RESVERATROL REVERSES BRAIN GLUTATHIONE SYSTEM INVOLVED NEURONAL LOSS AFTER IMMOBILIZATION STRESS

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ABSTRACT

Background: This study evaluated the antioxidant effects of Resveratrol on stress-induced neuronal loss in rat brain involving brain glutathione system.

Materials and Methods: The control rats received vehicle while another set of rats received 21 days restraint stress. The third and fourth group received similar intensity of restraint stress as well as either 10 or 20mg/kg dose of resveratrol respectively. The cognitive test included passive avoidance test. This was followed by estimation of reduced glutathione and glutathione reductase enzyme levels in brain homogenate and histomorphological study of hippocampus and medial prefrontal cortex.

Results: Restraint stress has resulted in poor retrieval of learning behaviour and resveratrol has enhanced retrieval of learning behaviour in stressed condition in passive avoidance task. Both reduced glutathione and glutathione reductase levels were reduced after restraint stress and resveratrol at both the doses has normalized their levels. Restraint stress has affected CA3, CA2 and dentate regions of the hippocampus and also medial prefrontal cortex. In all these areas resveratrol has minimized neuronal loss which were due to chronic stress.

Conclusion: From the results of the present study we conclude that stress induced oxidative damage involves brain glutathione system and which in turn could be one of the causes for neuronal loss and resveratrol suggests to protect the brain against stress in rat model.

KEY WORDS: Resveratrol, Restraint stress, Reduced glutathione, Glutathione reductase, Hippocampus.
MATERIALS AND METHODS

Animals and housing conditions: In-house bred male Wistar rats (weighing 220g±20) were selected for the study. The rats were maintained under controlled conditions of light (12 h light and 12 h dark cycle), temperature (22±3°C), and humidity (approximately 50±10%). All rats were maintained on the standard rat food and water ad libitum. For housing the rat plastic cages with paddy husk as bedding material was used. The institutional animal ethical committee has approved this research protocol.

Animal groups (n=6)

Group 1 (Control): Control and received sodium carboxymethylcellulose as vehicle
Group 2 (Stress): Received 21 days restraint stress (6h daily)
Group 3 (R10 + S): Received 21 days stress + resveratrol (10mg/kg body weight dose) for 28 days (Resveratrol was given a week prior to stress treatment)
Group 4 (R20 + S): Received 21 days stress + resveratrol (20mg/kg body weight dose) for 28 days (Resveratrol was given a week prior to stress treatment).

Stressing procedure: Rats were assigned to a restraint stress for 21 days in a wiremesh restrainer for six hours (2hrs X 3 times/day). The timings of the stress were different to avoid adaptation to the stress. The wire mesh restrainer had a wooden base and stainless steel wire mesh restrainers hinged to the base. The restrainers with dimensions of 11cm (L) x 8cm (B) x 8cm(H) was used to stress. This type of restrainer will only restrict the movements of the animal without causing any pain, discomfort or suffocation [5].

Resveratrol treatment: Although no studies have yet appeared regarding proper dosage of resveratrol for humans, the optimum beneficiary results were obtained from a dose ranging from 30 to 110 mg/day in human clinical trials [6]. Applying this dose to rat model and also from number of previous studies in rats [7], 10 mg and 20 mg/kg body weight dose were selected. Resveratrol suspended with 0.5% carboxy methyl cellulose was administered orally using oropharyngeal tube.

Chemicals: Resveratrol (Cat. No. 70675) was obtained from Cayman Chemicals, USA. All other chemicals and reagents were HPLC or analytical grade (Sigma, St. Louis, Mo.)

Passive avoidance test: To test the memory retention, rat in all the groups were subjected to passive avoidance test as described by Bures et al [8] & Cherian et al [9]. The test determines the ability of a rat to remember a foot shock delivered 24 h prior to the memory retention test. Passive avoidance apparatus consists of a wooden box with two compartments: (a) larger, bright compartment and (b) smaller, dark compartment both equipped with grid floor, which was attached to a shock source. The connection between the two compartments could be closed with a sliding door.

