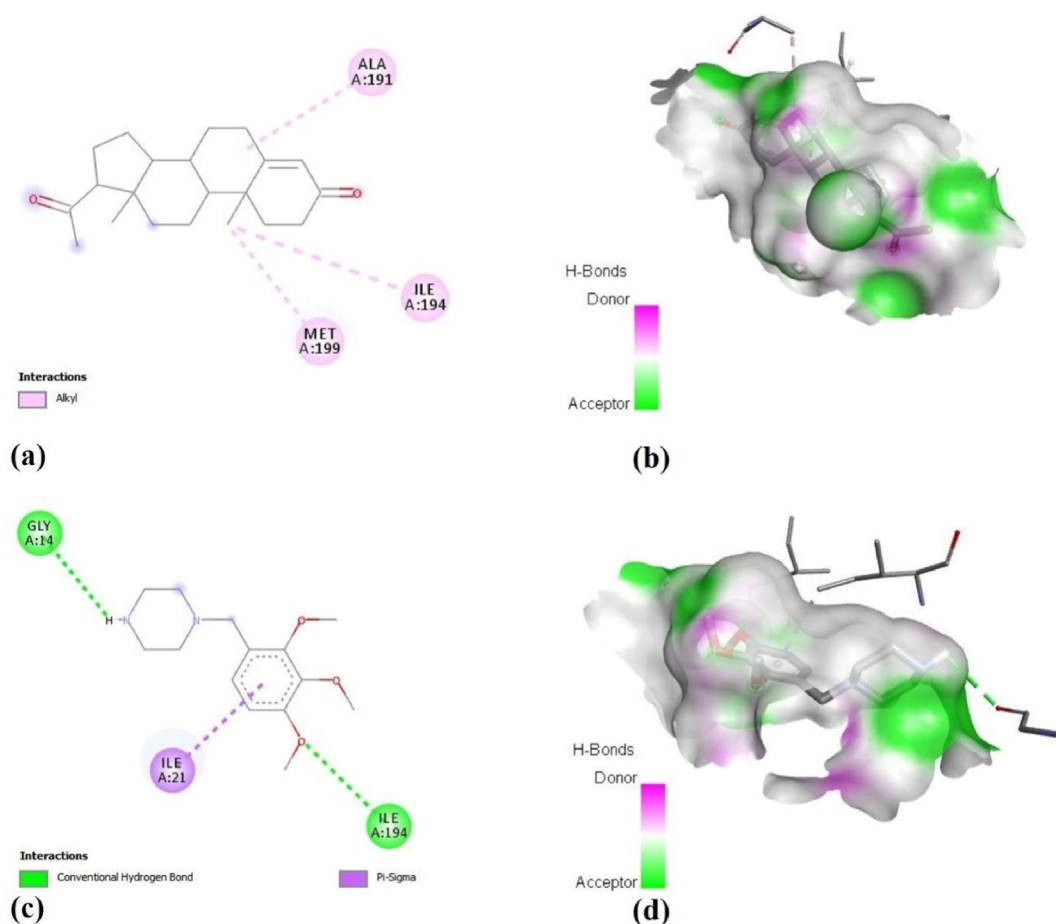
Fig. 2. Docking details of Progesterone [(a) 2D-interactions (b) 3D-interactions (H-bonding surface)] and Trimetazidine [(c) 2D-interactions (d) 3D-interactions (H-bonding surface)] against Enoyl reductase.

infarct volume (54.59 ± 7.51 mm^3), which was significantly ($P < 0.05$) different from the rats in other groups. Similarly, the percentage of swelling in the ischemic brain was highest (26.43 ± 2.90 %) in rats of the vehicle-treated I/R control group. The reduction in brain swelling after combination treatment (9.43 ± 0.64 %) was statistically significant– more than half to that of the I/R control group.

3.4. Scavenging of oxidative free radicals

The ipsilateral hemispheres of rats in the I/R control group had a significant change in oxidative stress biomarkers: SOD, Catalase, GSH, and LPO. The ischemic brain tissue had significantly less SOD activity and marked increase in LPO than sham-operated (non-ischemic) rats. The treatment, a combination of PG and TMZ was more effective than vehicle treatment at improving SOD activity and GSH levels, while it significantly reduced LPO in rats (Table 3). The change in catalase activity had no statistical significance amongst the treatment groups. The difference between the SOD activity (9.15 ± 0.25 IU) and MDA content (6.42 ± 0.33 nmol) of the combination treatment group was statistically ($P < 0.05$) significant as compared with PG or TMZ groups.

3.5. The activity of the myeloperoxidase (MPO) enzyme

The MPO activity, a biomarker for neutrophil infiltration and inflammatory reactivity, showed significant elevation in the brain due to reperfusion injury; and the pretreatment with PG and TMZ produced a significant decrease in MPO activity of the treated rats. The I/R control group had elevated MPO activity (0.49 ± 0.03 units) against the baseline (0.19 ± 0.02) seen in the sham-operated group after reperfusion injury.

