Canadian Journal of Physiology and Pharmacology

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Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2019-0188.R1
Manuscript Type:	Article
Date Submitted by the Author:	20-May-2019
Complete List of Authors:	Grewal, Amarjot; Chitkara College of Pharmacy, Pharmacology Singh, Nirmal; Punjabi University, Patiala-147002, Punjab, India Singh, Thakur Gurjeet; Chitkara College Of Pharmacy, Rajpura
Is the invited manuscript for consideration in a Special Issue:	Not applicable (regular submission)
Keyword:	Pharmacological Postconditioning, Global Cerebral Ischemia, Resveratrol, Sirtinol, oxidative stress

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Effects of Resveratrol Postconditioning on Cerebral Ischemia in mice: Role of the Sirtuin-1 (SIRT1) Pathway

Amarjot Kaur Grewal¹, Nirmal Singh² & Thakur Gurjeet Singh¹

¹Chitkara College of Pharmacy, Chitkara University, Rajpura, Distt. Patiala -140401, Punjab, India, amarjotkaurgrewal@yahoo.co.in

²Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, Punjab 147004, nirmal_puru@rediffmail.com

¹Chitkara College of Pharmacy, Chitkara University, Rajpura, Distt. Patiala -140401, Punjab, India, gurjeet.singh@chitkara.edu.in

Running title: Pharmacological Postconditioning & Cerebral Ischemia-Reperfusion

Address for Correspondence*:

**Dr. Thakur Gurjeet Singh,
Department of Pharmacology,
Assistant Dean & HOD,
Chitkara College of Pharmacy,
Chitkara University,
Patiala-140401, Punjab
+91-9815951171, Fax No. 01762-507085
gurjeet.singh@chitkara.edu.in
gurjeetthakur@gmail.com**

Abstract

Evidence has demonstrated that resveratrol preconditioning exhibits neuroprotection against cerebral IR injury. The current investigation aimed to explore whether pharmacological postconditioning, by administering resveratrol, after a sustained ischemia & prior to prolonged reperfusion abrogates cerebral IR injury. Cerebral ischemia-reperfusion-induced injury mice model was employed in this study to evaluate the neuroprotective effects of pharmacological postconditioning (pPoCo) with resveratrol (30 mg/kg; i.p.) administered 5 mins before reperfusion. We administered Sirtinol, a SIRT1/2 selective inhibitor (10 mg/kg; i.p.) 10 min before ischemia (17 min) and reperfusion (24 h), to elucidate whether the neuroprotection with resveratrol postconditioning depends on SIRT1 activation. Various biochemical, behavioral parameters and histopathological changes were assessed to examine the effect of pPoCo. Infarct size is estimated using TTC staining. It was established that resveratrol postconditioning abrogated the deleterious effects of IR injury expressed with regard to biochemical parameters of oxidative stress (TBARS, SOD, GSH), acetylcholinesterase activity, behavioural parameters (memory, motor coordination), infarct size and histopathological changes. Sirtinol significantly reversed the effect of resveratrol PoCo. We conclude that induced neuroprotective benefits of resveratrol postconditioning may be the consequence of SIRT1 activation and resveratrol can be considered, for further studies, as potential agent inducing pharmacological postconditioning in clinical situations.

Key words: Pharmacological Postconditioning, Global Cerebral Ischemia, Resveratrol, SIRT, Sirtinol.

Chemical compounds used in experimental design in this study:

Resveratrol (PubChem CID: 445154)

Sirtinol (PubChem CID: 5717148)

1. Introduction

Reperfusion is a strategy to salvage the brain after ischemia but is also inimical to brain leading to damaging cerebral ischemia-reperfusion injury (Lin et al. 2016). Various cerebroprotective preconditioning procedures like pharmacological or ischemic preconditioning have been proved to be effective in animal studies (Gidday 2006; Vijaykumar et al. 2016). However, the anticipation of ischemic episodes is often not possible in clinical set up; therefore, practicality of these preconditioning procedures is particularly limited (Bansal et al. 2013). Ischemic postconditioning has also been shown to render protection against cerebral IR injury, however, it is difficult to initiate it in clinical settings (Ledger et al. 2015). Hence, the idea of pharmacological postconditioning (pPoCo), which involves administration of bioactive pharmaceutical agents during or before reperfusion, seems to be more pragmatic and promising as it is achievable, effectual and safe in clinical milieu (Esposito et al. 2015).

