Exploring the possible role of date fruit (*Phoenix dactylifera*, L) extract in amelioration of stroke in rats

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ABSTRACT :

Background: Considerable morbidity and mortality are associated with stroke and approximately 5.7 million deaths reported due to stroke every year. Restoration of blood flow to ischemic brain is associated with generation of reactive oxygen radicals. Date fruits are an integral part of food and they possess antioxidant, neuroprotective, anti-stress properties.
Purpose: The present study investigates the effect of aqueous extract of sukari variety of date fruits on acute cerebral ischemia-reperfusion induced oxidative stress and histopathological alteration in rats.
Methods: Acute cerebral ischemia-reperfusion was produced in rats by occlusion of bilateral common carotid arteries for 30 minutes followed by reperfusion for 4 hrs. Various groups of rats i.e group 1 to 6 were subjected to pretreatment for 30 days followed by induction of ischemia-reperfusion 1 hour after last dose. Group 7 was given L-NAME just 30 minutes before surgery. However group 2, sham operated control underwent all the processes except surgery. At the end of study half of rats from all groups were sacrificed for estimation of various biomarkers in brain homogenates and rest were sacrificed for brain histopathological examination.
Results: The surgery group showed altered levels of superoxide dismutase, catalase and malondioalhyde when compared to sham control. Pretreatment for 30 days with extract prevented this reperfusion-induced rise in these biomarkers dose dependently. Histopathological examination of TTC stained coronal sections of brains showed a reduction in infarct area dose dependently by DFE. The results suggest that aqueous extract of sukari dates may be useful in treatment of cerebral reperfusion injury and may be useful in prevention of stroke. This protective effect may be attributed to its antioxidant properties.
Conclusions: Aqueous extract of sukari dates offer neuroprotective effect against ischemia-reperfusion induced injury and may offer potential benefits in the management of stroke.

Keywords: Sukari dates, Stroke, Antioxidant, Neuroprotection, Ischemia, Reperfusion

INTRODUCTION:

Stroke is a cerebrovascular disease in which the cerebral blood flow reduces leading to damage or death of brain cells. This interruption of the blood supply to a part of brain occurs by thrombus or embolus occlusion or by hemorrhage. Stroke is of two types, ischemic and hemorrhagic type and they are a most common cause of death worldwide. It is also a leading cause of death and neurological dysfunctions in the industrialized nations. Ischemic stroke accounts for approximately 80% of all strokes. There is an approximately 5.7 million deaths annually with 15 million new acute strokes added every year. Major approaches developed so far to treat acute ischemic stroke fall into 2 categories, thrombolytic therapy and neuroprotective therapy. However, treatment for stroke only limited to supportive care and management of complication. Several therapeutic strategies inhibiting excitatory amino acid receptor activation, calcium overload and oxidative stress have shown promising results in well controlled animal stroke models but failed to show efficacy in clinical studies. Despite substantial research into neuroprotection, there is no approved therapy that can reduce stroke size or cause [1-3].
In recent years there has been a spurt in the interest of scientists to explore fruits and vegetables in preventing or reducing the risk of many chronic diseases including stroke. The potential health benefits that fruits and vegetables offer are attributed to their polyphenol contents such as flavonoids. The flavonoids have been reported to effectively attenuate oxidative stress induced neuronal cell death in various experimental settings. There are several other studies suggesting the delay in the development of various neurodegenerative disorders by supplementation with antioxidants. One such dietary supplement is the date fruits (*Phoenix dactylifera* L.) which are important from nutritional and economic points of view. Date fruits consists of a fleshy pericarp and seed and have been cultivated in the Middle East since thousands of years. For the native

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arabs, dates are an integral part of their food and for muslims dates are of religious importance and are mentioned in many places in the Holy Quran and Hadith. The importance of the dates in nutrition is due to their rich contents of carbohydrates, salts and minerals, fibers, vitamins, fatty acids, amino acids and protein. Date fruits have also been reported to possess antioxidant, antimutagenic, antibacterial, antifungal, anti-tumoral, gastrprotective and neuroprotective effects. Various studies have also established the presence of neuroprotective agents such as melatonin, polyphenolic compounds, flavonoids, vitamin E etc in the date fruits [4-6].