The observed significant difference brought in by the combined treatment (0.23 ± 0.02 units), in comparison with the I/R control group and the single treatment groups, indicated reduced inflammation (Table 4).

3.6. Permeability of blood brain barrier

The pretreatment with PG, TMZ, or its combination cut the extravasation of EB in the brain to more than half of its content in the I/R control group (3.86 ± 0.53 μg/mL). The significant ($P < 0.05$) difference in the EB content of the treated group demonstrated reduced damage to the integrity of BBB in comparison with the I/R control group (Table 4).

3.7. DNA fragmentation

Laddering of DNA in the I/R group suggested fragmentation of DNA strands upon exposure to reperfusion injury; treatment groups showed reduced a pattern of DNA laddering, an indicator of DNA damage in the brain (Fig. 7).

3.8. Histological changes

The structural damage to brain tissue included the presence of neuronal loss, vacuolated spaces, and the number of pyknotic nuclei (Fig. 8A–E). The histological changes observed in the I/R control group were significantly different from the sham-operated group indicating tissue damage due to reperfusion injury. The pre-treatment with a combination of PG and TMZ restricted structural damage in tissue, which reflects in the significant difference of histological grades when compared with the I/R control group (Fig. 9).
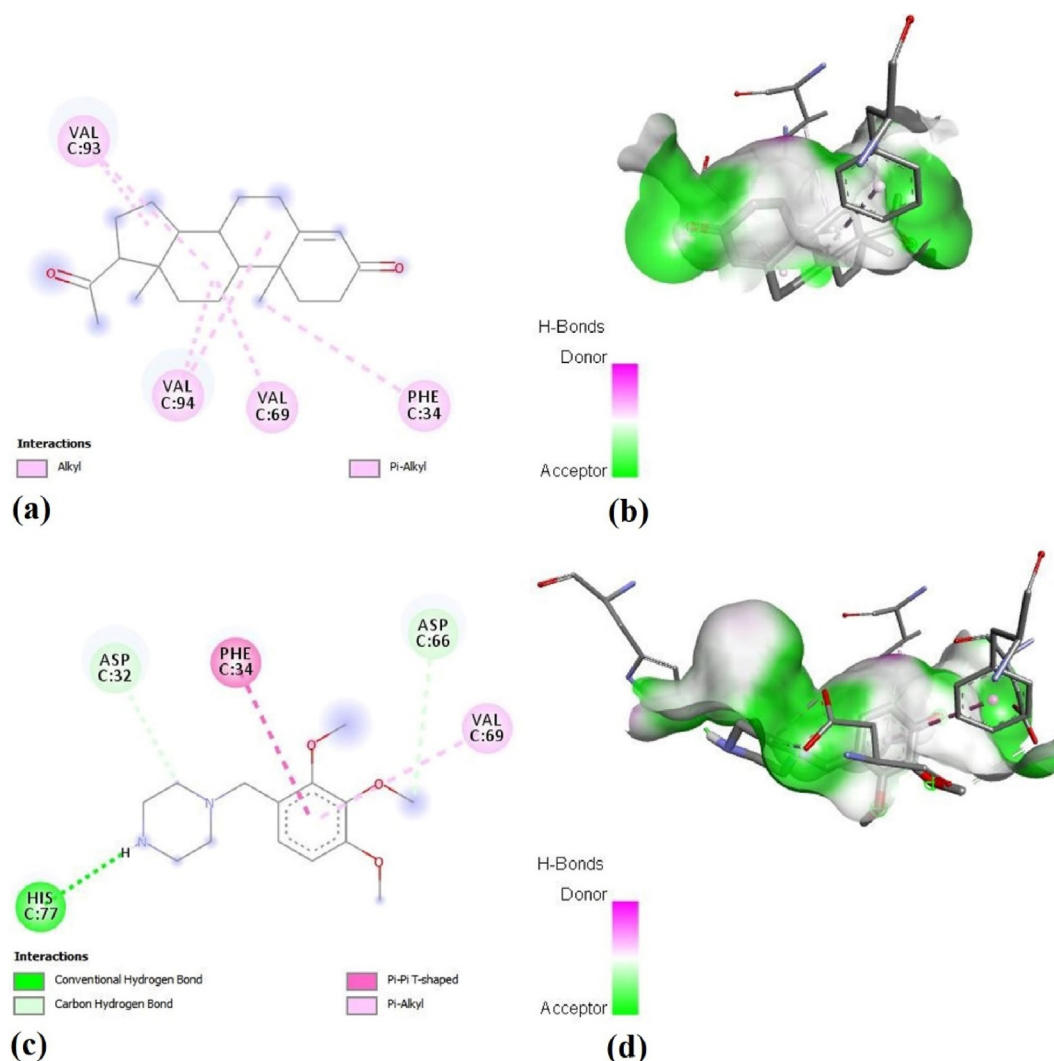
Fig. 3. Docking details of Progesterone [(a) 2D-interactions (b) 3D-interactions (H-bonding surface)] and trimetazidine [(c) 2D-interactions (d) 3D-interactions (H-bonding surface)] against Apoptotic Protease-activating factor 1.