Resveratrol preconditioning, in vitro & in mice models, has been shown to mimic the ischemic preconditioning neuroprotection (Della-Morte et al. 2009; Wang et al. 2014). Resveratrol has potent anti-inflammatory, anti-oxidant and anti-apoptotic activity. These defensive effects of resveratrol are mediated via multifarious target molecules and diverse signaling pathways (Gao et al. 2006; Lu et al. 2006; Tsai 2007; Yousuf et al. 2009; Shin et al. 2010).

The multiplex pathophysiology (Fann et al. 2013) of cerebral ischemia reperfusion injury includes impairment of metabolism, reactive oxygen species stress, inflammation, excitotoxicity and overload of calcium and apoptosis (Buckley et al. 2014; Chumboatong et al. 2017; Shu et al. 2018). As apoptosis play a significant role in injury, targeting apoptotic pathways to improve neuronal survival can reduce cerebral IR injury.

SIRT1, the histone deacetylase, is one of the major mediators of resveratrol pharmacological actions (Borra et al. 2005; Della-Morte et al. 2009). In various studies it was demonstrated that resveratrol has the efficacy to intensify the activity of SIRT1, which in turn, enhances the cellular processes dependent on its activity. Such processes include regulation of cell cycle, mobilization of adipocytes, differentiation of muscle cell, protection of axons and retardation of transcription dependent on NF-kB (Michan and Sinclair 2007).

Studies have revealed resveratrol preconditioning, before sustained ischemia, salvages brain from injury (Della-Morte et al. 2009; Wang et al. 2014). However, the resveratrol postconditioning induced protection acute mice model of stroke is yet to be explored. Such study may expand and promote the use of resveratrol as an add - on therapy of stroke to prevent reperfusion injury during stroke in clinical scenario. Therefore, this study was designed to explore the hypothesis that resveratrol administration before prolonged reperfusion and after major ischemia may render neuroprotection against IR injury by activating SIRT1.

Draft

Materials and methods

Experimental animals

This experimental study was conducted on Swiss albino male mice (body weight 25-30 g), which had *ad libitum* standard diet (rodent) and tap water. Chitkara College of pharmacy, Chitkara University, Punjab facilitated animal house for preservation of animals in 12 hour light/dark photoperiod. Animals were conserved according to CPCSEA guidelines, Ministry of Environment and Forests, Government of India guidelines (Reg. no. 1181/PO/ReBi/08/CPCSEA). Institutional Animal Ethics Committee of Chitkara University endorsed all the experimental proceedings of the current study. Animals were treated in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press).

Drugs and Chemicals

Resveratrol (PubChem CID: 445154), Sirtinol (PubChem CID: 5717148) were purchased from Sigma–Aldrich, India and all the freshly prepared solutions were used during estimations.

Global Cerebral Ischemia induction

Global cerebral ischemia was introduced by firstly anaesthetizing mice with chloral hydrate at dose 400 mg/kg, intraperitoneally following the method stated by Himori et al. (Norio et al. 1990) with some modification as reported by Rehni et al. (Rehni et al. 2008). The throat of ventrally placed mice was cut at midline. The carotid arteries both on right and left were sited and detached from adjacent tissue and also vagus nerve. After the cotton threads were passed under each of the carotid artery, the thread ends were pulled with same weight for induction of 17 min. global cerebral ischemia. The weight on threads was released after ischemia to initiate reperfusion of 24 h. Surgical part was sutured and sanitized. For the reperfusion intervention, pharmacological agents (i.e. pPoCo) Resveratrol (30 mg/kg; intraperitoneally) was administered 5 mins. before reperfusion of 24 hrs so that the drug would be in circulation at the time of onset of reperfusion.