In the Qassim region sukari variety of dates is very popular and in a previous study by authors have successfully shown the neuroprotective potential of the aqueous extract of this locally available variety of date fruits in chemically induced memory deficit in rats. It was also found in this study, the aqueous extract displayed strong antioxidant activity against DPPH free radicals. Encouraged by these findings we undertook the present study to explore the cerebroprotective ability of sukari dates extract in cerebral infarction in rats.

2. Materials and Methods

i.Drugs and chemicals

Date fruit extract, thiopentone sodium, 2% triphenyl tetrazolium chloride (Sigma Aldrich, USA), phosphate buffered saline (pH 7.4), L-NAME (Santa Cruz Biotechnology, Germany). All the other chemicals used in the study were of analytical grade.

ii.Animals

Male Sprague dawley rats weighing 250-300 gm were procured from central animal facility of the Institute. The rats were housed in polypropylene cages lined with husk, maintained in controlled temperature(24±1°C) and humidity(55±5%) with 12-h light and dark cycle. They were provided standard diet and water *ad libitium*.

Rats were acclimatized for a minimum period of 1 week prior to the initiation of study. The experimental protocol was approved by Institutional Animal Ethical Committee, College of Pharmacy, Qassim University.

iii. Preparation and administration of Investigational drug, date fruits

Fresh fruits of Sukari dates (P. dactylifera L.) were purchased from the local market and samples of these dates were kept frozen for future reference. Date fruits were separated from seeds and washed to remove any adhering dust particles and dried at room temperature. The aqueous extract of the date fruit was prepared by grinding the pulp in a mechanical set with distilled water. It was centrifuged at 4°C for 20 minutes at 4000g, and the supernatant was collected, lyophilized and stored at -20°C until use. Most of the date fruit components were extracted in water as mentioned in the study by Vayalil. Thus prepared aqueous date fruit extract (DFE) was used for administration in experimental animals by oral gavage. Every time fresh solution of the fruits was prepared before administration [4].

iv.Experimental design

Induction of global ischemia

The surgical technique used for the induction of cerebral ischemia was bilateral common carotid artery occlusion (BCCAO) followed by reperfusion: Rats were anesthetized by thiopentone sodium (40 mg/kg) i.p. Under anesthesia, midline incision was given, bilateral common carotid arteries were identified and separated carefully from vago-sympathetic nerve. BCCAO was performed by thread for 30 mins and reperfusion was allowed for 4 h by removing the thread with the help of knot releasers. Body temperature was maintained at 37 ± 0.5° C throughout the surgical procedure [7-10].

Grouping of the animals

The rats were divided into the following groups each consisting of 8,

Group 1: Normal control (saline 2 ml/kg, PO)
Group 2: Sham operated control (saline 2 ml/kg, PO)
Group 3: Ischemic reperfusion (I/R) (saline 2 ml/kg, PO)
Group 4: Date fruit extract (DFE, 200 mg/kg, PO)
Group 5: Date fruit extract (DFE, 400 mg/kg, PO)
Group 6: Date fruit extract (DFE, 800 mg/kg, PO)
Group 7: L-NAME (15 mg/kg, IP)

All the groups except group 7 were subjected to respective treatment as above for a period of 30 days whereas group7 was given L-NAME 30 mins before surgery. At the end of treatment period and 30 mins after last dose, all the rats were subjected to BCCAO. However group 2 underwent all the processes except BCCAO.