4. Discussion

We found that combination treatment of PG and TMZ provided neuroprotection by reducing 1) infarct volume, 2) oxidative stress, and 3) inflammation in ischemia-reperfusion injury. The outcomes indicate that the combination of two drugs, which target multiple mechanisms of ischemia-reperfusion injury, complement each other to inhibit inflammation and reduce lipid peroxidation. Earlier reports emphasized that meeting the energy demand of cells, reducing oxidative stress, and limiting inflammatory reactivity is crucial to yield additive or even synergistic effects in ischemia-reperfusion injury (Kalogeris et al., 2014; Chomova and Zitnanova, 2016; Rink and Khanna, 2011; Hedayatpour et al., 2018; Toung et al., 2004).

We demonstrated a decrease in infarct volume of treated rats after simultaneous treatment of PG and TMZ. The cerebral infarction is a result of impaired mitochondrial function and deficient oxidative respiratory enzyme activity (Rink and Khanna, 2011; Longa et al., 1989; Bederson et al., 1986; Thiyagarajan and Sharma, 2004). TMZ restores energy metabolism in mitochondria (Kantor et al., 2000; Nowak et al., 2006; Onay-Besikci and Ozkan, 2008), and the improvement in metabolic energy utilization resulted in a reduction in infarct volume following reperfusion injury (Iqbal et al., 2002; Dhote and Balaraman, 2008). PG also attenuates mitochondrial dysfunction by its free radical scavenging

ability and contributes to a decrease in neuronal cell death (Andrabi et al., 2017; Roof et al., 1997).

The use of molecular docking is a rational approach to model the drug-protein interaction that characterizes the behavior of a drug in the binding sites of the target proteins. We docked PG and TMZ on multiple targets of energy homeostasis; TMZ fits best with β-ketothiolase (also referred to as 3-KAT), with minimum binding energy indicating a high affinity for the enzyme. PG had very low binding energy for enoyl reductase suggesting greater conformational compatibility leading to alteration in fatty acid oxidation activity and mitochondrial functions responsible for a significant decrease in infarct volume (Kantor et al., 2000; Iqbal et al., 2002; Nowak et al., 2006; Sayeed and Stein, 2009, Ishrat et al., 2009).

The ischemia followed by reperfusion generates loads of free radicals that induce neurotoxicity and impair most of the neuronal functions, especially the peroxynitrite (Kalogeris et al., 2014; Cuzzocrea et al., 2001). The dysbalance in generation and detoxification of free radicals injure components of neuronal cells such as structural proteins, DNA, and cellular membranes (Kalogeris et al., 2014; Chen et al., 2011). The decreased scavenging activity of SOD and elevated MDA content indicates elevated FFA metabolism and impaired fuel utilization (Chomova and Zitnanova, 2016; Watson et al., 2006).

