Journal of Advanced Zoology

ISSN: 0253-7214

*Volume **44** Special Issue-**2** **Year 2023** Page **3580:3602***

Beneficial Effect of Oryzanol on Transient Middle Artery Occlusion Induced Ischemic Stroke in Atherosclerotic Rats. Improvement in Behavioural and Biochemical Parameters

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Article History

Received: 08 Aug 2023

Revised: 29 Sept 2023

Accepted: 29 Oct 2023

Abstract:

The present study was designed to determine the beneficial effect of oryzanol treatment in the ischemic stroke in atherosclerotic rats. Atherosclerosis was induced in rats using high fat diet (containing 20% ground nut oil, 0.5% cholesterol, 1% cholic acid) for 32 days. Ischemic stroke was induced in the atherosclerotic rat (AT rats) on 33rd day using tMCAO model. In the oryzanol treatment group, oryzanol (100 mg/kg, po) was administered on the very first day (day rats were fed with high fat diet), ischemic stroke was induced on 33rd day and oryzanol treatment was continued after the induction of ischemia from 34th day to 40th day. The neurological score was determined for 7days with gap of 24 hrs between the testing procedure. Rats were sacrificed followed by the blood collection and excision of whole brain for the determination of various parameters including brain damage (infarct volume, brain hemisphere weight difference, and Na^+-K^+-ATPase activity) and oxidative stress parameter (SOD activity, reduced GSH level, MDA level, nitrite level and LDH level). It was observed that ischemia-reperfusion (IR rats) in atherosclerotic rats increased the neurological score, increase in infarct volume, brain hemisphere weight difference and reduced activity of Na^+-K^+-ATPase. Further IR rats showed the decreased

activity of SOD activity and GSH, whereas the level of MDA, nitrite and LDH activity was found to be increased in the atherosclerotic-IR rats. Further it was observed that the oryzanol treatment in the atherosclerotic counteracted the high fat induced rise in the TG, LDL, and VLDL level and increased the level of HDL in the treated animals. Further the administration of oryzanol improved the neurological score, reduce the infarct volume, brain hemisphere weight difference and improve the activity of Na^+-K^+-ATPase. Oryzanol treatment further improved the SOD activity, increased the level of GSH, reduced the level of MDA, nitrite and LDH activity in atherosclerotic-IR rats.

Keywords: Oryzanol, ischemia, stroke, atherosclerosis, infarction, brain

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1.0.Introduction

Stroke is a neurological deficit characterized by the presence of the acute focal injury resulting in the symptoms including dizziness, headache, hemianopia, numbness, weakness, etc. (Sacco et al., 2013; Becker, 2005). Stroke is a major cause of morbidity and mortality in the individual across the world (Tran et al., 2010). It has been reported that the prevalence of stroke is greater in the elderly individuals suggesting elderly population at the risk (Bramlett and Dietrich, 2004).

Atherosclerosis is the condition characterized by the arterial thickens due to the building up of cholesterol (Kazim and Ibrahim, 2011). The development of atherosclerotic plaques results in the restriction of the supply of blood or molecular O_2 to the brain resulting in the risk of development of ischemic stroke (Khan et al., 2011) and the later accounts for 80% of cases (Della-Morte et al., 2012). The goal is to maintain the blood supply to the ischemic region, however the restoration of blood supply to the ischemic region is responsible for the emergence of reperfusion injury and thus account for stroke in 50-70% of patients (Lin et al., 2016). The main aim of the reperfusion is to restore the supply of the oxygen to the ischemic tissue, but the rapid reperfusion also imposes the deleterious effect on the brain function known as “reperfusion injury”, responsible for the worsening of the conditions and the outcomes in the stroke patients (Lin et al., 2016). Thus, the ideal pharmacotherapeutics for the stroke include the restoration of the oxygen supply with minimize reperfusion injury. Pharmacotherapy generally aims to resolve the cerebral ischemia, decreases the atherosclerotic plaques, prevention of platelet aggregation and clotting, thrombolysis, etc (Bogousslavsky and Piechowski Jozwiak, 2006). Cholesterol-lowering therapy is considered mainly in the prevention of primary stroke (Steultjens and Dekker, 2003).

Gamma-oryzanol (γ-oryzanol) is the naturally occurring and main active constituent of rice bran oil (RBO). γ-oryzanol has been shown to confer various pharmacological effects including as anti-diabetic, cholesterol-lowering, anti-inflammatory, antioxidant, etc (Seetharamaiah and Prabhakar, 1986; Ramazani et al., 2021). Oryzanol is a mixture of ferulic

acid ester with phytosterol or triterpene alcohols (Metwally et al., 1974). γ-OZ has been shown to reduce the LPS-induced adhesion molecule, mRNA expression, inhibit NF-kB, leukocyte adhesion, reduce the risk of atherogenesis, aggregation of platelets, increases the muscle mass, improvement vascular functions, etc. (Patel and Naik, 2004). It has also been reported that OZ reduces serum cholesterol, triglyceride and low-density lipoproteins (LDL) level in hypercholesterolemic patients (Berger et al., 2005; Ghatak and Panchal, 2011). OZ provide protection to the cells and cellular molecules against the oxidative damage confer by the reactive oxygen species (ROS) (Kaliora and Dedoussis, 2007). These lines of evidence suggesting the worth of exploration of OZ against the transient middle cerebral artery occluded ischemic stroke in atherosclerotic rats.

2.0. Material and Methods

2.1. Animals

Adult Sprague Dawley rats (either sex, 250-300g) were used in the study. Rats were maintained in the central animal house of Nirma University, Ahmedabad at 21± 23^{o}C and 12:12 h light/dark cycle in polypropylene cages with free access to food and water *ad libitum*. The rats were fed with the Amrut Rat Diet supplied by Pranav Agro Industry Ltd. All the analyses were carried out between 09:00-15:00h. Procedures followed in the present study were approved by the Institutional Animal Ethics Committee (IAEC) of Institute of Pharmacy, Nirma University, Ahmedabad (*IPS/PCOL/MPH11-12/1011* dated 10.08.2011). The care of rats was taken as per guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India. Animals were acclimatized for one week before starting the experiment.

2.2. Drug

Cholesterol and cholic acid were purchased from Chemdyes Corporation, Rajkot, India. Oryzanol was purchased from Shah Enterprises Mumbai, India. Oryzanol (100mg/kg) was prepared using 4% w/v tween-80.