v. Histological and biochemical evaluation [7-15]

a. Measurement of Infarct size

Four rats from each group were sacrificed by cervical dislocation 24 hrs after the surgery to isolate brain. The isolated brains were placed in a freezer at -20°C for up to 20 minutes to facilitate coronal section cutting of 2 mm thickness. The sections were put in a glass petridish containing 2% 2, 3, 5-triphenyl tetrazolium chloride (TTC) solution, and each section was covered with cover slips in such a way that the top and bottom surfaces of the section were in contact with the glass. The sections were allowed to stain for 30 minutes at 37°C in water bath. After 30 minutes of staining, sections were removed from TTC solution and placed in 4% formalin solution for fixation. To prevent distortion, brain slices were kept flat in the petridish overnight. The stained sections were analysed for total, ipsilateral and infracted area using computer software Adobe Photoshop 7. The infarct volume was calculated by subtracting the correlated intact volume of the ipsilateral hemisphere from the total volume of the contralateral hemisphere.

b. Estimation of oxidative stress markers

The remaining four rats from each group were sacrificed by decapitation under anesthesia and brains were isolated, homogenized in 1 gm/10ml of phosphate buffered solution, PBS having pH 7.8 using a homogenizer (High Speed-RQ-127A) and centrifuged at 2°C -8°C at 15000 rpm for 10 minutes. The supernatant was used for the estimation of various endogenous oxidative enzymes.

1. Assay of SOD Activity

The test was carried out by adding 0.5 ml of carbonate buffer, 0.1ml EDTA and 1.0ml of epinephrine. The optical density of formed adrenochrome was read at 480nm for 3-min. at intervals of 30 sec. SOD calibration curve was prepared by taking 0.01 U/ml, 0.1 U/ml, 1 U/ml, and 10 U/ml concentrations as standard solutions. The enzyme activity was expressed in terms of U/min/mg of protein.

Calculation of SOD activity:

The rate of increase in absorbance units (A) per 30 seconds up to 3 minutes for the control and for the test sample/s was/were measured by using following formula.

$$\frac{A_{480nm}\ @\ 3\ min\ -\ A_{480nm}\ @\ 0\ min}{3\ min} = A_{480nm}/min$$

The % inhibition for the test sample(s) was measured by using following formula:

$$\frac{[(A_{480nm}/min)^{ctrl} - (A_{480nm}/min)^{test}] \times 100}{(A_{480nm}/min)^{ctrl}} = \% \text{ Inhibition}$$

One unit of SOD inhibits the rate of increase in absorbance at 550 nm by 50% under the conditions of the assay. The percent inhibition of test sample was correlated with SOD activity using an SOD standard curve.

2. Assay of Catalase activity

150 µl of 30mM H_2O_2 was taken and 3 ml volume. 50µl of sample was added to this solution. The decreased in absorbance was read at 240nm for 2.5 min. at interval of 15sec. The results were expressed as mean absorbance of catalase activity.

Calculation of Catalase:

The amount of catalase was calculated using extinction co-efficient of H_2O_2, 0.041/µmole/cm^2 and has been reported as µmoles of H_2O_2 utilized/min/mg of protein.

3. Reduced glutathione (GSH)

200 µL of brain homogenate was mixed with equal volume of 10 % TCA and centrifuged at 2700 rpm for 15 min.100 µL of the clear supernatant was reacted with 0.89 ml of 1.0 M Tris Buffer (pH 8.2) and 0.02 M EDTA. To this 10 µL 5, 5'- dithiobis-2-nitrobenzoic acid was added in succession. The intensity of the resulting yellow color was read spectrophotometrically at 412 nm. Reduced GSH was used as a standard.

4. Assay of Lipid Peroxidation

1 ml sample was mixed with 0.2ml 8.1 % sodium dodecyl sulfate, 1.5 ml 20 % glacial acetic acid solution and pH was adjusted to 3.5 using 1 N NaOH. Then 1.5 ml 0.8 % of thiobarbituric acid (TBA) was added. Blank was prepared by substituting the TBA solution with distill water. The resultant mixture was heated in a hot water bath at 95°C for 1hr. The intensity of pink color developed was read against a blank at 532 nm. The amounts of MDA (TBA reactive material) was calculated using molar extinction co-efficient 1.56 x 10^5 M Cm^{-1}' and has been reported as an nmoles of MDA/mg of protein.