Behavioural parameter assessments

Morris Water Maze (MWM) test for memory evaluation

Animal's memory assessment was conducted by employing the Morris Water Maze (MWM).

Acquisition trial

Every mouse undergoes four trials per day giving 5min. of relaxation between each trial, the trial underwent for four successive days. Each day the starting position of the training trial was altered and in all trials the target quadrant considered was Q4. On day fourth Escape Latency Time (ELT) of individual animal was recorded as time required by the animal for finding the concealed platform that indicated memory and learning acquisition. Ischemia reperfusion injury was induced after measuring ELT.

Retrieval trial

On day fifth, all mouse explored the water maze for 120s without the presence of escape platform in the maze. Each animal underwent trial for four times and in every trial, the starting quadrant was not the same. Apart from the target quadrant, the time for how long the animal stayed in rest of the quadrants was recorded; also time for retrieval was recorded as how long the animal remained in the target quadrant (Q4) seeking the missing platform (Morris 1984; Parle 2004).

Elevated Plus Maze (EPM) test for Short-term memory evaluation

Animal's assessment for short-term memory was completed with the help of plus maze. Far from the central platform, animals were put at the end of open arm of the maze to walk through it. The first trial was carried out on Day2. This exposure to the EPM was carried out again on the day 3 and 4 to record Latency time (TLT), which the time is required by rodent to get into the closed arm through open arm with their four legs. On day 4, after the 3rd training trial mice were subjected to IR injury. Memory acquisition and learning was assessed from day 4 TLT. Memory retrieval was indicated by day 5 TLT (Itoh et al. 1990; Pateliya et al. 2008).

Motor coordination assessment

Rota Rod test

Rota rod instrument helped to estimate gripping ability on a spinning rod that depicted the motor co-ordination of animals. Variation from fall off time as compared to control animals indicated motor in coordination. The rodents were put through trials before IR injury, on day 4, to finally select the animals which have the ability to remain, for 5 mins , on rotating rod. The rota rod test was repeated on day 5 after IR injury (Jones et al. 1968; Pateliya et al. 2008).

Inclined beam walking test

Walking test on inclined beam evaluated the front and back legs motor co-ordination. Varying grades (0 to 4) indicated the variation in the motor performance. On day 4, before IR injury, the test was carried out to select the rodent which is able to move easily (grade 0) on the beam. The test was repeated on day 5 after IR injury (Yonemori et al. 1998; Rehni et al. 2008).

Assessment of Cerebral Infarct Size

On fifth day, after ischemia and reperfusion, brain of sacrificed animals were extracted and kept whole night at - 4°C. Frozen brain was incised into uniform slices and kept for 20 minutes incubation in 1% triphenylterazolium chloride (TTC) at 37°C in 0.2 M tris buffer (pH 7.4). A plastic grid (transparent) with 100 squares in 1 cm² was set over the glass plate on which the brain slices were already laid for calculating area (average) of each brain slice by counting the number of squares on both sides, likewise the total squares that fall under monotonous yellow part (Non-stained) were also enumerated and considered as infarcted area and represented in terms of % age weight and % age volume (Bochelen et al. 1999; Joshi et al. 2004; Kumar et al. 2014)

Hematoxylin & Eosin Staining

After ischemia and 24 hrs of reperfusion the removed samples of brain were fixed (for 48 hrs), embedded in paraffin wax, sliced (coronal sections; 4µm thick), stained with hematoxylin & eosin dye and observed under a light microscope.

Biochemical analysis

Tissue Preparation

For biochemical analysis, 24 h after evaluation of behavioral parameters, brain was removed and rinsed with ice-cold isotonic saline. This was followed by homogenization of brain tissue and centrifugation at 2,000×g for 15 min. Supernatant collected was subjected to biochemical estimations.