2.3. Induction of focal cerebral ischemia in atherosclerotic rats

Atherosclerosis was induced by feeding the rats with the high fat diet (containing 20% ground nut oil, 0.5% cholesterol, 1% cholic acid) for 32 days. Atherosclerosis was confirmed after 32 days of induction. The lipid profile was estimated according to the method of Krishna et al. (2010)

Polyamide monofilaments (Ethicon NW-3318 size 4.0) were cut into 28-30 mm long filaments. The 30 mm long filaments were first made blunt using direct flame. Then they were kept immersed in 0.1% w/v poly-l-lysine solution for ten minutes. Filaments were exposed to a temperature of 60˚C for half an hour in a hot air oven to facilitate drying. The filaments were wrapped into aluminium foil then transferred into an airtight container for storage in a cool and dry place (Belayev et al., 1996)

Animals were anaesthetized using single i.p. injection of chloral hydrate (350 mg/kg) and were placed on the operating table. Skin hair at neck region was removed and the left common carotid artery (CCA) was exposed through midline incision. The occipital artery branches of the external carotid artery (ECA) were then isolated and tied using a cotton thread. The ECA was dissected further distally and tied using cotton thread along with the

terminal lingual and maxillary artery branches, which were then divided. The internal carotid artery (ICA) was isolated and carefully separated from the adjacent vagus nerve Cotton thread was tied loosely around the ECA stump near the bifurcation. The ICA and CCA both were temporarily occluded by a fine vessel clip. The entry of blood from CCA and ICA was blocked to prevent bleeding from the incision in the ECA stump. Small incision was made on ECA stump and blunt Poly-L-lysine coated 4-0 polyamide monofilament was inserted through that ECA puncture. Loosely bound cotton thread around ECA was tightened to prevent blood loss from ECA puncture site. Fine vessel clip from ICA was removed and suture was gently advanced from ECA puncture site to ICA lumen. After a variable length of suture had been inserted into the ECA stump, resistance was felt and a slight curving of the suture or stretching of the ICA was observed, indicating that the tip of suture had passed the MCA origin and reached the proximal segment of the anterior cerebral artery (ACA), which has a smaller diameter. At this point, the intraluminal suture had blocked the origin of the MCA, occluding all sources of blood flow from the ICA, ACA and posterior cerebral artery (PCA). Finally, fine vessel clip from CCA was removed to restore blood flow. The midline incision was closed, leaving 10mm of the suture protruding so it could be withdrawn to allow reperfusion. Reperfusion of the MCA blood flow was done after 90 minutes of the surgery under mild ether anaesthesia. The 20 mm of suture was pulled back until resistance was felt, indicating that the tip cleared (Wang-Fischer, 2008). The ACA-ICA lumen and was in the ECA stump, and then trimmed. The animal was then transferred to a fresh cage with free access to food and water.

2.4. Measurement of neurological deficit score, cerebral infarction, and brain edema

Neurological deficit was graded using Longa's scoring system (Longa et al., 1989). The scoring scale was as follows: 0, no apparent neurological deficit; 1, contralateral forelimb flexion; 2, circling motion toward the paretic side when attempting to walk; 3, falling to the lateral side when pushed gently; 4, no spontaneous locomotion and depressed levels of consciousness. Neurological examination was observed on 24 hours, 48 hours and 72 hours after the induction of ischemia and reperfusion as per method described below (Menzies and Hoffand Betz, 1992). Rats with a neurological score of 0 were excluded from further experiments (Ruan et al., 2017; Longa et al., 1989).

Cerberal infarct was determined according to the method of Alessandrini et al. (1999). Rats were sacrificed with an overdose of chloral hydrate after evalution of the neurological deficit score. Brain tissues were immediately removed and weighed to obtain brain tissue wet weight. Then, they were placed in the refrigerator under -20°C for about 20 min, cut into five coronal slices continuously from front to back with a blade, and immersed into 4% TTC at 37°C in 0.2 M tris buffer (pH 7.4) for 30 min in the dark. The brain slices were turned over for several times, so that they could be evenly exposed to the dye. The TTC stained sections, where the viable cerebral tissue was stained red while the infarct cerebral tissue remained pale, were photographed with a digital camera and the infarct area of each section was measured using Image-Pro plus (version 6.0) analyzer software.

Brain edema was determined by evaluating the brain water content according to the wet-dry method (Hatashita et al., 1988). In brief, after measurement of infarct volume, all of the samples including both infarct and non-infarct sections were dried in an oven at 110°C for 24

h and weighed again to obtain the dry weight. Then, brain edema was calculated as follows: brain water content (%) = [(wet weight-dry weight)/wet weight] × 100.

Brain infarct volume, brain was removed immediately from euthanized animal with greater care for prevention of damage to brain territory. Then it was rinsed with 20% chilled sucrose solution and kept intact in to glass petridish for storage at 15˚C to -20˚C (Ahmad, 1997).

2.5. Blood sample collection

The fasting blood samples was obtained from each rat via cardiac puncture and was collected into a tube containing lithium heparin as anticoagulant. Plasma was obtained after centrifugation at 3500 rpm for 15minutes. The plasma was collected into a plain tube and stored at -20°C until the analysis.

2.6. Lipid Profile

The plasma concentration of TC, TG, HDL-cholesterol and LDL-cholesterol were measured using spectrophotometric methods. Laboratory kit reagents (Randox Laboratory Ltd, UK) were used for all biochemical analysis and their absorbance were read using a UV-Vis spectrophotometer (DREL 3000 HACH).

2.7. Determination of Oxidative stress parameters

SOD Activity: In brief, 0.1 ml of sample was mixed with the reaction mixtures containing sodium carbonate (1 mL, 50 mM), nitroblue tetrazolium (0.4 mL, 25 µm), and hydroxylamine hydrochloride (0.2 mL, 0.1 mM) followed by the determination of absorbance at 560 nm (Misra and Fridovich, 1972).

Reduced Glutathione (GSH) Activity: In brief, 0.5 mL plasma was mixed with 0.2 M Tris buffer (pH of 8.2), and 0.1 mL of 0.01 M Ellman's reagent (5,5′-dithiobis-(2-nitro-benzoic acid) (DTNB). The mixture was then centrifuged at 3000 g for 15 min followed by the collection of supernatant and determination of absorbance at 412 nm (Ellman, 1959).

Lipid peroxidation assay: In brief, 0.2 mL of plasma was mixed with 0.2 mL SDS, 1.5 mL acetic acid, and 1.5 mL TBA. The volume was made upto 4 ml using water. The solution was then heated at 95°C for 60 min on water bath followed by colling at room temperature. After cooling, 1 mL water and 5 mL n-butanol/pyridine mixture were added. The resultant solution was shaken vigorously and centrifuged at 4000 rpm for 10 min. The organic layer was separated followed by the determination of absorbance at 532 nm (Ohkawa et al., 1979).

Nitrite level: In brief, 100 µl of supernatant from the brain homogenates were incubated with 100 µl of the Griess reagent (1% sulfanilamide in 5% o-phosphoric acid and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride) at room temperature for 10 min. The absorbance was measured at 546 nm (Green et al., 1982).

Lactate dehydrogenase (LDH) activity: In brief, 0.2 mL of plasma was mixed with the 100 mM potassium phosphate buffer (pH 7.2), 4.1 mM a-ketoglutaric acid (pH 7.2) and 0.I7 mM reduced nicotinamide adenine dinucleotide (NADH) Followed by the volume make upto 4 ml using buffer solution. The solution was kept undisturbed for 10 min at 37°C followed by the determination of absorbance at 340 nm (Schatz and Segal, 1969).