Statistical Analysis

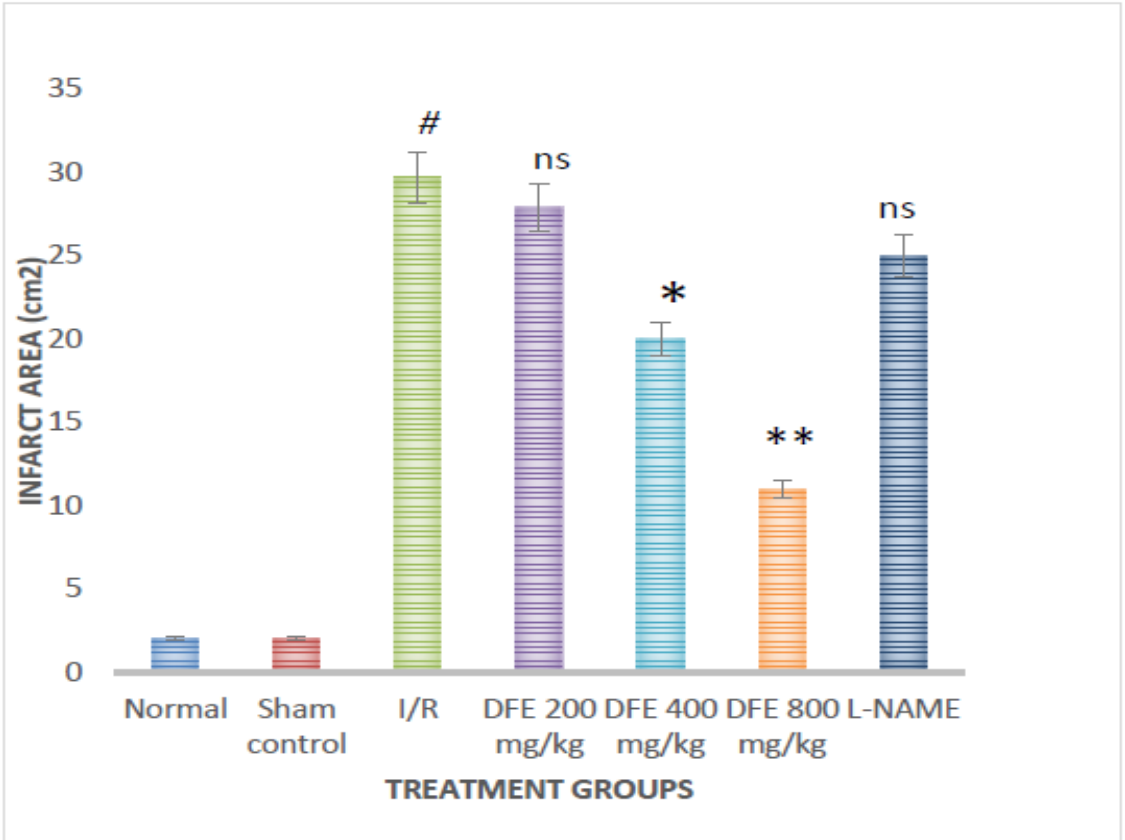
All the results were expressed as mean ± SEM. The data were analyzed using ANOVA followed by tukey's multiple comparison post hoc test. p <0.05 were considered as significant. Graphpad prism demo version software was used for this purpose.