Combined treatment not only restored GSH content and SOD activity but also reduced lipid peroxidation in the current study. TMZ reduces
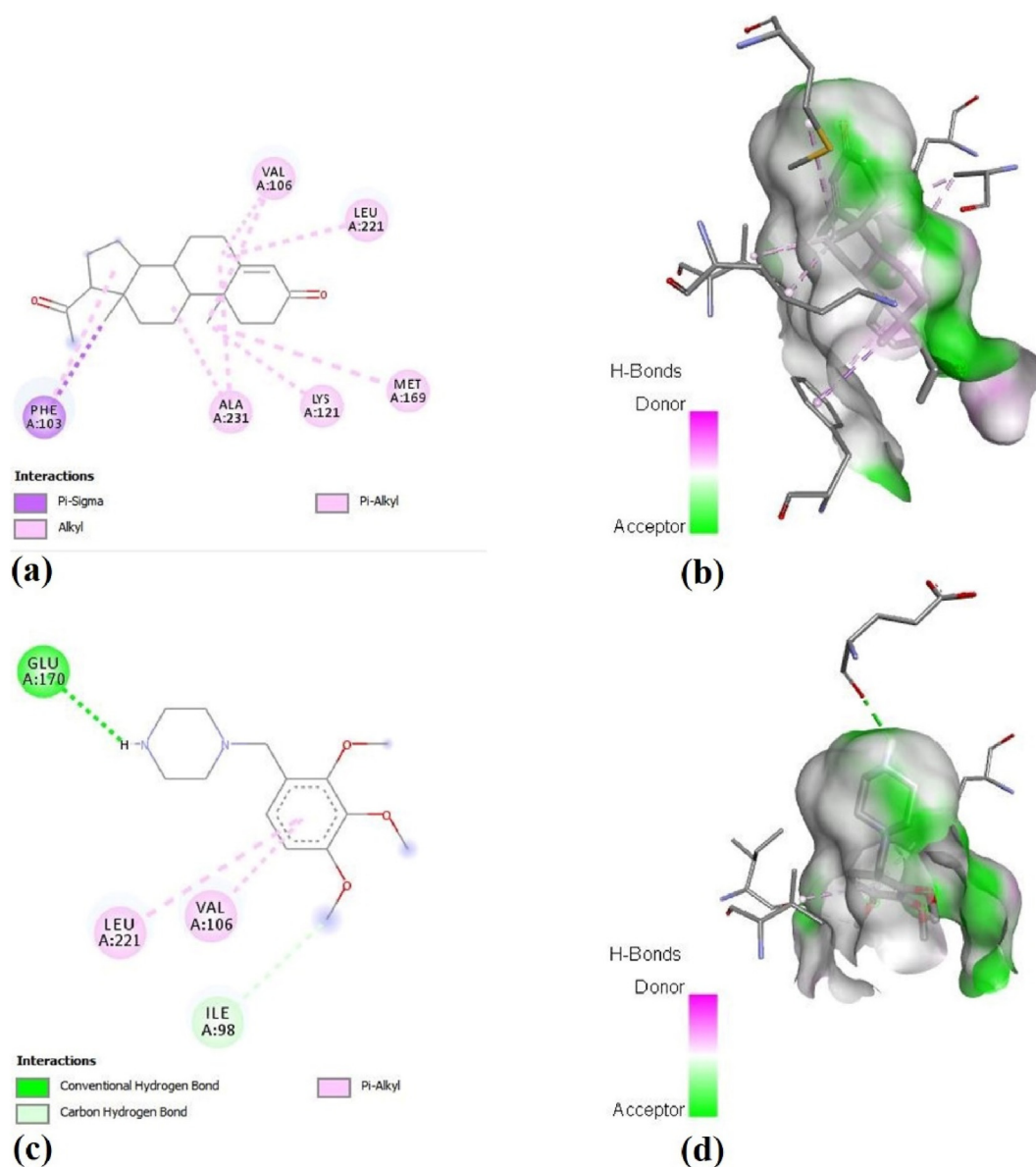
Fig. 4. Docking details of progesterone [(a) 2D-interactions (b) 3D-interactions (H-bonding surface)] and trimetazidine [(c) 2D-interactions (d) 3D-interactions (H-bonding surface)] against rho kinase.

Table 2
Neurological deficit.

Treatment	Neurological Score	Interquartile Range	
		25% Percentile	75% Percentile
I/R	3.5	3.00	4.00
PG	2.5	1.00	3.00
TMZ	2.5	1.75	3.25
PG + TMZ	2.5	1.00	3.00

Table legend text: Neurological deficit was assessed after 22 h of reperfusion. The values represented as median of scores and respective interquartile range; n = 6. Ischemia-reperfusion injury (I/R); Progesterone PG (PG; 8 mg/kg) trimetazidine (TMZ; 25 mg/kg) group, compared with I/R; $P < 0.05$.

lipid peroxidation by attenuating β-oxidation through inhibition of 3-KAT coenzyme and ensures glucose uptake in the brain (Watson et al., 2006; Nowak et al., 2006; Onay-Besikci and Ozkan, 2008). The improvement in SOD activity was better with combined treatment than the individual treatment with PG or TMZ, whereas lipid peroxidation had

significantly decreased than PG alone. This antioxidant activity contributes to neuroprotection in reperfusion injury (Dhote and Balaraman, 2008; Iqbal et al., 2002; Watson et al., 2006; Roof et al., 1997).

Reperfusion injury instigates the pro-inflammatory mediators via lipid peroxidation to trigger brain damage (Ziebell and Morganti-Kossmann, 2010; Kawabori and Yenari, 2015). The infiltration of neutrophils, following free radical generation, is crucial to exacerbate neuronal injury (Rink and Khanna, 2011; Kalogeris et al., 2014). The activation of infiltrated neutrophils stimulates myeloperoxidase activity in inflamed tissue (Kawabori and Yenari, 2015; Bradley et al., 1982). We demonstrated a decrease in myeloperoxidase activity of treated rats in the current study. PG and TMZ suppress interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), responsible for activating microglia, and instigating inflammatory damage in the brain (Iqbal et al., 2002; Zhou et al., 2012; Ishrat et al., 2009). The observed inhibition of inflammation by PG and TMZ is in harmony with earlier reports (Andrabi et al., 2017; Roof et al., 1997; Iqbal et al., 2002; Zhou et al., 2012).