Evaluation of brain biochemical parameters (TBARS, GSH, SOD, AChE)

On day 5 various biochemical evaluations were carried out. The assessment of brain lipid peroxidation from the supernatant fraction was measured as the amount of TBARS by Ohkawa method (Ohkawa et al. 1979) acetylcholinesterase brain activity using Ellman et al method was estimated (Ellman et al. 1961) reduced glutathione level was assessed by Beutler method and expressed as $\mu\text{M}/\text{mg}$ of tissue (Beutler 1963), SOD activity (superoxide dismutase) was assessed spectrophotometrically via technique reported by Misra & Fridovich (1972).

Experimental design

After dividing mice (male), randomly, in five groups of 8 mice each (N=40): Sham Control (group 1), IR control (group 2), Sirtinol *per se* (group 3), Resveratrol PoCo (group 4) and Sirtinol + Resveratrol (group 5); rodents were subjected to biochemical analysis & behavioral tests. In order to ensure that pharmacological agent is in circulation before the commencement of reperfusion injury, Resveratrol (30 mg/kg; i.p.) was delivered 5 mins before reperfusion of 24 h. Sirtinol, a SIRT1/2 selective inhibitor, (10 mg/kg; i.p.) was administered 10 mins. before the induction of IR injury.

Statistical analysis

The result data obtained was represented as mean $\pm$ standard error of mean (S.E.M.) followed with statistical model tests ANOVA (one way) as well as post hoc Tukey's test for multiple comparisons. $p < 0.05$ level was considered as statistically significant level of variation among different groups. Non parametric Wilcoxon rank sum test was used in statistical result analysis of inclined beam walking test.

Results

Effect of pharmacological interventions on memory evaluated using Morris water maze (MWM)

Hippocampus is the most affected area during IR injury of brain. The evidenced changes due to the oxidative damages in hippocampus can promote learning impairment (Schmidt et al. 2014). Hence, in the current study the effect on memory was assessed using Morris water maze and plus maze. From day 1 to day 4, acquisition (learning) trials were carried out in animals of each group using MWM. A descending trend in escape latency time (ELT) was observed in the rodents exposed to acquisition trials indicating normal learning abilities. Day 5 Memory retrieval tests results of sham control group showed notable ($p<0.05$) increase in time spent in Q4 (target) quadrant as compared to other quadrants signifying normal memory acquisition and retrieval. Cerebral IR injury remarkably ($p<0.05$) reduced day 5 TSTQ, indicating memory impairment as compared to sham control group. Resveratrol PoCo remarkably ($p<0.05$) prolonged the day 5 TSTQ as compared to IR group indicating memory improvement. Sirtinol pretreatment in Sirtinol+Resveratrol PoCo group remarkably ($p<0.05$) abrogated the effect of Resveratrol PoCo on TSTQ. No notable affect of Sirtinol *per se* treatment was observed on changes in TSTQ induced after IR injury (Figure 1a).

Effect of pharmacological interventions on Impairment of Short-Term Memory using elevated plus maze

The transfer latency time (TLT), recorded using plus maze test, is one of the variables of memory and learning. TLT is remarkably ($p<0.05$) prolonged after cerebral IR injury, in the current study, as compared to sham control group. Resveratrol PoCo significantly ($p<0.05$) reversed the detrimental effect of IR injury on learning and memory indicated by the resulting decrease in TLT as compared to IR group. Sirtinol pretreatment in Sirtinol+Resveratrol PoCo group remarkably ($p<0.05$) abrogated the effect of Resveratrol PoCo on TLT. No notable affect of Sirtinol *per se* treatment was observed on changes in TLT induced by IR injury (Figure 1b).

Effect of Pharmacological interventions on motor performance

Effect on fall down time using rotarod test

Cerebral IR injury is associated with impairment of balance and motor coordination, which, in present study, was investigated using inclined beam test and rota rod test (Grewal et al. 2013; Moshfegh and Setorki 2017). For evaluation of neuroprotective effects resveratrol PoCo on motor performance, the fall down time, after ischemia of 17 min and reperfusion for 24 h, was observed. The fall down time of IR control group was decreased in comparison with that of sham group ($p < 0.05$) indicating motor performance impairment. Resveratrol PoCo resulted in prominent ($p < 0.05$) increase in fall down time as compared to IR group. In Sirtinol+Resveratrol PoCo group, the protective effect of Resveratrol PoCo on motor impairment, estimated by fall down time, was notably abolished by Sirtinol pretreatment. Sirtinol *per se* did not cause impairment of motor performance (Figure 2a).