2.8. Brain *Na^+ K^+-ATPase activity*

250 µl of Tris–HCl (184 mM; PH 7.5), 50 µl of 600 mM NaCl, 50 µl of 50 mM of KCl, 50 µl of 1mM sodium EDTA, and 50 µl of 80 mM ATP were mixed and incubated at 37°C for 10 min in an eppendorf tube. To this, 25 µl of 10% homogenate was added. The mixture was incubated again at 37°C for 1h and 10% TCA was added to stop the reaction. The mixture

was centrifuged at 3500 rpm for 10 min. In 200 µl of supernatant 925 µl distilled water, 125 µl ammonium molybdate and 50l µl 1-amino-2-napthol-4- sulphonic acid (ANSA) were added. The mixture was incubated for 10 min at 37°C followed by the determination of absorbance at 640 nm (Rao and Deshpande, 2005; Babua and Ramanathan, 2011).

2.9. Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) when found appropriate followed by Tukey's *post hoc* test using Graphpad Prism software (version 9.0.0). Values were expressed as Mean ± S.E.M. $p < 0.05$ was considered as statistically significant.

2.10. Experimental Groups

The animals were divided in randomized way into five groups as follows (n=8 in each group):

a) **Normal Control**: 4% tween-80 was given as vehicle.

b) **Atherosclerotic Control**: The high fat diet was provided to the animal for 4 weeks.

c) **Atherosclerotic Sham operated**: The high fat diet was provided to the animal for 4 weeks and after induction of atherosclerosis Sham operation will be performed.

d) **Atherosclerotic + MCAO**: The high fat diet was provided to the animal for 4 weeks and after induction of atherosclerosis MCAO will be performed.

e) **Atherosclerotic + MCAO + Oryzanol (100 mg/kg/day):** The high fat diet was provided to the animal for 4 weeks and the animal will be treated with oryzanol and after induction of atherosclerosis MCAO will be performed.

Day-1 to Day-32: Induction of Atherosclerosis by using High fat diet (diet containing 20% ground nut oil, 0.5% cholesterol,1% cholic acid).

Day-32: Conformation of Atherosclerosis by estimation of biochemical parameters (i.e. Total cholesterol, LDL-C, HDL-C Triglyceride etc.)

Day 33: Induction of Cerebral Ischemia/ Reperfusion using MCAO method (or)
Sham operation

Day 40: Blood was collected, animals were sacrificed, and brain was excised.

3. Results

3.1. Infarction volume

Oryzanol treatment to the I/R rats decreased the infarct volume significantly as compared to the atherosclerosis IR group (shown in *Figure 1* and *Table 1*).

Table 1: Effect of oryzanol on brain infarct volume

Sr. No.	Groups	Brain infarct volume (mm^3)
1	Atherosclerotic IR Control	157.6±46.25
2	Atherosclerotic IR O-100	105.3±14.16*

Values are expressed as Mean±SEM. n=8 in each group. *P<0.05 significant difference from atherosclerotic IR group.

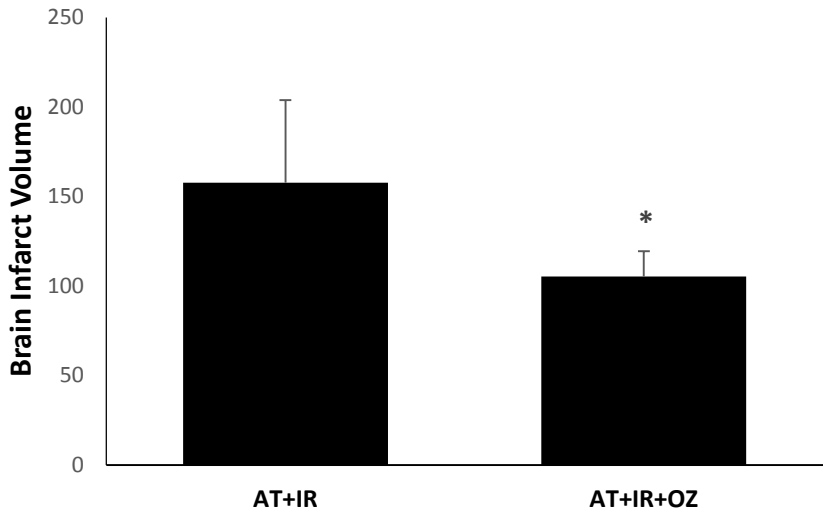


Figure 1: Effect of Oryzanol treatment on Brain infarct volume

Values are expressed as Mean±SEM. n=8 in each group. *P<0.05 significant difference from atherosclerotic IR group.

3.2. Brain water content

Oryzanol treatment decreased the brain hemisphere weight difference significantly compared to its respective control (*shown in Figure-2 and Table-2*). One way ANOVA revealed the significant effect of oryzanol treatment on the brain hemisphere weight difference ($F_{4,35}$=6.75, P<0.001). IR group has increased brain water content significantly as compared to the sham-operated group (P<0.05). Oryzanol treatment significantly reduced the brain hemisphere weight difference in AT-IR group (P<0.05) (Atherosclerotic IR Control*:* 0.049±0.0029 *vs.* Atherosclerotic IR O-100: 0.019±0.0011).

Table 2: Effect of oryzanol on brain hemisphere weight difference

Sr. No.	Groups	Brain hemispheres weight difference (gm/100gm of body weight)
1	Control (NC)	0.011±0.0014
2	Atherosclerotic Control (AT)	0.019±0.0008
3	Atherosclerotic Sham Control (AT+S)	0.023±0.0010
4	Atherosclerotic IR Control (AT+IR)	0.049±0.0029#
5	Atherosclerotic IR O-100 (AT+IR+O)	0.019±0.0011$

Values are expressed as Mean±SEM. n= 8 in each group. #P<0.05 significant difference from atherosclerotic sham group; $P<0.05 significant difference from atherosclerotic IR group.

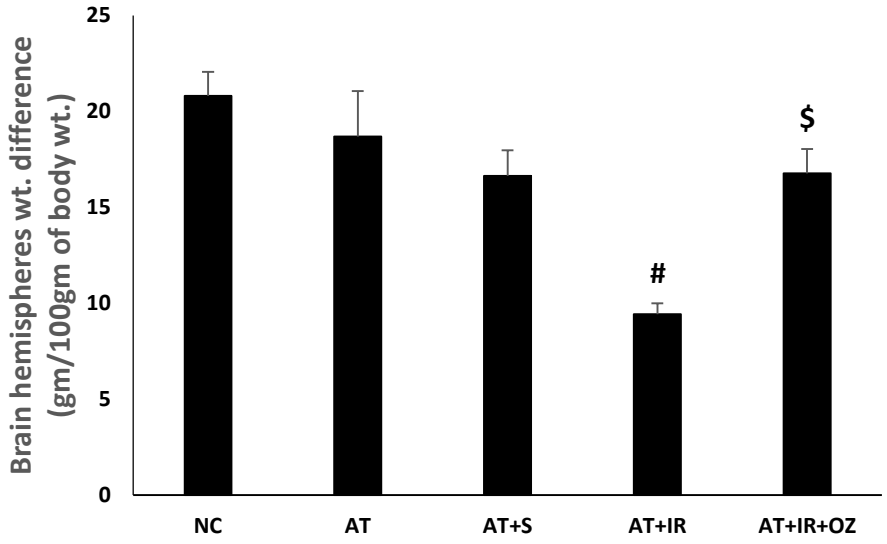


Figure 2: Effect of oryzanol on brain hemisphere weight difference

Values are expressed as Mean±SEM. n= 8 in each group. #P<0.05 significant difference from atherosclerotic sham group; $P<0.05 significant difference from atherosclerotic IR group.