Neuroinflammation contributes to secondary brain injury, and it shatters the integrity of endothelial cell junctions to damage BBB (Hinson
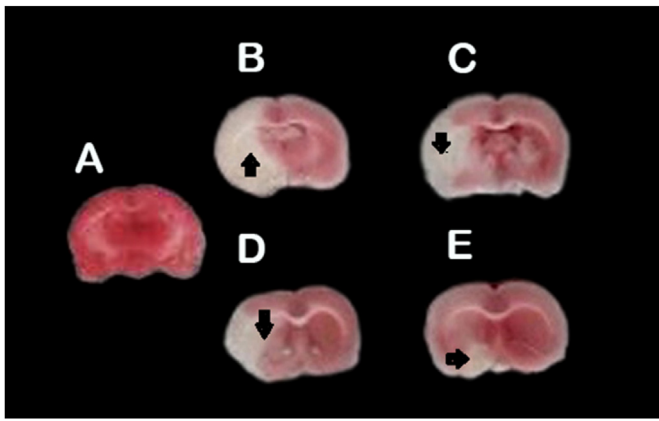
Fig. 5. Infarct area in brain slice after I/R injury:
Unstained (white colour) region indicates infarction which is marked by arrow displaying infarction in the brain and stained (red colour) indicates uninfarcted region in the brain. The slices represent, **A:** Sham operated rat with no or very small infarct area; **B:** I/R control rat with large infracted tissue area; **C:** Progesterone treated rat with slight reduction in infarct area; **D:** Trimetazidine treated I/R rat showed reduced cellular infarct; **E:** Progesterone and trimetazidine combination treated I/R rat had smallest infarct area indicated by an arrow. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

et al., 2015; Fukuda and Badaut, 2012, Sekaran et al., 2019). The treatment with the combination of PG and TMZ reduced the extravasation of EB dye, indicating a decrease in the destruction of BBB. The enhanced BBB permeability ensures passive diffusion of water and results in swelling; neutrophils infiltrate and release cytokines (Ishrat et al., 2010; Rosenberg and Yang, 2007, El Ali et al., 2011). PG decreases water diffusion (Guo et al., 2006; Ishrat et al., 2010), and TMZ blocks cytokine release (Zhou et al., 2012; Tritto et al., 2005) to restore BBB integrity.

The mitochondrial energy homeostasis regulates the rate and extent of apoptosis in reperfusion injury (Christophe and Nicolas, 2006). DNA fragmentation, a qualitative measure of apoptosis, is associated with an energy deficit, altered membrane permeability, and excitotoxicity (Yang et al., 2018; Christophe and Nicolas, 2006; Bradley et al., 1982). Activation of ROCK, a signaling protein in apoptosis, also regulates the integrity of BBB and induces structural damage (Yan et al., 2015; Kim

et al., 2012; El Ali et al., 2011; Christophe and Nicolas, 2006). Moreover, caspase enzymes activation, implicated in cell death, gets triggered by oligomerization of Apaf-1 and Rho-kinase signaling (Yan et al., 2015; Christophe and Nicolas, 2006; Noshita et al., 2001). With a molecular docking study, we demonstrated that PG had a strong binding affinity for both enzymes: enoyl reductase and Rho-kinase.

Similarly, TMZ exhibits high Gibb's free energy due to the best fit conformer with these enzymes (Trott and Olson, 2010). Both PG and TMZ reduced apoptosis by modulating energy homeostasis and suppressing apoptotic protein (Luoma et al., 2011; Ishrat et al., 2009; Kantor et al., 2000)., indicated by reduced DNA fragmentation which strongly correlates in-silico outcomes with in-vivo observations of the current investigation. Hence, the co-administration of PG and TMZ would complement reducing the fragmentation of DNA strands (Luoma et al., 2011; Dhote and Balaraman, 2007, 2008; Iqbal et al., 2002). Histological changes indicating restriction of structural damage in the brain slices also substantiated the observed outcomes of reduced injury through anti-apoptotic actions.

Our outcome implies inhibition of inflammation through the decreased release of cytokines and neutrophil activation, although expression of IL-6 and TNF-α might reflect the impact on inflammation more clearly; however, myeloperoxidase activity, used here, is also an index of inflammation in chronic disorders (Deng et al., 2018). Similarly, the lactate/pyruvate ratio would give more insight into altered energy metabolism.