The experiment included three parts, i) an exploration test, ii) an aversive stimulation and learning phase (passive avoidance acquisition), and iii) retention test.

i) Exploration test: On the first day of test, rat was placed in the centre of the illuminated large compartment facing away from the entrance to the dark small compartment, for exploration. The door between the two compartments remained closed.
open at this time. The rat was allowed to explore both compartments for 5 minutes. This is followed by three test trials of 5 min each. At the end of the trial, the rat was placed in the home cage, where it remained during an inter-interval of 5 minutes. In each trial, fraction of time spent in each compartment was noted.

ii) Aversive stimulation and learning phase (passive avoidance acquisition): At the end of 3rd test trial, as soon as the animal stepped into dark compartment, a foot shock was delivered through the grid floor (50 Hz, 1.5 mA, for 1 second). The rat was held additional 10 sec, to allow the animal to form an association between the properties of the chamber and foot shock. The rat was then returned to its home cage.

iii) Retention test: The memory retention test was done 24 hr after foot shock. The rat was placed in the bright compartment and the time taken (the step-through latency) for it to enter the dark compartment for the first time was recorded using a stop watch. A maximum of 300 sec were given for the rat to explore. Normal rats avoid entering the dark chamber, where they received shock on previous day, suppressing their normal behaviour of exploring the dark compartment. Decreased latency to enter the dark compartment will suggest poor memory retention.

Antioxidant systems measurement in brain:
Tissue processing: Biochemical tests were carried out 24 h after the last day of stressing. The whole brain was removed rapidly and rinsed with 0.1M/L saline phosphate buffer (pH 7.4). Tissue was weighed and homogenized (1:10w/v) in 0.1M/L saline phosphate buffer. The homogenate was centrifuged at 10,000 X g for 20 min at 4°C and aliquots of supernatant were separated and used for following biochemical estimations.

Assay of reduced glutathione (GSH): Tissue GSH concentration was estimated according to the method described by Ellman [10].

Principle of the assay: The general thiol reagent, 5-5′-dithiobis [2-nitrobenzoic acid] (DTNB, Ellman’s Reagent) reacts with GSH to form the 412 nm chromophore, 5-thionitrobenzoic acid (TNB) and GSH-TNB. The GSH-TNB is subsequently reduced by glutathione reductase and b-nicotinamide adenine dinucleotide phosphate (NADPH), releasing a second TNB molecule and recycling the GSH, thus amplifying the response. Any oxidised (GSSG) initially present in the reaction mixture or formed from the mixed disulphide reaction is rapidly reduced to GSH.

Assay procedure: One millilitre of supernatant was precipitated with 1 ml of metaphosphoric acid and cold digested at 4°C for 1 h. The samples were centrifuged at 1,200g for 15 min at 4°C. To 1ml of this supernatant, 0.2 ml of phosphate buffer and 0.2 ml of 5, 5′ dithio-bis-2-nitrobenzoic acid (DTNB) was added. The yellow colour that developed was read immediately at 412 nm using a Systronic-117 UV-Visible spectrophotometer. The values were expressed in mg/gm protein. The total protein concentration of tissues was measured by the method of Lowry et al [11].

Assay of glutathione reductase (GSH-Rd): The GSH-Rd activity was measured using the method originally described by Moron et al [12].

Principle of the assay: This assay is based on the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) by NADPH in the presence of glutathione reductase. The reaction is measured by the decrease in absorbance at 340 nm using an extinction coefficient of 1.36x104 (mol/l)-1 cm-1. The activity of glutathione reductase was used as an indicator for oxidative stress.

Assay procedure: The reaction mixture consisted of 1.6 ml of 0.067 M potassium phosphate buffer (pH 6.6), 0.12 ml of 0.06% NADPH, 0.12 ml 1.15% GSSG, 0.1ml of enzyme source and water in a final volume of 2 ml. All mixtures and solutions were prepared at room temperature. Control cuvettes then received 180µL of deionized water while sample cuvettes received 60µL of deionized water and 120 µL of GSSG solution. NADPH oxidation was followed for 5 min and was recorded using a Systronic-117 spectrophotometer. The reduction of GSSG to GSH was determined indirectly by the measurement of the consumption of NADPH, as demonstrated by a decrease in absorbance at 340 nm as a function of time. The enzyme activity was calculated using extinction coefficient of...
RESULTS

Histological study of Dentategyrus, hippocampus and medial pre-frontal cortex: Each rat was deeply anesthetized with ether and secured on a dissection board, and its chest cavity was opened to expose the heart. About 15 mL of 0.9% saline was perfused through the left ventricle at the rate of 1 mL/min. This was followed by perfusion of 10% formalin at the same flow rate. The animal was decapitated and the brain was removed and kept in 10% formalin for 48 h (post-fixation). Paraffin blocks were made in an embedding bath. Coronal sections of 5-μm thickness were cut in the dorsal hippocampus using a rotary microtome (Jung Biocut II P45, Leica, Germany). Ten sections from each rat were mounted serially on air-dried gelatinized slides.