3.3. Neurological assessment

The neurological score was determined at 1st, 2nd, 3rd, 4th, 5th, 6th and 7th day after the induction of ischemia in respective groups (*shown in Figure 3*). Atherosclerotic IR control group showed severe injuries and neurological deficits. Oryzanol treatment decreased the neurological score in treated animals.

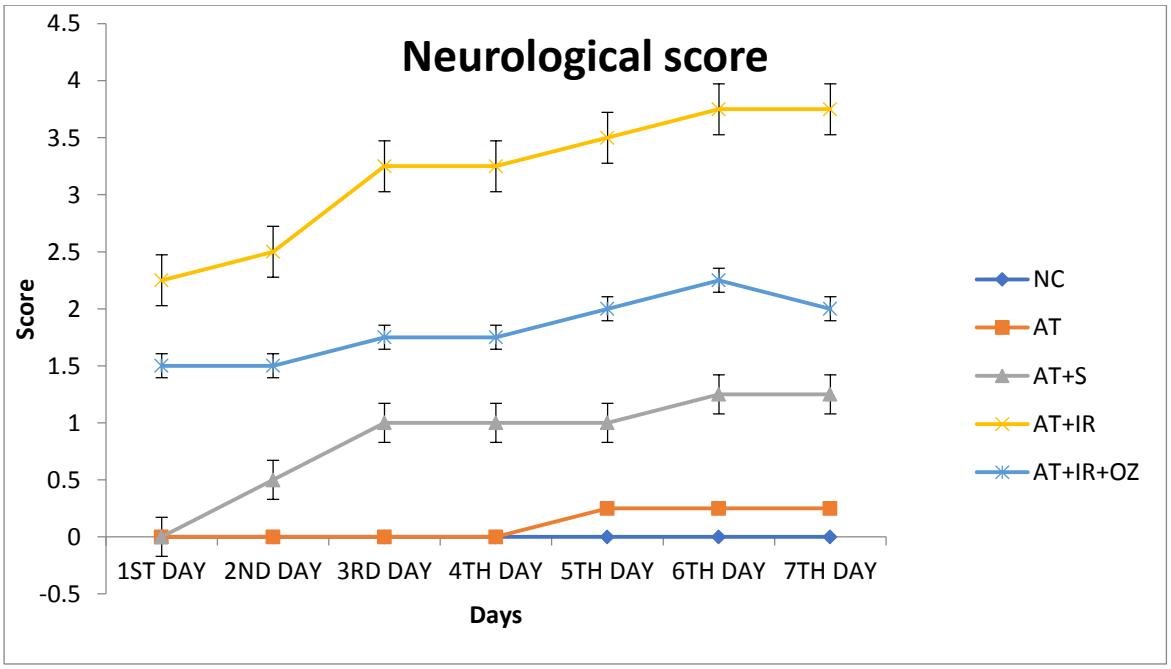


Figure 3: Effect of Oryzanol treatment on Neurological score Level in Atherosclerotic – IR mice

Values are expressed as Mean±SEM. n=8 in each group.

AT+IR- Atherosclerotic MCAO group**; AT+IR+OZ-** Atherosclerotic MCAO group with oryzanol treatment of 100mg/kg/day.

3.4. Oryzanol treatment increases Na^+/K^+-ATPase activity in the ischemic brain

The Na^+/K^+-ATPase activity revealed the effect of focal cerebral ischemia/reperfusion. Effect of various treatments on the Na^+/K^+-ATPase activity is *shown in Figure 4 and Table 3.* One way ANOVA revealed the significant effect of oryzanol treatment on Na^+/K^+-ATPase activity ($F_{4,35}$=20.6, P<0.001). It was observed that Na^+/K^+-ATPase activity decreased in I/R group was significantly as compared to sham-operated rats (P<0.05) (Atherosclerotic Sham Control: 16.64±1.33 *vs.* Atherosclerotic IR Control: 9.42±0.57). Oryzanol treatment of I/R rats increased the Na^+/K^+-ATPase activity significantly as compared to I/R group (P<0.05) (Atherosclerotic IR Control: 9.42±0.57 *vs.* Atherosclerotic IR O-100: 16.77±1.27).

Table 3: Effect of oryzanol on Na^+/K^+ ATPase Level

Sr. No.	Groups	Na^+/K^+ ATPase LEVEL (nmole of Pi liberating /hr/mg of protein)
1	Control (NC)	20.80±1.26
2	Atherosclerotic Control (AT)	18.68±2.37
3	Atherosclerotic Sham Control (AT+S)	16.64±1.33
4	Atherosclerotic IR Control (AT+IR)	9.42±0.57$^{\#\#}$
5	Atherosclerotic IR O-100 (AT+IR+O)	16.77±1.27$^{\$}$

Values are expressed as Mean±SEM. n= 8 in each group. $^{\#\#}$P<0.01 significant difference from AT+ S group; $^{\$}$P<0.05 significant difference from atherosclerotic IR group.

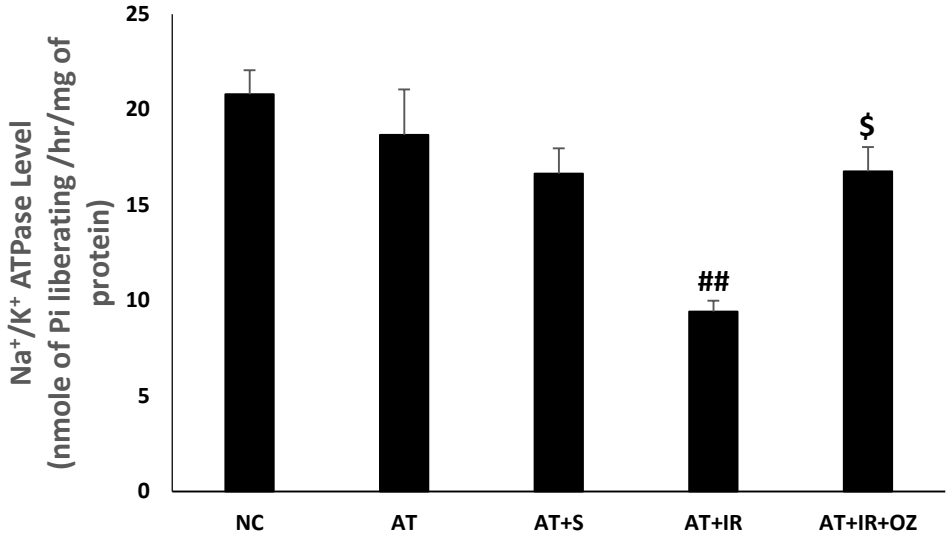


Figure 4: Effect of oryzanol on Na^+/K^+ ATPase Level

Values are expressed as Mean±SEM. n= 8 in each group. $^{\#\#}$P<0.01 significant difference from atherosclerotic sham group; $^{\$}$P<0.05 significant difference from atherosclerotic IR group.