This investigation demonstrated a significant decrease in inflammation and oxidative stress with the combination of PG and TMZ in ischemia-reperfusion injury. The combination regulated, to some extent, the metabolic energy fuel, oxidative stress, inflammation, and apoptosis to provide neuroprotection. The data suggests interlinked roles of oxidative stress and inflammation in reperfusion injury (Rink and Khanna, 2011; Ziebell and Morganti-Kossmann, 2010); it also underlines the significance of mitochondrial energy balance and apoptosis to restrict brain damage (Chomova and Zitnanova, 2016; Christophe and Nicolas, 2006). One reason for neuroprotection is that both agents cross BBB readily and distribute in large portions of the brain to influence various events of ischemia-reperfusion injury (Iqbal et al., 2002; Ishrat et al., 2010, Andrabi et al., 2017). We carefully selected the doses of PG (8 mg/kg) and TMZ (25 mg/kg) to avoid the ceiling neuroprotective effect achieved by any individual treatment. Earlier, studies reported the use of multiple-dose levels of both agents for pharmacological actions in various animal studies (Ishrat et al., 2010; Sayeed and Stein, 2009; Iqbal et al., 2002; Chen et al.,
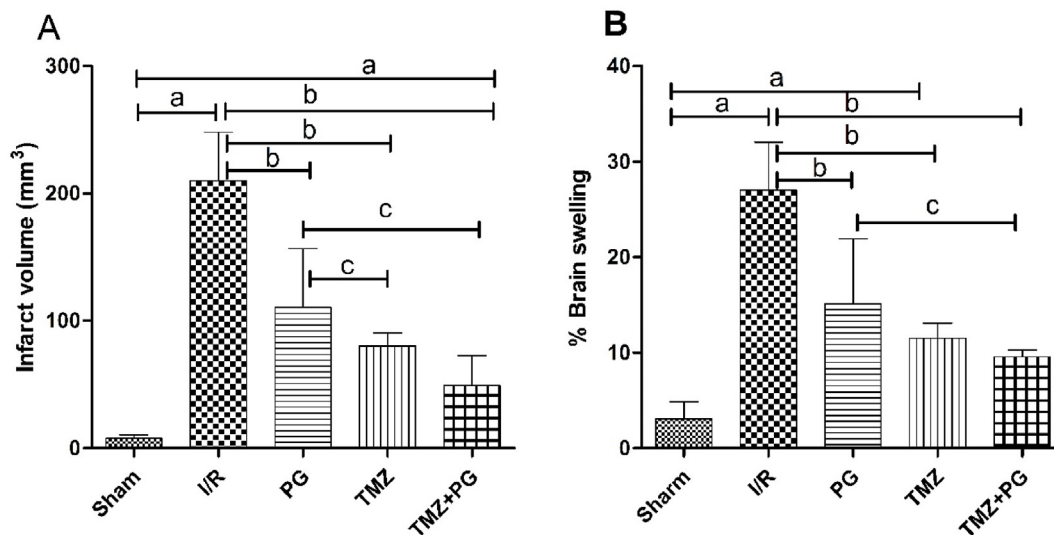

Fig. 6. Infarct volume and Brain swelling
Data represented as mean ± SEM (n = 6); Sham operated (SO); Ischemia reperfusion injury (I/R); Progesterone PG (PG; 8 mg/kg), trimetazidine (TMZ; 25 mg/kg) group. Significant difference is [a]$P < 0.05$ vs SO group; [b]$P < 0.05$ vs I/R group; [c]$P < 0.05$ vs PG group.

Table 3
Effect of treatment on oxidative stress markers.

Groups	SOD (IU/mg of protein)	CAT (H$_2$O$_2$ consu/min/mg of protein)	GSH (µg/mg of protein)	MDA (nmol/mg of protein)
SO				
Ipsilateral	9.03 ± 0.34	232.35 ± 5.16	21.39 ± 1.19	4.97 ± 0.62
Contralateral	9.22 ± 0.89	244.30 ± 11.77	21.67 ± 3.98	5.14 ± 0.39
I/R				
Ipsilateral	4.17 ± 0.38[a]	185.39 ± 22.92	5.20 ± 0.22[a]	12.94 ± 0.67[a]
Contralateral	6.48 ± 0.54	218.75 ± 21.49	15.69 ± 0.26	9.35 ± 0.66[a]
PG				
Ipsilateral	8.00 ± 0.19[b]	149.41 ± 12.69	9.97 ± 0.82[ab]	8.63 ± 0.41[a,b]
Contralateral	7.40 ± 0.50	190.8 ± 10.92	16.8 ± 3.51	7.91 ± 0.39[a]
TMZ				
Ipsilateral	7.19 ± 0.14[b]	206.2 ± 12.10	10.11 ± 0.31[ab]	7.73 ± 0.44[a,b]
Contralateral	7.24 ± 0.86	197.6 ± 9.64	11.34 ± 2.31	7.17 ± 1.00
PG + TMZ				
Ipsilateral	9.19 ± 0.25[b,c,d]	279.56 ± 20.36	14.84 ± 0.87[ab]	6.42 ± 0.33[a,b,d]
Contralateral	8.65 ± 0.53	209.16 ± 15.79	15.03 ± 1.57	6.60 ± 0.39[b]

Table legend text: Data represented as mean ± SEM (n = 6); Sham operated (SO); Ischemia reperfusion injury (I/R); Progesterone PG (PG; 8 mg/kg) trimetazidine (TMZ; 25 mg/kg) group. Significant difference is [a]$P < 0.05$ vs SO group; [b]$P < 0.05$ vs I/R group; [c]$P < 0.05$ vs PG group; [d]$P < 0.05$ vs TMZ group.