Effect on motor in-coordination score using inclined beam-walking test

Cerebral IR injury caused functional neurological deficit, indicated by the increase in score of motor in coordination ($p < 0.05$), evaluated by inclined beam walking test, as compared to sham control group. The results of current study show that Resveratrol PoCo exhibited notably significant ($p < 0.05$) improvement in scores of neurological deficit indicating improvement in motor in coordination. Sirtinol pretreatment in Sirtinol+Resveratrol PoCo group remarkably ($p < 0.05$) abrogated the effect of Resveratrol PoCo on motor incoordination score. Sirtinol *per se* did not cause impairment of motor coordination (Figure 2b).

Effect of Pharmacological interventions on cerebral infarct size

Qualitative evaluation of cerebral infarction after surgical and pharmacological interventions has been shown in figure 3A-3E. Quantitative assessment of cerebral infarct size measured by volume and weight method indicated significant increase ($p < 0.05$) in infarction after IR injury in comparison with sham control group. Our results exhibit that resveratrol administration before the beginning of reperfusion (PoCo) remarkably ($p < 0.05$) decreased infarct size. Pre treatment with Sirtinol crucially ($p < 0.05$) abrogated the effect of Resveratrol PoCo in Sirtinol +Resveratrol PoCo group. No significant effect was observed with Sirtinol *per se* treatment on IR induced change in infarct size. (Figure 3A-3E; 3K and 3L).

Effect of interventions on histopathological changes

The histopathological changes in the hippocampal CA1 and cerebral cortex region were assessed by the H&E staining in all the groups. The increase in intercellular space due to neuronal cell

shrinkage and pycnotic nuclei was exhibited in the IR control group; Sirtinol *per se* group and Sirtinol + Resveratrol postconditioning group. In resveratrol postconditioning group, these neuronal damages were reduced significantly as compared to ischemic control group (Figure 3F-3J).

Effect of Pharmacological interventions on thiobarbituric acid reactive substances (TBARS), GSH levels and SOD activity.

Lipid peroxidation, which is the oxidative degradation of lipids, by ROS damages cerebral neurons during ischemic stroke (Li and Yang. 2016). To assess the effects of Resveratrol PoCo on lipid peroxidation induced by IR injury, TBARS levels in cerebral tissue were measured. After 17 min ischemia and 24 h reperfusion, TBARS levels were significantly ($p<0.05$) raised compared to sham control group. Resveratrol PoCo resulted in remarkable ($p<0.05$) decrease in concentration of TBARS indicating attenuation of IR injury induced lipid peroxidation. Sirtinol pretreatment notably ($p<0.05$) abrogated the protective effect of Resveratrol PoCo in Sirtinol +Resveratrol PoCo group. No notable affect of Sirtinol *per se* treatment on IR- induced changes in the level of TBARS was observed.

Cerebral ischemia increases ROS both in rodent disease models and human patients. Reperfusion may induce the second phase of ischemia/reperfusion injury and precipitate the generation of ROS that is fueled by the reintroduction of oxygen molecules to the ischemic tissue (Li and Yang. 2016). Hence, the antioxidant activities of enzymes such as GSH and SOD were measured, in the present study, to assess the protective effect of Resveratrol postconditioning. The data showed that IR injury remarkably ($p<0.05$) decreased GSH levels and SOD activity as compared to sham control group. The neuroprotective effect of Resveratrol PoCo was established by the results obtained which indicated the remarkable ($p<0.05$) increase in GSH levels and SOD activity. Sirtinol pretreatment notably ($p<0.05$) abrogated the protective effect of Resveratrol PoCo in Sirtinol +Resveratrol PoCo group. No significant affect of Sirtinol *per se* treatment on IR-induced changes in the GSH levels and SOD activity was observed (Figure 4a, 4b and 4c).