3.5. Serum Lipid Parameters

Lipid levels were measured in order to determine the effect of the oryzanol treatment on lipid level (*shown in Figure 5 and Table 4*). One way ANOVA revealed the significant effect of oryzanol treatment on lipid parameters TG level ($F_{4,35}$=2.5, P<0.001); LDL level ($F_{4,35}$=4.5, P<0.001); HDL level ($F_{4,35}$=5.67, P<0.001) and VLDL level ($F_{4,35}$=5.89, P<0.001). Oryzanol treatment significantly reduced the level of TG (P<0.001) (Atherosclerotic IR Control: 225.9±13.4 vs. Atherosclerotic IR O-100: 128.1±12.5), LDL (P<0.001) (Atherosclerotic IR Control: 41.90±3.4 vs. Atherosclerotic IR O-100: 21.67±1.2), and VLDL (P<0.001) (Atherosclerotic IR Control: 45.17±2.7 vs. Atherosclerotic IR O-100: 25.62±2.5) and significantly increased the level of HDL (P<0.001) (Atherosclerotic IR Control: 18.86±3.1 vs. Atherosclerotic IR O-100: 29.83±1.6) as compared to its respective control.

Table 4: Effect of Oryzanol on lipid parameter

Sr. No.	Groups	TG (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	VLDL (mg/dl)
1	Control (NC)	82.14±19.2	14.32±1.6	33.60±1.5	16.43±3.8
2	Atherosclerotic Control (AT)	240±13.9	39.63±3.7	22.50±3.2	48.01±2.8
3	Atherosclerotic Sham Control (AT+S)	244.9±24.2	40.59±2.1	21.43±2.2	48.98±4.8
4	Atherosclerotic IR Control (AT+IR)	225.9±13.4	41.90±3.4	18.86±3.1	45.17±2.7
5	Atherosclerotic IR O-100 (AT+IR+O)	128.1±12.5***	21.67±1.2***	29.83±1.6***	25.62±2.5***

Values are expressed as Mean±SEM. n=8 in each group. ***P<0.001 significant difference from atherosclerotic IR group.

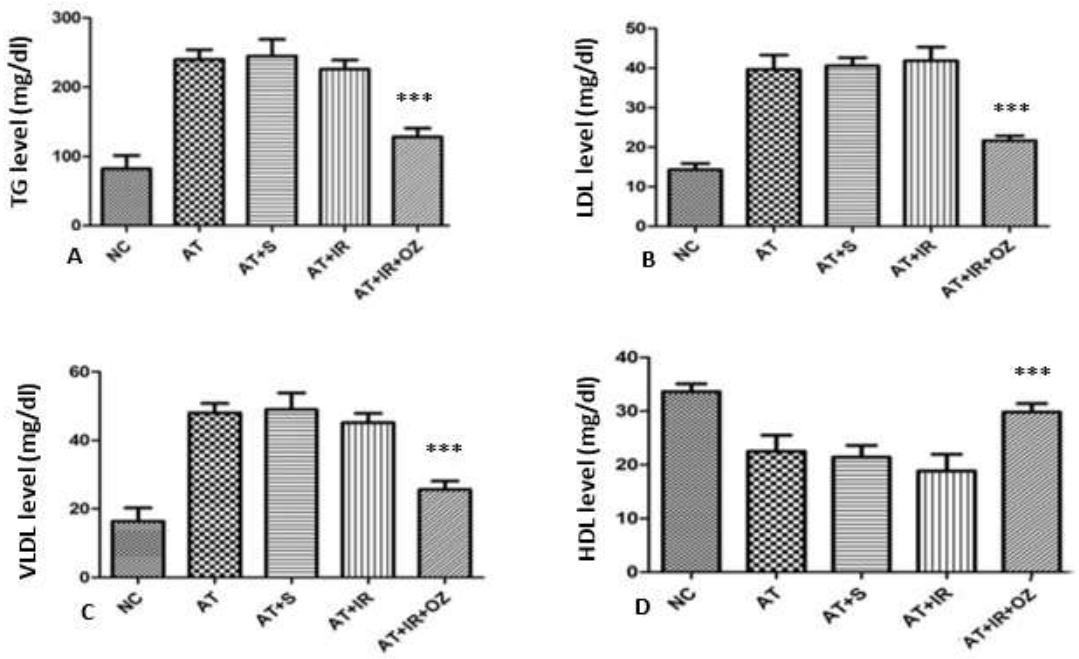


Figure 5: Effect of Oryzanol on various lipid parameter (A). TG level; (B). LDL Level; (C). VLDL level and (D). HDL Level

Values are expressed as Mean±SEM. n=8 in each group. ***P<0.001 significant difference from AT+IR group.

3.6. Effect of oryzanol treatment on various oxidative stress parameters

The effect of oryzanol treatments on the oxidative stress parameters are shown in *Figure 6* and *Table 5*. One way ANOVA revealed the significant effect of oryzanol treatment on SOD activity ($F_{4,35}$=4.36, P=0.0028); reduced GSH activity ($F_{4,35}$=4.00, P=0.0036); MDA level ($F_{4,35}$=6.50, P=0.007); nitrite level ($F_{4,35}$=6.00, P=0.001) and LDH level ($F_{4,35}$=4.92, P<0.001) Oryzanol treatment in I/R group (Atherosclerotic IR O-100: 2.302±0.28) significantly increased the SOD activity as compared to its respective control (Atherosclerotic IR Control: 1.043±0.31) (P=0.01). Oryzanol treatment in I/R group (Atherosclerotic IR O-100: 5.710±0.15) significantly increased the GSH activity as compared to its respective control (Atherosclerotic IR Control: 2.567±0.47) (P<0.001). Oryzanol treatment in I/R group (Atherosclerotic IR O-100: 2.867±0.23) significantly decreased the MDA level as compared to its respective control (Atherosclerotic IR Control: 5.128±0.53) (P<0.001). Oryzanol treatment in I/R group (Atherosclerotic IR O-100: 16.16±0.53) significantly decreased the nitrite level as compared to its respective control (Atherosclerotic IR Control: 24.79±0.87) (P<0.01). Oryzanol treatment in I/R group (Atherosclerotic IR O-100: 1187±68.82) significantly decreased the nitrite level as compared to its respective control (Atherosclerotic IR Control: 2302±100.4) (P<0.001).

Table 5: Effect of oryzanol various oxidative stress biomarker

Sr. No.	Groups	SOD Level (µg /mg)	GSH (µg/mg of protein)	MDA level (nmoles/mg)	Nitrite level (µmoles NO/mg)	LDH Level (U/L)
1	Control (NC)	4.686±0.20	6.637±0.15	2.442±0.24	10.10±1.15	1112±28.53
2	Atherosclerotic Control (AT)	3.936±0.47	4.790±0.90	3.454±0.54	16.07±1.37	1322±35.16
3	Atherosclerotic Sham Control (AT+S)	2.499±0.35	3.688±0.45	3.74±0.40	18.58±1.29	1322±43.80
4	Atherosclerotic IR Control (AT+IR)	1.043±0.31	2.567±0.47	5.128±0.53	24.79±0.87	2302±100.4
5	Atherosclerotic IR O-100 (AT+IR+O)	2.302±0.28**	5.710±0.15***	2.867±0.23***	16.16±0.53**	1187±68.82***

Values are expressed as Mean±SEM. n= 8 in each group. **P<0.01 and ***P<0.001 significant difference from AT+IR group.