Table 4
Effect of treatment MPO and Blood Brain Barrier permeability estimation.

Groups	MPO units/mg of protein	Evans blue µg/mL
SO		
Ipsilateral	0.19 ± 0.02	0.97 ± 0.19
Contralateral	0.20 ± 0.03	0.87 ± 0.14
I/R		
Ipsilateral	0.49 ± 0.03[a]	3.86 ± 0.53[a]
Contralateral	0.38 ± 0.03	2.79 ± 0.92
PG		
Ipsilateral	0.37 ± 0.02[a,b]	1.53 ± 0.17[ab]
Contralateral	0.29 ± 0.03	1.19 ± .013
TMZ		
Ipsilateral	0.36 ± 0.03[a,b]	1.54 ± 0.13[ab]
Contralateral	0.27 ± 0.04	1.50 ± 0.1
PG + TMZ		
Ipsilateral	0.23 ± 0.02[b,c,d]	1.35 ± 0.18[ab]
Contralateral	0.21 ± 0.04	1.31 ± 0.15

Table legend text Data represented as mean ± SEM (n = 6); Sham operated (SO); Ischemia reperfusion injury (I/R); Progesterone PG (PG; 8 mg/kg) trimetazidine (TMZ; 25 mg/kg) group. Significant difference is [a]$P < 0.05$ vs SO group; [b]$P < 0.05$ vs I/R group; [c]$P < 0.05$ vs PG group; [d]$P < 0.05$ vs TMZ group.

Fig. 7. DNA damage and fragmentation
Gel electrophoresis of ipsilateral hemisphere of brain; Lane 1, 1-kb DNA standard; Lane 2, Sham control; Lane 3, IR control, indicates apoptotic cellular injury in the form laddering pattern; Lane 4, Progesterone treated, no fragmented DNA; Lane 5, Trimetazidine, absence of laddering of DNA stands; Lane 6, combination treated rat brain without any apparent DNA breaks.

1999; Napoli Di, 2008; Jain et al., 2010). This combined intervention improved antioxidation, attenuated inflammation, restored vascular permeability, and reduced apoptosis.

Interestingly, this treatment schedule failed to induce any marked improvement in the neurological outcome, suggesting that 24 h duration is insufficient to assess neurological recovery in the MCAO model of transient focal ischemia-reperfusion injury. This study adds two vital pieces of information to the existing knowledge; first, the docking of PG and TMZ validates the correlation of therapeutic effect and the binding with molecular targets of apoptosis and energy homeostasis. Second, the cognitive and behavioral recovery would be incomplete if the post-injury treatment is not extended beyond 24 h in the MCAO model of reperfusion injury.

In conclusion, the combination of PG and TMZ complemented the neuroprotective effects of individual agents to provide better outcomes.
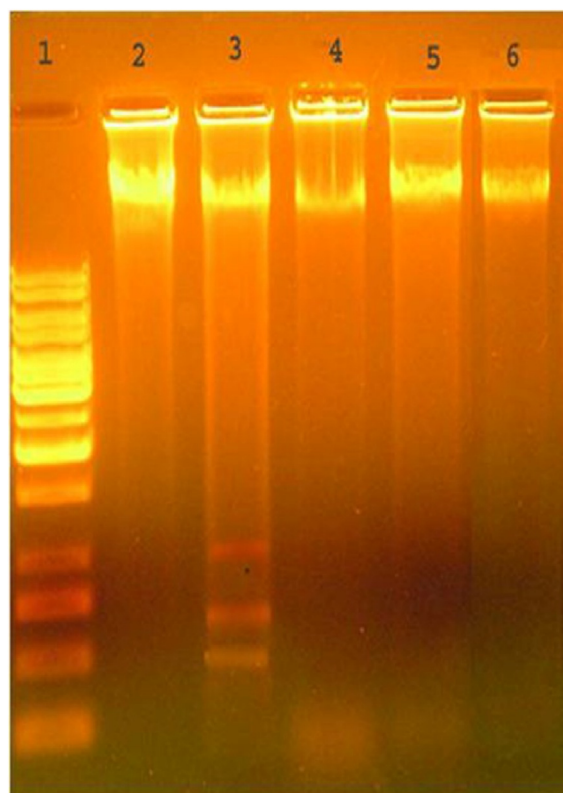
We report restoration of energy homeostasis and inhibition of inflammation is critical to reducing the extent of cerebral injury. In addition, the use of in-silico modeling techniques adds value to observed beneficial effects on cellular apoptosis and energy utilization mechanisms after combined treatment. Reduction in DNA fragmentation and histology of structural changes, although qualitative, suggest the therapeutic potential of PG and TMZ combination in cerebral reperfusion injury. Further investigation of this combination approach in the post-ischemic recovery model will allow exploration of its effect on post-recovery neurological and cognitive outcomes in ischemia-reperfusion injury.