Results

i.Effect of DFE on infract size

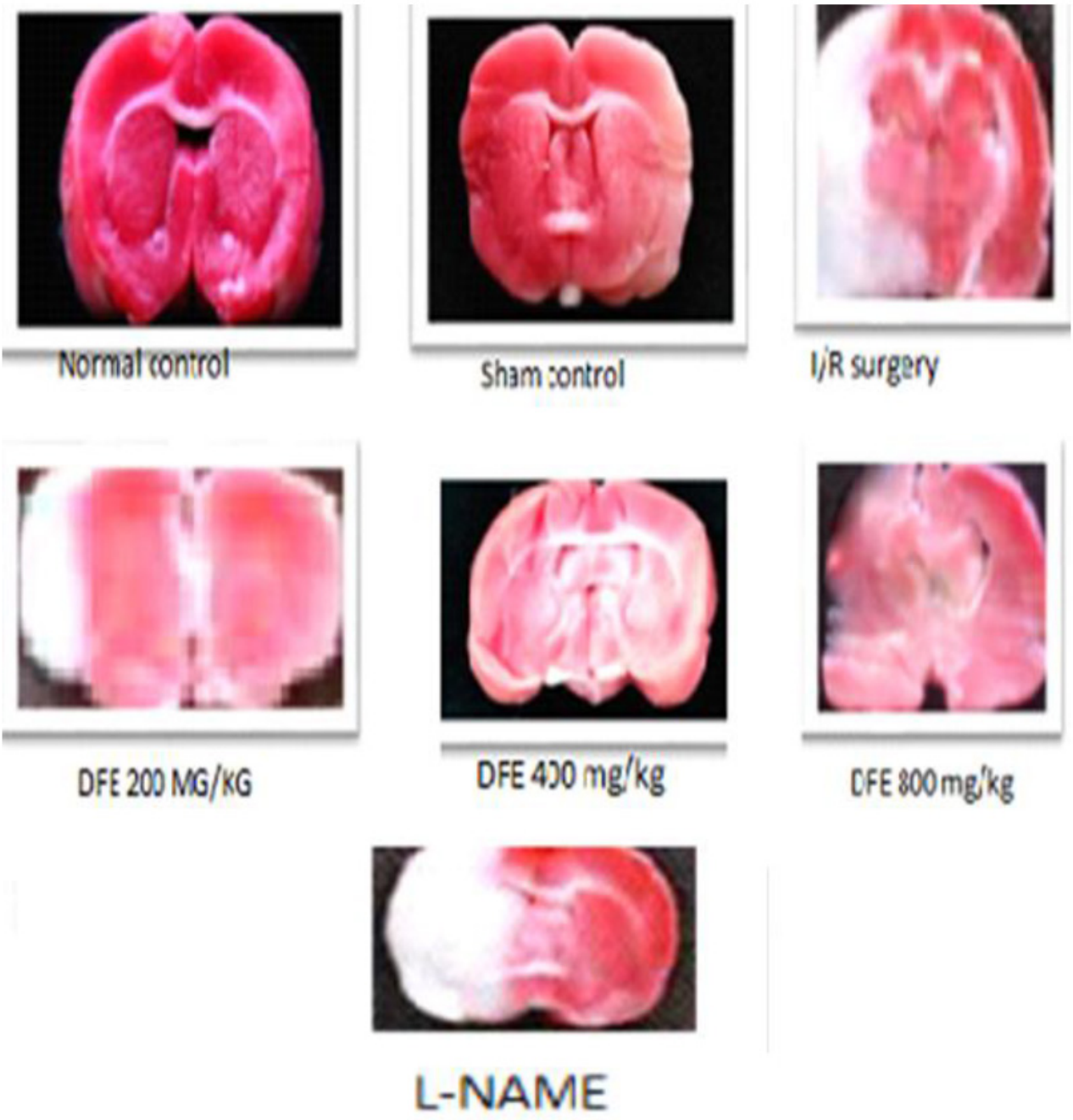
Surgery group showed highly significant infarcted brain area compared to normal control group. The DFE treated groups exhibited dose dependent reduction in infarction volume though the effect produced by 200 mg/kg was not statistically significant. L.NAME treated group also did show any significant effect on infarction volume.

Fig1. Effect of different doses of DFE following pretreatment for 30 days on infarct size in rat brain.



Statistical analysis by one-way ANOVA followed by tukey's test. Values are expressed as mean ± S.E.M (n = 4). ns- statistically non-significant , *<0.05, **p < ***0.01,*** ***p < 0.001 when compared to*** ischemic/reperfusion alone group, #<0.05 when compared to sham operated control.

Fig 2. . Effect of different doses of DFE following pretreatment for 30 days on infarct size in coronal sections of rat brains following TTC staining



Representative TTC stained coronal sections of rat brain slices following bilateral common carotid artery occlusion (BCCAO) for 30 mins and 4 hrs of reperfusion in sham operated control, I/R surgery and DFE and L-NAME pretreated groups followed.