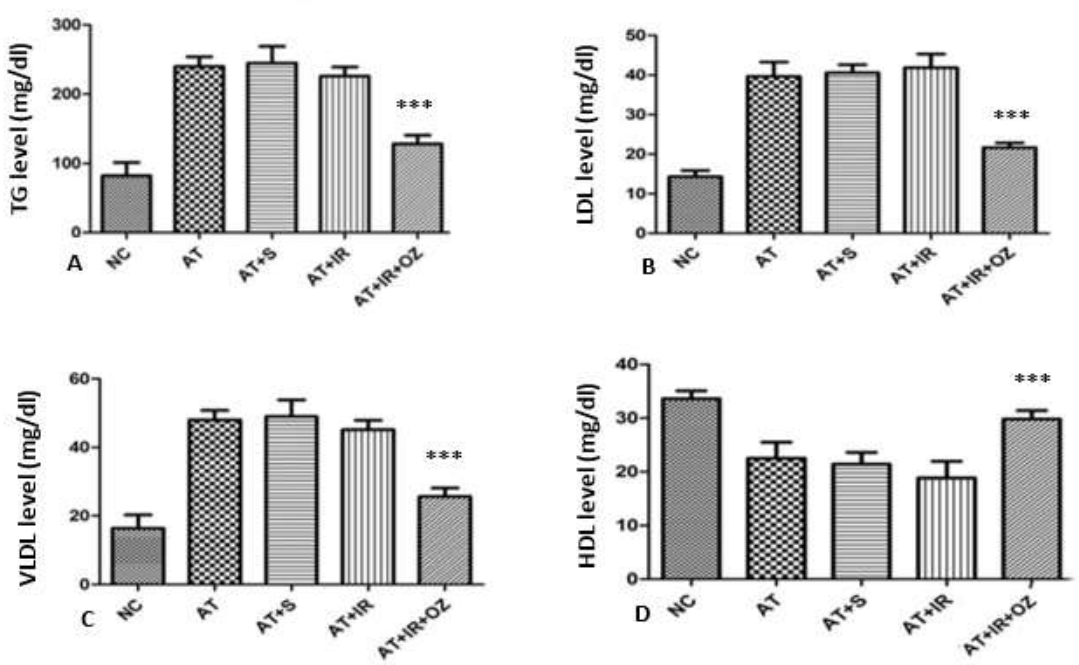


Figure 5: Effect of Oryzanol on various lipid parameter (A). TG level; (B). LDL Level; (C). VLDL level and (D). HDL Level

Values are expressed as Mean±SEM. n=8 in each group. ***P<0.001 significant difference from AT+IR group.

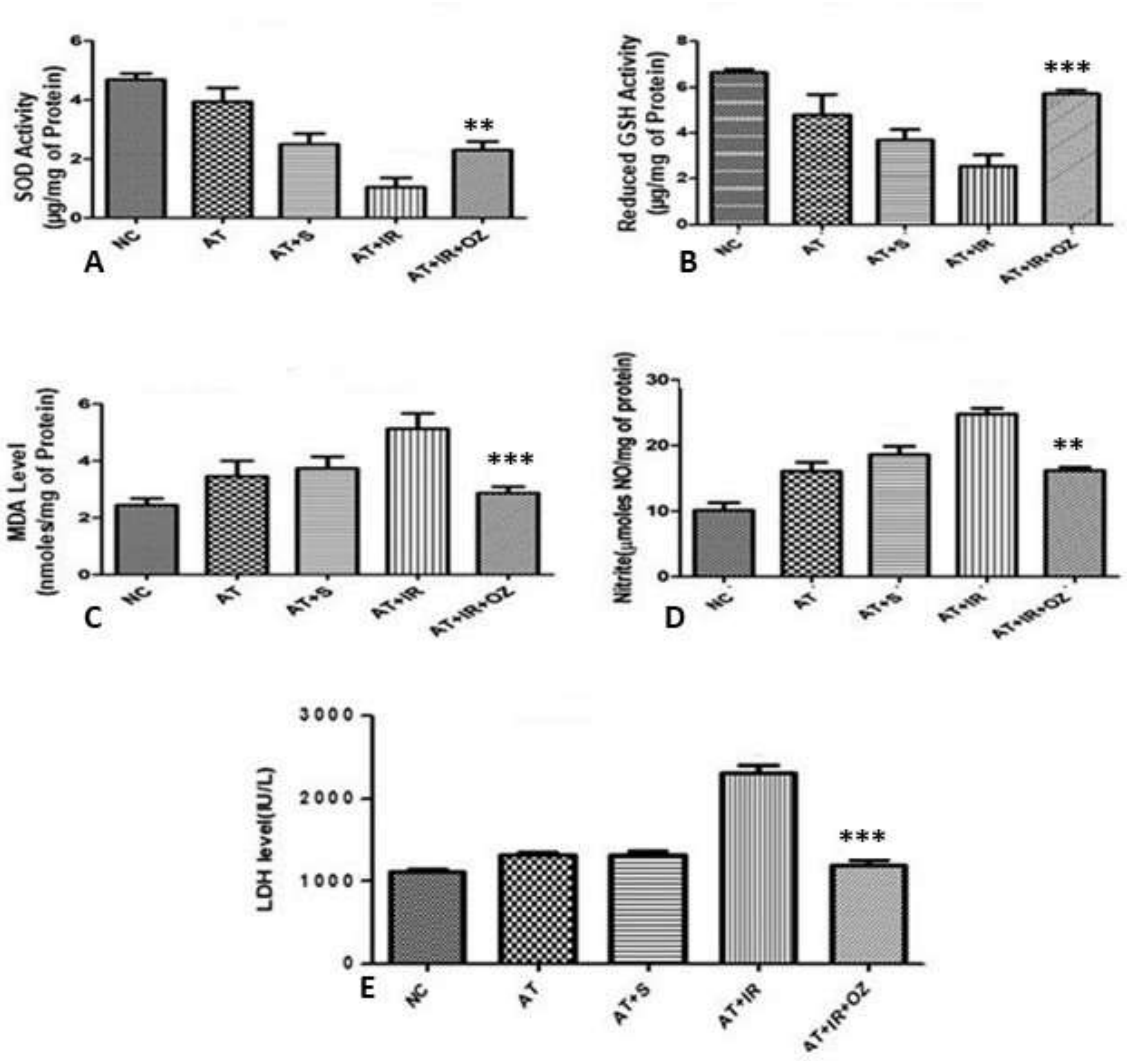
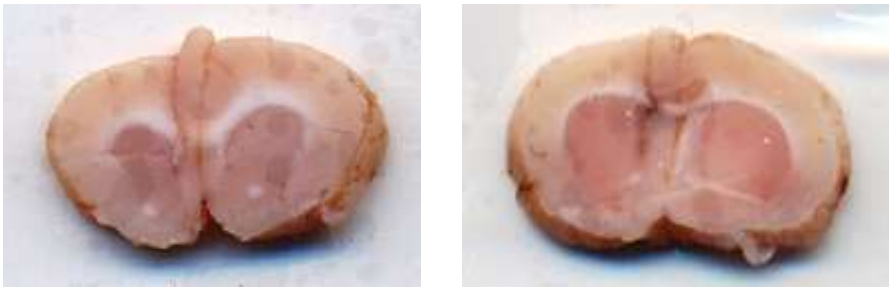