Effect of Pharmacological interventions on brain Acetylcholinesterase (AChE) activity

AChE activity is increased due to aggravation of oxidative stress (Melo et al, 2003; Milatovic et al. 2006). ACh is a neurotransmitter involved in memory, learning and synaptic

neurotransmission, the effect of pharmacological interventions on acetylcholinesterase enzyme activity was evaluated as this enzyme is involved in the metabolism of ACh. Global ischemia and reperfusion injury significantly ($p < 0.05$) augmented the acetylcholinesterase activity in comparison to the sham control group. The results of our study showed the significant ($p < 0.05$) decrease in AChE activity as compared to IR control group indicating improvement in memory, learning and synaptic transmission with Resveratrol PoCo. Sirtinol pretreatment notably ($p < 0.05$) abrogated the effect of Resveratrol PoCo on cerebral AChE activity in Sirtinol +Resveratrol PoCo group. No notable affect of sirtinol *per se* treatment was observed on alterations in cerebral AChE activity induced with IR injury (Figure 5).

Discussion

In the current study, the effect of pharmacological postconditioning with resveratrol on brain was investigated and the mechanisms for the neuroprotection by resveratrol postconditioning were explored. Cerebral global ischemia induced neuronal damage, encountered clinically in situations like cardiopulmonary arrest, drowning, is mimicked in the global cerebral ischemia animal model used in this study (Neumann et al. 2013). Oxidative stress, viewed as a key factor in the inception of IR damage, influences the levels of TBARS, antioxidants and ROS scavenging enzymes (Akhtar et al. 2008; De Vries et al. 2013; Aşçı et al. 2016). Moreover, AChE activity is increased due to aggravation of oxidative stress (Milatovic et al. 2006). Evidence from literature review suggests a role for ROS on increase of AChE activity as a result of lipid peroxidation decreasing cell membrane order and ultimately leading to the exposure of more active sites of the enzyme (Melo et al, 2003). The magnitude of neuronal injury induced by IR was estimated using TTC staining (Bochelen et al. 1999; Joshi et al. 2004).

The changes observed due to oxidative damages affects hippocampus the most leading to memory impairment. (Schmidt et al. 2014). Hence, in the current study the effect on memory was assessed using plus maze and Morris water maze. Also, cerebral IR injury is associated with impairment of balance and motor coordination, which, in present study, was investigated using inclined beam test and rota rod test (Grewal et al. 2013; Moshfegh and Setorki 2017). Reperfusion (24h) proceeded by cerebral global ischemia (17mins.), in our study, culminated in significant cerebral injury demonstrated in the form of aggravation of motor in coordination, increase in memory impairment and cerebral infarction. The increase in extent of TBARS, AChE

activity and depleted levels of SOD, catalase and reduced GSH indicated the increase in oxidative stress. The alteration in the above mentioned parameters are comparable to the results of similar studies carried out in other labs (Gupta et al. 2003; Ozerol et al. 2009; Gaur and Kumar 2012; Reddy et al. 2013; Gulati and Singh 2014; Asci et al. 2016). Initiating the reperfusion rapidly is the most effectual treatment to reduce damage from IR injury. Nonetheless, reperfusion is a potential threat also causing additional injury (Zhao and Vinten-Johansen 2006; Sanderson et al. 2013).

Postconditioning is an innovative phenomenon functional in experimental cerebral (focal & global) ischemia. pPoCo has proved to be better approach to induce neuroprotection in clinical setting (Zhao et al. 2012). Also, for cases in which cerebral blood vessels are not available for iPoCo, a pharmacologic stimulus can be applied subsequent to the ischemic event to attenuate neuronal injury but the application interlude may limit the benefits of pPoCo (Ovize 2010). Because of the narrow time window as demonstrated in experimental studies, one has to consider that such a drug, for induction of postconditioning, should be administered before reperfusion so that it is circulating in blood at the time of onset of reflow (Andreasson 2008). Literature is replete with studies involving neuroprotection with pharmacological agents administered at the start of reperfusion (Sicard et al. 2009; Du et al. 2010; Tong et al. 2011; Hein et al. 2013; Bouhidel et al. 2014; Zhang et al. 2016). However, it has been debated whether this approach can be described as 'pharmacological postconditioning' or 'post-reperfusion treatment' (Sandu and Schaller 2010). Therefore, in our study protocol, resveratrol (30 mg/kg; i.p) was administered 5 mins before reperfusion to induce pharmacological postconditioning.