The sections were stained with cresyl violet stain. One hundred milligrams of cresyl violet was dissolved in 100 mL of distilled water. To this, 0.5 mL of 10% acetic acid was added to give a pH of 3.5–3.8. The stain was filtered before use [13].

Scoring: Quantification was done using Nikon trinocular microscope (H600L) under 40X magnification using imaging software NIS Elements Br version 4.30. For various regions of the hippocampus 250 μm length area was selected, for dentate gyrus 240 μm² area and for medial prefrontal cortex 300 μm² area was selected for quantification of normal neurons. Slides from different groups of rats were decoded to avoid manual bias while counting the cells.

Statistical Analysis: The data were expressed as mean ± SD. The significance of differences among the groups were assessed using one way analysis of Variance (ANOVA) test followed by Bonferroni’s multiple comparison test. P values < 0.05 were considered as significant.

The general health conditions of the rats during stressing/treatment period were monitored carefully; body weights were noted at frequent interval to ensure that no other ill health affects the results. No such effects were observed among all the rats tested.

Passive avoidance test: Rats who received restraint stress took significantly (p<0.001) lesser time to enter the dark compartment when compared to control. This shorter latency indicates poor retrieval of learning behaviour. Resveratrol treatment in stressed rat did not show any increase in time interval to enter the dark compartment compared to control as the latency to enter the dark compartment were significantly less (p<0.001) when compared to control. This would indicate a poor retrieval of learning behaviour in stressed rats even after resveratrol treatment. However, resveratrol at both doses has enhanced latency to enter the dark compartment in stressed rats compared to rats who received only stress (Fig.1).

Reduced glutathione (GSH) level in brain: There is a significant (p<0.001) reduction in reduced glutathione level (GSH) in stressed rat brain homogenate compared to the control. Resveratrol treatment (both doses) in stressed rats reversed the oxidative damage by elevating reduced glutathione level significantly (p<0.001). Resveratrol treatment at 10mg/kg dose in stressed rats has shown an increase in the reduced glutathione level at par with control (as there is no statistical significant difference between their values), (Fig.2).

Fig. 1: Observations of passive avoidance test by rats subjected to restraint stress and resveratrol treatment.
Fig. 2: Brain reduced Glutathione level (mg/gm protein) in rats.

Values are expressed as mean ± SD (n=6). Comparison between Control Vs others ** = p<0.01, *** = p<0.001. Comparison between Stress Vs others @@@ = p<0.001.

**Glutathione reductase (GSSG-Rd) activity in brain:** The activity of brain glutathione reductase enzyme was significantly (p<0.001) decreased in stressed rats compared to control. Resveratrol treatment (both doses) in stressed rats has significantly (p<0.001) increased glutathione reductase activity compared to rats who received only stress claiming the antioxidant potential of resveratrol in stressed conditions. Resveratrol treatment at 10mg/kg dose in stressed rats has shown an increase in the glutathione reductase level at par with control (as there is no statistical significant difference between their values), (Fig. 3).

Fig. 3: Brain Glutathione reductase activity (nmol NADPH oxidized/min/mg protein) in rats.

Values are expressed as mean ± SD (n=6). Comparison between Control Vs others ***=p<0.001. Comparison between Stress Vs others @@@ = p<0.001. Comparison between R10+SVs R20+S CCC =p<0.001.

**Neuronal assay of Dentate Gyrus (DG):** The expression of number of healthy neurons in DG has reduced significantly (p<0.001) in stressed rats compared to control indicating a neuronal loss. The number of neurons were significantly (p<0.001) more in stressed rats who received resveratrol compared to the group which received stress alone. Rats who received stress and resveratrol 10mg/kg dose expressed higher (p<0.01) number of neurons compared to rats who received stress and 20mg/kg dose of resveratrol (Fig. 4, 5a & 5b) suggesting 10mg/kg dose acting better.

Fig. 4: Resveratrol and their combination induced changes in the neuronal numbers in 240sqµm² area in Dentate Gyrus (DG).
**Fig. 5:** Histomicrographic pictures of dentate gyrus under 40X, Cresyl violet staining. Note degenerated neurons indicated by red arrow in stressed group of rats.

**Fig. 6:** Resveratrol and their combination induced changes in the neuronal numbers in 250µm length area in hippocampal CornuAmmonis (CA4, CA3, CA2, CA1). Values are expressed as mean ± SD (n=6). Comparison between Control Vs others ** = p<0.01, *** = p<0.001. Comparison between Stress Vs others @@ = p<0.01, @@@ = p<0.001.

**Fig. 7:** Histo micrographic pictures of hippocampus under 40X, Cresyl violet staining. Scale bar indicates 50µm length.