BCCAO occlusion for 30 min caused marked congestion of blood vessels which were further augmented by reperfusion due to lymphocytic proliferation and neuronal necrosis. In the present study, TTC staining (Fig 2) showed a significant increase in cerebral infarction in ischemic reperfusion (I/R) rats as compared to sham control group. DFE treated groups showed dose dependent reduction in the infarction volume with the highest reduction in infarction volume observed with DFE 800 mg/kg. L-NAME, however did not have any significant effect on infarction volume.

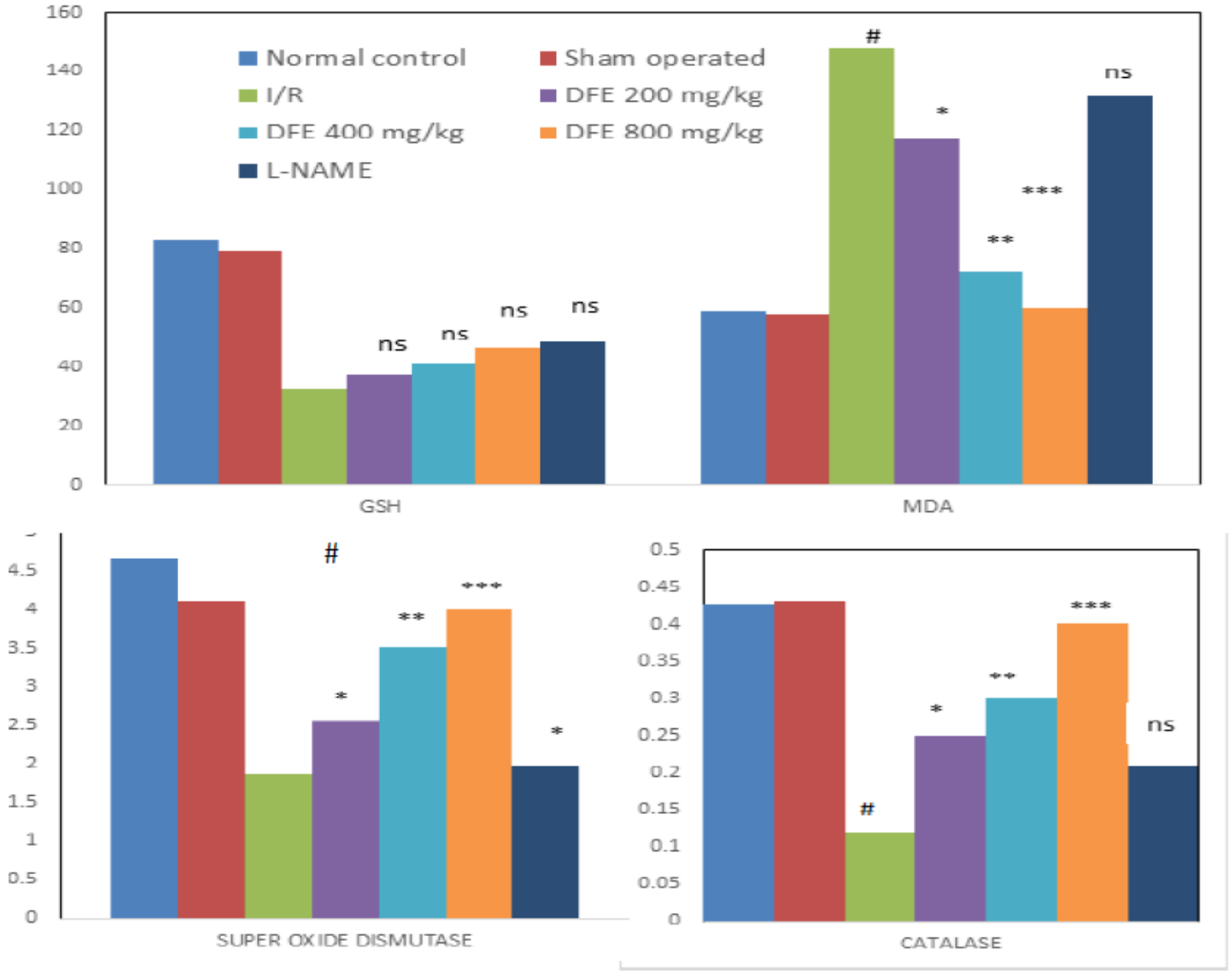
ii. Effect of different doses of DFE on markers of oxidative stress