Various studies have demonstrated the neuroprotection through the resveratrol preconditioning (Huang et al. 2001; Sinha et al. 2002; Inoue et al. 2003; Zamin et al. 2006; Rava et al. 2006). As discussed earlier, because of the more beneficial effects of pPoCo there was a need to investigate reperfusion strategy via induction of resveratrol postconditioning and to explore the mechanisms involved in triggering the adaptive endogenous protective signals in brain during reperfusion injury. pPoCo with resveratrol significantly abrogated the injury induced by oxidative stress and decreased the AChE activity. There was significant improvement of memory, learning along with amelioration of infarct size & motor impairment as a consequence of resveratrol postconditioning. Also, prior treatment Sirtinol (selective inhibitor of SIRT1) has significantly attenuated this protective effect of resveratrol postconditioning on brain.

Sirtuins belonging to histone deacetylases (HDAC) Class III share homology with yeast silent information regulator 2. Seven sirtuins have been defined in the mammals till date. Except SIRT 4, all sirtuins possess NAD⁺ dependent deacetylase activity. Their lucrative role has been reported in varied pathological conditions including inflammation, cancer, stroke, cardiovascular diseases, diabetes mellitus and neurodegenerative diseases (Turkmen et al. 2014; O'Callaghan and Vassilopoulos, 2017; Mendes et al. 2017; Ajami et al. 2017) and they have been proved to be promising targets to withstand IR injury of various organs (Pantazi et al. 2013; Shalwala et al. 2014; Khader et al. 2016; Zhang et al. 2017; Nikseresht et al. 2018; Yin et al. 2018). Results from other labs have demonstrated the involvement of Sirtuin (SIRT1) activation in neuroprotective state resulting from resveratrol pretreatment (Raval et al. 2006; Della-Morte et al. 2009; Albani et al. 2009; Morris et al. 2011). However, the involvement of SIRT1 in resveratrol induced pharmacological postconditioning mechanism has not yet been established.

As mentioned above, the treatment with sirtinol before induction of IR injury has abrogated the beneficial effect of pPoCo with resveratrol. Hence, our hypothesis of possible crucial role of SIRT1 activation in neuroprotection via pPoCo with resveratrol is designated by the results of our study, though, more exhaustive studies like evaluation of SIRT1 expression in neuronal cells are required to validate these findings which are the limitations of this study.

Acknowledgements

The authors are grateful to the Chitkara College of Pharmacy, Chitkara University, Rajpura, Patiala, Punjab, India for providing the necessary facilities to carry out the research work.

Financial Support and Sponsorship

Nil.

Conflicts of Interest

There are no conflicts of interest.

Author contributions

Conceived and designed the experiments: Ms. Amarjot Kaur Grewal & Dr. Thakur Gurjeet Singh

Performed the experiments: Ms. Amarjot Kaur Grewal

Analysis and interpretation, data collection: Dr. Thakur Gurjeet Singh

Wrote the manuscript: Ms. Amarjot Kaur Grewal & Dr. Thakur Gurjeet Singh

Critically reviewed the article: Dr. Nirmal Singh

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Figure 1a: Effect of resveratrol postconditioning and interventions on ischemia and reperfusion induced impairment of memory in mice on day 5 [Assessed using Morris water maze test].

[Values are mean $\pm$ SEM. a= $P<0.05$ vs. time spent in target quadrant i.e. Q-4 in sham group on day 5; b= $P<0.05$ vs. time spent in target quadrant i.e. Q-4 in control group on day 5; c= $P<0.05$ vs. time spent in target quadrant i.e. Q-4 in resveratrol postconditioning group on day 5]

Figure 1b: Effect of resveratrol postconditioning and interventions on ischemia and reperfusion induced impairment of memory in mice [Assessed using elevated plus maze test].