Figure 6: Effect of oryzanol on various oxidative stress biomarker (A). SOD activity; (B). Reduced GSH activity; (C). MDA level; (D). Nitrite level; (E) LDH level

Values are expressed as Mean±SEM. n= 8 in each group. $^{**}P<0.01$ and $^{***}P<0.001$ significant difference from AT+IR group.

Control



A **B**

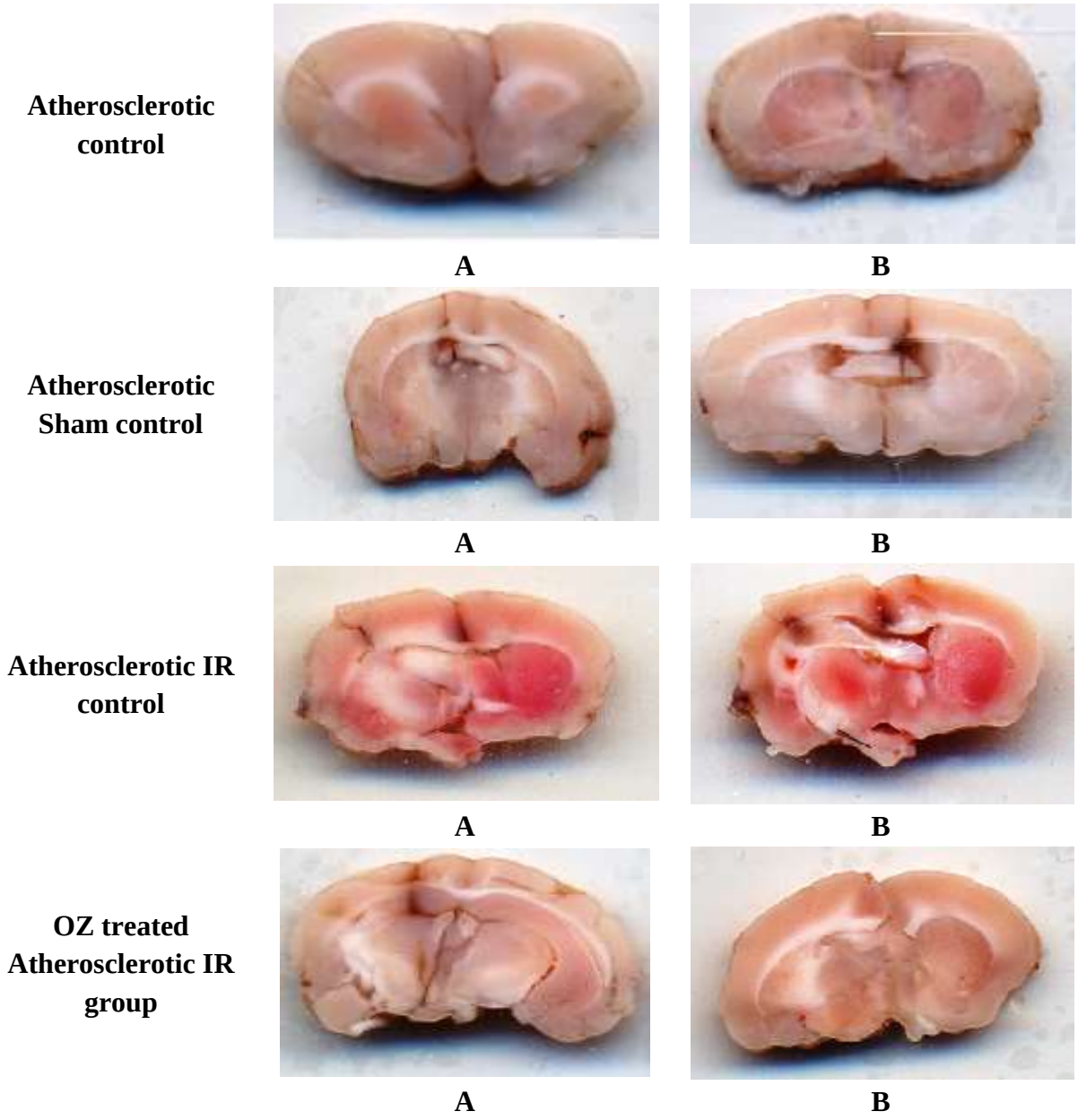


Figure 7: Effect of various treatments on brain tissue

4.0. Discussion

Ischemic stroke is the condition characterize by the reduced supply of oxygen to the particular region of brain (Alberts, 2003). Preclinical models of stroke enabled the evaluation of potential therapeutics that may confer neuroprotection against stroke (Carmichael 2005). Endovascular techniques are minimally invasive and are used generally for the induction of stroke in the experimental animals (Schmid-Elsaesser et al., 1998). In this technique, middle cerebral artery is occluded by passing suture through the artery to block the blood flow, resulting in the generation of infarction. The occlusion is followed by the retraction of suture, responsible for the reperfusion and mediated injury in that particular region (Gerriets et al., 2004). The extent of injury following this is determined by measuring the infarct volume (Lin et al., 1993). In the present study, the infarct volume increases in the atherosclerotic I/R rat as compared to its respective control. Further the oryzanol treatment in the atherosclerotic I/R

rat significantly decreased the infarct volume as compared to its respective control. Thus, the present study suggested the neuroprotective effect of oryzanol treatment in atherosclerotic I/R rats. To determine the degree of damage over a period neurological function was determined. It has been reported that the stroke s characterized by the presence of learning and memory impairments. Previous studies have suggested that the occlusion of unilateral middle cerebral artery (MCAO) induces contralateral neurological deficits in the experimental animals (Schaar et al., 2010). The neurological deficits are followed by the development of compensatory behavior to perform the daily activities despite of the developed neurological impairments (Allred and Jones, 2008). Further the unilateral brain damage results in the symmetric deficits also which can be evaluated by the tests of asymmetry (Hua et al., 2002). Therefore, it is important to assess the neurological functions after the induction of stroke and after the treatment of test agent in the induced animals (Schaar et al., 2010). Neurological functions following stroke was assessed using Bederson scale which includes the use of a grading scale of 0-5 to assess behavioral deficits after stroke (Hua et al., 2002). In the present findings the atherosclerotic I/R rats will have significantly more neurological deficits than non-ischemic animals, resulting in a higher score. Further the administration of oryzanol to the atherosclerotic I/R rats resulted in the significant reduction of neurological score in treated animals.