**Fig. 8:** Resveratrol and their combination induced changes in the neuronal numbers in 300µm² area in medial prefrontal cortex. Values are expressed as mean ± SD (n=6). Comparison between Control Vs others *** = p<0.001. Comparison between Stress Vs others @@@ = p<0.001.

**Fig. 9:** Histo micrographic pictures of medial prefrontal cortex (MFC) under 40X, Cresyl violet staining.
Neuronal assay of various regions of the Hippocampus: Restraint stress has not affected (p>0.05) the neuronal population of CA4 and CA1 regions of the hippocampus. However CA3 region showed a severe (p<0.001) decline in neuronal number followed by CA2 region (p<0.01) in stressed rats compared to control counterparts.

Resveratrol at 10mg/kg dose in stressed rats enhanced (p<0.001) the expression of normal neurons in CA3 region and also in CA2 region to a lesser (p<0.01) extent. Resveratrol at 20mg/kg dose in stressed rats also enhanced (p<0.01) the expression of normal neurons in CA3 region but not (p>0.05) at CA2 region (Fig.6, 7a & 7b).

Neuronal assay of medial prefrontal cortex: Restraint stress has expressed significantly (p<0.001) lesser number of neurons in stressed rats compared to control. Resveratrol at both doses in stressed rats showed a significant (p<0.001) higher number of neurons compared to rats who received stress alone (Fig.8, 9a & 9b).

DISCUSSION

The results of the present study revealed poor retrieval of learning behaviour in passive avoidance task after restraint stress. Though the restraint stress-induced cognitive dysfunction was reported in many animal studies most of them assessed hippocampal dependent spatial memory using variety of maze tests. The present study was designed to evaluate the cognitive function in an unconditioned learning environment and our results further demonstrates the involvement of hippocampal as well as prefrontal cortex neurons involving in it. Chronic restraint stress induced cognitive dysfunction involving hippocampus [14] and prefrontal cortex [15],[16] in humans and animal models were well established [17]. There are number of hypotheses addressing the mechanism of stress induced cognitive dysfunction. It includes oxidative damage, altered glucocorticoid receptor expression [18], altered neurotransmitter and synaptic proteins [19], neuronal Ca²⁺ homeostasis disturbance [20], altered dendritic morphology [21] and many more. Among the various factors affecting the stress-induced cognitive dysfunction, the involvement of in built antioxidant defence system in brain is gaining lot of focus in the recent years. It is because of the fact, that in age related dementia and in Alzheimer’s disease the neuronal loss was mainly due to loss of antioxidant defence system [22] in the brain specially in hippocampus [23] and prefrontal cortex [24]. For these reason natural antioxidants crossing blood brain barrier like resveratrol was used as therapeutic strategy. Resveratrol has exerted its antioxidant potential in enhancing cognitive function against various neuronal insults [25],[26],[27],[28],[29],[30]. In the present study though resveratrol has not enhanced retrieval of learning behaviour to the normalcy, but it was better than the performance of stressed rats. Number of studies in the past using different study models have demonstrated resveratrol’s ability to prevent cognitive loss. The mild difference observed in the results of various study models, may be due to sensitivity of rats for each study design. In the present study the neuroprotective role of resveratrol against restraint stress-induced cognitive dysfunction involving brain glutathione system is further elaborated.

Reactive oxygen species (ROS) generated by chronic restraint stress significantly hampers the in-built antioxidant system in rat brain [1]. In the present study chronic restraint stress has caused a decline in reduced glutathione level in rat brain homogenate and this effect was reversed by resveratrol treatment. Glutathione (GSH) exerts an important function in defence against membrane peroxidation and also by reducing hydrogen peroxide with glutathione peroxidase. In cells GSH is retained in their reduced form by the glutathione reductase and in turn reduces other metabolites and enzyme systems as well as reacting directly with oxidants. Reduced glutathione is essential for the cellular detoxification of ROS in brain cells [31]. Immobilization stress causing a decrease in the brain levels of glutathione [32] was reported. In this study resveratrol has significantly elevated the brain glutathione level in stressed rats. Similar protective effect of resveratrol was observed after traumatic brain injury by expressing elevated reduced glutathione level [33]. The study by Ma et al[25] and Tiwari & Chopra[26], also show elevated brain glutathione level after resveratrol treatment against vascular dementia and
alcohol induced toxicity models respectively. Now this study further confirms antioxidant role against stress-induced free-radical generation.