[Values are mean $\pm$ SEM. a= $P<0.05$ vs. day 2 TLT of sham group; b= $P<0.05$ vs. day 2 TLT of control group; c= $P<0.05$ vs. day 2 TLT of resveratrol postconditioning group]

Figure 2a: Effect of resveratrol postconditioning and interventions on ischemia and reperfusion induced impairment of motor coordination in mice [Assessed using rota rod test].

[Values are mean $\pm$ standard error of means (S.E.M.) a = $P<0.05$ vs. Sham group; b = $P<0.05$ vs. Ischemic Control group; c= $p<0.05$ vs. resveratrol postconditioning group]

Figure 2b: Effect of resveratrol postconditioning and interventions on ischemia reperfusion induced changes in motor performance (Score of motor performance) in mice using inclined beam walk test.

[Values are mean $\pm$ standard error of means (S.E.M.); a = $P<0.05$ vs. Sham group; b = $P<0.05$ vs. Ischemic Control group; c= $p<0.05$ vs. resveratrol postconditioning group]

Figure 3 A-3J: The characteristic changes in infarct size (assessed using TTC staining) and histopathology (assessed using HE staining) in brain after ischemia (17 min.) & reperfusion (24 h) in mice

Black arrows ( ) depict increased intercellular space due to cell shrinkage and pyknosis

A & F: Sham Control; B & G: IR Control; C & H: Sirtinol per se; D & I: Resveratrol Postconditioning; E & J: Sirtinol+ Resveratrol Postconditioning

Figure 3 K: Effect of resveratrol postconditioning and interventions on ischemia and reperfusion induced increase in cerebral infarct size in mice measured by volume method.

[Values are mean $\pm$ SEM. a= $P<0.05$ vs. sham group; b= $P<0.05$ vs. Ischemic control group; c= $p<0.05$ vs. resveratrol postconditioning group].

Figure 3L: Effect of resveratrol postconditioning and interventions on ischemia and reperfusion induced increase in cerebral infarct size in mice measured by weight method.

[Values are mean $\pm$ SEM. a= $P<0.05$ vs. sham group; b= $P<0.05$ vs. Ischemic control group; c= $p<0.05$ vs. resveratrol postconditioning group].

Figure 4a: Effect of resveratrol postconditioning and interventions on ischemia and reperfusion induced increase in TBARS in mice.

[Values are mean $\pm$ SEM. a= $P<0.05$ vs. sham group; b= $P<0.05$ vs. Ischemic control group; c= $p<0.05$ vs. resveratrol postconditioning group].

Figure 4b: Effect of resveratrol postconditioning and interventions on ischemia and reperfusion induced decrease in the level of GSH.

[Values are mean $\pm$ SEM. a= $P<0.05$ vs. sham; b= $P<0.05$ vs. Ischemic control; c= $p<0.05$ vs. resveratrol postconditioning Control].

Figure 4c: Effect of resveratrol postconditioning and interventions on ischemia and reperfusion induced decrease in the activity of SOD.

[Values are mean $\pm$ SEM. a= $P < 0.05$ vs. sham group; b= $P < 0.05$ vs. Ischemic control group; c= $p < 0.05$ vs. resveratrol postconditioning group].

Figure 5: Effect of resveratrol postconditioning and interventions on ischemia and reperfusion induced increase in brain Acetylcholinesterase (AChE) activity

[Values are mean $\pm$ SEM. a= $P < 0.05$ vs. sham group; b= $P < 0.05$ vs. Ischemic control group; c= $p < 0.05$ vs. resveratrol postconditioning group].

Draft

Figure 1a **Figure 1b**

Figure 2a

Figure 2b

Figure 3 A-L

Figure 4a

Figure 4b

Figure 4c

Figure 5

Effects of resveratrol Postconditioning on Cerebral Ischemia in mice: Role of the Sirtuin-1 (SIRT1) Pathway.