Atherosclerosis is considered as the main cause of stroke. The presence of large plaques in the proximal segment of the aorta increases the risk of ischemic stroke by three different mechanisms including perfusion failure, local thrombus formation and occlusion of penetrating arteries (Segura et al., 2001). The occlusion results in the development of stroke and cerebral territories supplied by their perforating vessels (Komotar et al., 2005). In the present study, oryzanol treatment has been shown to confer protective effect against I/R injury in atherosclerotic rat. Oryzanol has shown cholesterol lowering property and found to decrease LDL cholesterol and total serum cholesterol level and increases the level of HDL by increasing the absorption of dietary cholesterol and by increasing the conversion of cholesterol to fecal bile acid and sterols (Patel and Naik 2004). In the present study it was observed that the administration of oryzanol (100 mg/kg, p.o) decreases the cholesterol level in atherosclerotic I/R rats significantly as compared to atherosclerotic animals. Oryzanol (100 mg/kg p.o) treatment further reduces the LDL level in atherosclerotic I/R rats significantly as compared to the atherosclerotic rats. Serum HDL level is inversely correlated with risk of development of atherosclerosis because HDL cholesterol confer protection against oxidative modification of LDL which plays a key role in the initiation and progression of atherosclerosis (Sarkar and Rautaray, 2008). HDL cholesterol level also increased in some extent in the oryzanol treated IR rats. The results obtained suggested that the neuroprotective effect of oryzanol might be due to its cholesterol lowering effect.

Ischemic stroke mediated tissue injury is responsible for the production and release of ROS and proinflammatory mediators (Amantea et al., 2009). Previous findings have shown that both occlusion and reperfusion results in the production ROS (Peters et al., 1998; Yamato et al., 2003; Abe et al., 2015) and the later is responsible for the oxidation, modification, and degradation of protein molecules (Garrison and Jayko, 1962; Garrison, 1987). Extensive protein oxidation has been shown to be occur in the ischemic stroke in the preclinical models (Li et al., 2005). Beside protein oxidation, ROS is also responsible for the lipid peroxidation

by attacking the carbon-carbon double bonds, producing lipid radicals which further promote the generation more lipid radicals (Yin et al., 2011; Halliwell and Chirico, 1993). Lipid peroxidation results in the production of malondialdehyde (MDA) and the later served as a marker of lipid peroxidation (Picaud et al., 2004). Increased lipid peroxidation has been found in the animal model of stroke (Bromont et al., 1989; Yamamoto et al., 1983). Lipid peroxidation has been shown to play an important role in the pathogenesis of ischemic stroke and it results in the production of lipid peroxides responsible for the damage to biological membranes (Rahman, 2007). In the present study, MDA level (nmoles/mg of protein) in the brain tissue of the atherosclerotic IR group. Administration of oryzanol decreases the level of MDA in atherosclerosis I/R rats as compared to atherosclerotic sham rats.

Cerebral ischemia/reperfusion injury is thus responsible for the production of ROS that confer oxidative damage (Oliver et al., 1990) and neuronal cell death (Kapoor et al., 2019; Kho et al., 2018; Love, 1999; Nikonenko et al., 2009; Wang et al., 2006). Oxidative stress is also responsible for the down regulation of the endogenous antioxidants. GSH is a major endogenous antioxidant responsible for the scavenging (Galano and Alvarez-Idaboy, 2011) and detoxification of ROS (Orian et al., 2018). In these reactions, GSH is oxidized to glutathione disulfide (GSSG) and the later is converted into GSH by the action of enzyme glutathione reductase (Couto et al., 2016). Cerebral ischemia/reperfusion is also responsible for the depletion of GSH level (Wu et al., 2019; Abdel-Fattah et al., 2018), and therefore maintaining the normal neuronal GSH levels confer neuronal protection against oxidative stress. The present findings demonstrated that the level of GSH decreases in atherosclerotic I/R rats as compared to the atherosclerotic rat. Further, oryzanol treatment has been shown to increase the level of GSH in atherosclerosis IR rats significantly as compared to Atherosclerosis IR non treated rats. SOD is another endogenous antioxidant that has been shown to provide protection against the oxidative damage (Francis et al., 1997). The results suggested the protection against ischemia-reperfusion injury in rats by reducing edema, reducing ROS production. The results of the present study suggested that the SOD level was reduced in atherosclerotic rats and atherosclerotic I/R rats as compared to control group. Treatment of atherosclerotic IR rats with oryzanol further increases the level of SOD in them respectively.

Ischemic stroke is characterized by the loss of blood and oxygen flow to a particular region of brain. Ischemia is responsible for the decrease levels and the activity of Na^+ K^+ ATPase (Nicholls et al., 2000; Mintorovitch et al., 1994) further responsible for the increased influx of sodium ions. The results obtained suggested that Na^+ K^+ ATPase activity reduced significantly in atherosclerotic I/R rats and the administration of oryzanol (100 mg/kg p.o) increases the activity of Na^+-K^+ ATPase in atherosclerotic I/R rats respectively. It has been reported that the damage to the cell membrane results in the release of L-Lactate dehydrogenase (LDH) (Stoddart, 2011) which is further responsible for the conversion of pyruvate to L-lactate (Decker and Lohmann-Matthes, 1988; Nachlas et al., 1960). Levels of lactate has been shown to increase during hypoxic and ischemic condition (Lampl et al. 1990; Hugu et al. 1998). LDH thus used as a marker of tissue hypoxia (Fullerton et al. 1988). The increased level of lactate has been shown to confer the acidosis and neuronal cell damage (Siesjo and Siesjo 1996). In present study, ischemic condition increases the level of LDH

respectively. Further administration of oryzanol to the atherosclerotic I/R rats reduced the lactate level significantly as compared to its respective control.

NO has been shown to play a dual role in the pathogenesis of cerebral ischemia. Ischemic injury to the brain or its tissue resulted in the activation of inducible NOS and neuronal NOS responsible for the increased production of NO in the neurons. NO then combines with superoxide ions to produce cytotoxic peroxynitrite ions which further confer the damage to cellular macromolecules and promotes neuronal apoptosis (Kashihara et al., 2010; Halliwell, 1992; Huie and Padmaja, 1993; Lepoivre et al., 1994; Moncada and Higgs, 1993). In the present study increased nitrite level was found in atherosclerotic rats and atherosclerosis I/R rat as compared to control. Administration of oryzanol to the atherosclerosis I/R rat further reduced the level of nitrite significantly as compared to its respective control.

In conclusion, oryzanol treatment has been shown to confer the neuroprotective effects in atherosclerosis I/R rat respectively. The neuroprotective effect of oryzanol might be due to its lipid lowering activity. Oryzanol treatment further reduces the level of nitrite and lactate in response to the I/R injury. Oryzanol treatment reduces the oxidative damage, suppress the lipid peroxidation and supports the endogenous antioxidants. In context of these observations, the present study suggested the beneficial effect of oryzanol against the I/R injury.

Funding Support: None

Conflict of Interest: None

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