I/R surgery group showed significant decrease in SOD, catalase, GSH and an increase in MDA levels as compared to the normal control. Treatment of groups 4, 5 and 6 with 200 mg, 400 mg and 800 mg/kg DFE respectively for 30 days resulted in a dose dependent increase in SOD and catalase levels with a very significant effect being observed with DFE 800 mg/kg. DFE group also normalized MDA levels dose dependently. None of the doses of DFE showed any significant effect on GSH. Similarly L-NAME treatment did not affect significantly any of the parameters altered by BACCAO and reperfusion.

Discussion

The present study aimed at investigating the potential beneficial effects of DFE on ischemia-induced oxidative stress as well as histological alterations. The study confirms the previous reports that cerebral postischemic reperfusion is

Fig 3. Effect of 30 days of treatment by DFE on SD, CAT, GSH and MDA following BACCAO and reperfusion.



Effect of different doses of DFE on markers of oxidative stress. Statistical analysis by one-way ANOVA followed by tukey's test. Values are expressed as mean ± S.E.M (n = 4). ns- statistically non significant , *<0.05, **p < 0.01, ***p < 0.001 when compared to ischemic/reperfusion (I/R) alone group, #<0.05 when compared to sham operated control

associated with generation of free radicals. The analysis of biochemical parameters proved that BCCAO followed by reperfusion results in tissue injury. Increased generation of free radicals during injury initiates lipid peroxidation which reflects as increased level of TBARS. Polymorph nuclear leukocytes are known to be involved in cerebral reperfusion injury are found to be accumulated in brain following cerebral ischemia. The activated neutrophils then act as a source of free radicals leading to activation of antioxidant enzyme systems in the brain. These events causes continued interplay of endogenous mechanisms resulting in increased membrane and cell damage [7-9].

The measurement of antioxidant enzymes after reperfusion is important in determining the effectiveness of neuroprotective agents [11-12]. Therefore the antioxidants like CAT, GSH, and SOD, which served as oxidative indices in brains of the ischemic rats were examined. Decrease in the levels of CAT, GSH, and SOD were observed in brains of the ischemic rats that indicates participation of superoxide radical which is a known toxic hydroxyl radical with H_2O_2 (Haber–Weiss reaction). These in turn decrease the SOD through a modification in histidine residue located in the active site of the enzyme. On the other hand this overproduction of H_2O_2 can be inactivated by catalase enzyme and thereby leading to reduction in CAT causing depletion in these enzyme levels in brains of ischemic rats. This is due to consumption of these defensive enzymes due to scavenging of the rapidly generated hydrogen peroxide and lipid peroxides. In the present study date fruit extract was found to elevate the activity of SOD and CAT in ischemic brain thereby reducing infarction volume. The severe neuronal loss (cerebral infarctions) was observed in the histopathological brain sections of the I/R group. The reduction in infarct size was very significantly prevented by the administration of DFE at a dose of 800 mg/kg. The result of the present study showed no significant effect by L-NAME (a NO synthase inhibitor), treated group contradicting the many published reports [17-20].

Conclusion:

Current findings indicate that oxidative stress is a predictable outcome of bilateral common carotid artery ligation induced ischemia/reperfusion. The biomarkers of this offending pathological mechanisms was effectively alleviated by DFE by restoring the levels of antioxidant enzymes and lowering the level of MDA in the brain and validated its worth as being an effective neuroprotective agent. These findings suggest a potential role of DFE as a protective agent against oxidative stress induced cellular damage like in stroke.

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