CRediT authorship contribution statement

Vipin Dhote: Conceptualization, Methodology. **Avinash Singh Mandloi:** Investigation. **Pradeep Kumar Singour:** Methodology. **Manisha Kawadkar:** Data curation, Software. **Aditya Ganeshpurkar:** Writing – review & editing. **Manoj P. Jadhav:** Methodology, Software.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
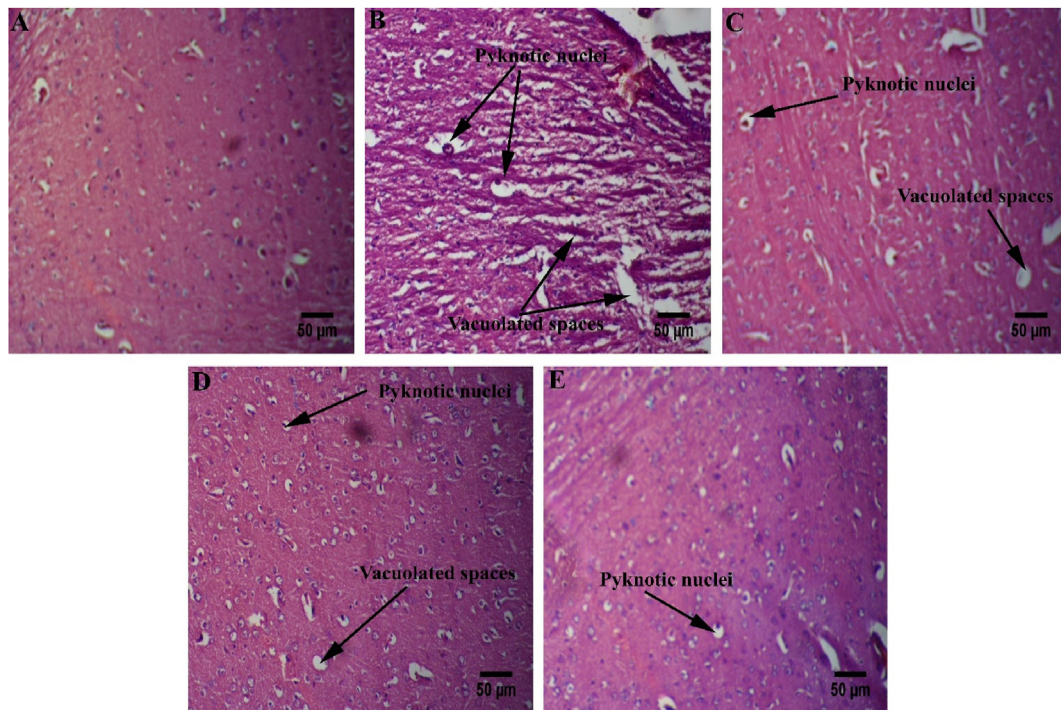
Fig. 8. Histopathology of Brain tissue

Photomicrographs (100X) of H& E stained brain slides after reperfusion injury; **A:** Sham operated showing no neuronal loss; **B:** Vehicle treated IR showing vacuolation and neuronal loss; **C:** Progesterone treated with improved neuronal architecture, few vacuolations and partial neuronal loss; **D:** Trimetazidine treated with indications of overall recovery, partial neuronal loss; **E:** PG + TMZ treated with marked improvement in cellular structure with minimum neuronal loss.
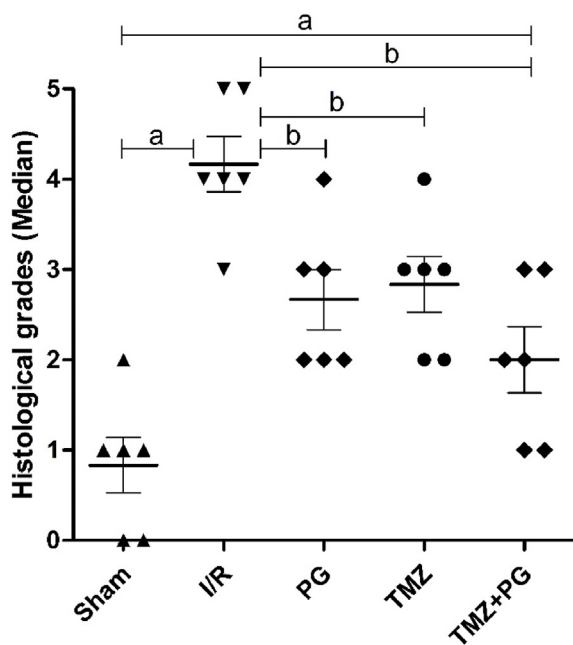

Fig. 9. Graphical presentation of histological changes

The values represented as median of scores (n = 6); Sham operated (SO); Ischemia reperfusion injury (I/R); Progesterone PG (PG; 8 mg/kg), trimetazidine (TMZ; 25 mg/kg) group.

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