The function of glutathione reductase enzyme is to produce reduced glutathione from oxidised glutathione, maintaining high ratio of reduce to oxidised form intracellularly. In the present study chronic restraint stress expressed a severe decline in this enzyme level. Resveratrol at 10mg/kg dose has protected this oxidative damage by bringing the glutathione reductase level to the normalcy. Resveratrol has exerted its antioxidant potential in obese mice associated with increase in cerebral oxidative stress [34].

Elevation in reduced glutathione and glutathione reductase enzyme observed in this study can be attributed to free radical scavenging properties of resveratrol. Being a well-known antioxidant resveratrol could inhibit free radical generation in brain and spinal cord [35],[36]. It is known to hinder the lipid peroxidation [13] and inhibits apoptotic cell death produced by oxidative stress [37]. It has been well claimed that resveratrol could inhibit mitochondria-induced production of ROS in rat brain [38], protect DNA from oxidative damage in stroke-prone hypertensive rats [39] and could prevent neuronal loss after ischemia/reperfusion injury.

The impact of oxidative stress to nervous tissue is numerous, as nervous tissue is particularly vulnerable to oxidative stress due to its high rate of oxygen consumption. Stress-induced oxidative damage involves mitochondrial dysfunction, dysregulation of Ca2+ homeostasis[40], damage to neuronal stem cells (defective neurogenesis) [41], induction of signaling events in apoptotic cell death [42]. Oxidative stress eventually leads to morphological changes and finally neuronal atrophy/death [43]. Hence in the present study we further focussed on neuronal morphology of the areas concerned with cognition.

The present study reveals a selective and quantitative neuronal loss affecting DG, CA3, CA2 and MPFC. Restraint stress induced neuronal loss in animal models is an established fact. Each neuronal population has a unique molecular composition that determines its level of vulnerability to oxidative stress. Results of the study by Tong et al[44] on animal model conclude that oxidative stress due to traumatic brain injury specifically involves CA2, CA3 regions of the hippocampus and DG. The region specific loss of neurons after restraint stress is known to involve many factors of which high density of corticosteroid receptors in DG and CA3 region which are vulnerable to glucocorticoids released due to stress. This would cause inhibition of glucose transport and elevation of cytoplasmic calcium concentration. The loss of neurons observed is consistent with breakdown of brain glutathione system in the rat brain. DG is an area where active neurogenesis continues throughout the adulthood in humans as well as animals. Functionally the projection of axons of granule cells of DG into CA3 region of hippocampus is a primary circuit for spatial memory. The turnover of granule cells of the DG in adult life is required for hippocampal function in spatial memory [45]. In our study restraint stress has shown neuronal loss in the dentate gyrus. Resveratrol in stressed rats has minimized this cytotoxic effect.

Long lasting stress inhibits proliferation and survival capacity of newly born neuronal stem cells [46]. The neuronal stem cells arising from DG differentiates and begin to migrate to the granule cell layer. This neurogenesis and survival are regulated negatively by glucocorticoids (as in stress), excitatory amino acids and opioids. Defective neurogenesis [47] or loss of adult neurons [48] is always associated with decline in learning abilities as well as memory retention as observed in the present study.

Medial prefrontal cortex (MPFC) is involved in both memory and decision making. But its functions like learning and memory consolidation depends on its connection with hippocampus [49]. Quinn et al[50] concluded that MPFC is necessary for both recent and remote memory. Both MPFC and hippocampus is necessary for consolidation of memory after learning. It is also known that MFC is involved in retrieval of memory after a task for subsequent days [49]. In the present study model the foot shock escaping task were continued for 5 days, observing the animal’s ability to retrieve the memory during 5 days and also after a week. Loss of neurons in the MPFC with declined retrieval capability was observed after chronic stress which was reversed by resveratrol. From the earlier
studies the interaction between hippocampus and MPFC is necessary for consolidation of memory after learning. Quantitative loss of neurons in both the regions can be attributed to cognitive decline.

**CONCLUSION**

To conclude, the chronic restraint stress induced cognitive decline in the present study can be attributed to break down of brain glutathione system and region specific neuronal loss. However estimation of reduced glutathione and glutathione reductase and other specific enzymes in hippocampal and frontal cortex region would have thrown more light on this observation. In this study resveratrol at all the three (behavioural, biochemical and morphological) levels showed beneficial effect. Hence in clinically diagnosed condition of stress, resveratrol would be a therapeutic option, as stress is integral part of current day’s life style.

**Conflicts of Interests:** None

**REFERENCES**


[23]. Padurariu M, Ciobica A, Mavroudis I, Fotiou D, Baloyannis S Hippocampal Neuronal Loss In The...
Ca1 And Ca3 Areas Of Alzheimer’s Disease Patients. Psychiatria Danubina 2012; 24(2):152-158.


