Int J Diabetes & Metabolism (2009) 17:105-109

Alloxan diabetes-induced oxidative stress and impairment of oxidative defense system in rat brain: neuroprotective effects of *cichorium intybus*

Nayeemunnisa Ahmed
Department of Pharmacology, Al-Ameen College of Pharmacy, Bangalore, India

Abstract
Background/Objectives: Diabetes mellitus impairs glucose homeostasis causing neurological disorders due to perturbation in utilization of glucose. The mechanisms responsible for failure of glycaemic control in diabetes need to be thoroughly elucidated and hence the present study was initiated. **Material and Methods**: Diabetes was induced in albino rat models with alloxan monohydrate (40 mg/Kg intravenously). Oxidative damage, impairment of oxidative defense and neuronal activity were investigated in cerebral hemispheres 48 h after alloxan administration. **Results:** Diabetes caused an elevation ($p < 0.001$) of blood glucose, protein carbonyl content (PrC) and lipid peroxidation. The brain level of the antioxidant enzyme, catalase (CAT), reduced glutathione (GSH) and acetyl cholinesterase (AChE) exhibited significant decline in alloxan-diabetes. Feeding with dried powder leaves of *Cihorium intybus* decreased blood glucose level to near normal level. **Conclusion**: Impaired glucose metabolism in the brain was the key factor responsible for the elevated oxidative damage leading to brain dysfunction in diabetes.

Keywords: *Alloxan diabetes, brain dysfunction, glucose homeostasis, Oxidative damage, Neuroprotection, Cichorium intybus, GSH, PrC, Catalase, Lipid peroxidation, Isozyme induction, AChE, Albino rats.*

Introduction

A number of plant extracts have been shown to be effective in age-related nervous disorders. However, experimental evidence on the protective and curative effects of *Cichorium intybus* (Chicory, Indian Kasni) in age-related diabetes in the brain is entirely lacking, though the cardioprotective effects of chicory treatment have been reported. [1] Keeping this in view, the present study examined the protective effects of *Cichorium intybus* in short and long-term diabetes as *Cichorium intybus* is used in Indian unani system of medicine to treat diabetes-induced hyperglycaemia and metabolic disorders.

Material and Methods

Plant Material

Fresh *Cichorium intybus* leaves were collected from chicory farm in Vaniyambadi, Tamilnadu, India and shade dried for 21 days. The dried leaves were finely powdered and kept in air tight glass containers before use. The plant was authenticated by a scientist at Bangalore University, Bangalore and a voucher specimen was deposited in departmental herbarium. Institute-bred Wistar rats ranging in weight from 250-285 were used for the study. These animals were housed in polypropylene cages at laboratory temperature (26 ± 2^0C) and fed standard pellet diet (Hindustan Lever Ltd., Bombay, India). Water was available *ad libitum*. The study protocol was approved by Institutional Animal Ethics Committee.

Received on: 31/12/2007
Accepted on: 15/10/2009

Correspondence to: Nayeemunnisa, Department of Pharmacology, Al-Ameen College of Pharmacy, Bangalore -27, India.
Email: nayeema201@yahoo.com

Induction of Diabetes

Diabetes was induced by intravenous injection of a freshly prepared aqueous solution of alloxan monohydrate (40mg/kg body weight). Blood was extracted from the tail vein for glucose analysis and rats with fasting glucose ranging from 210-220 mg/dl, showing clear signs of polyuria, polyphagia and polydipsia were considered diabetic and were analyzed 48 hours after alloxan treatment. Animals with fasting blood glucose less than 200 mg/dl were rejected.

The rats were divided into the following groups of 9 animals each:

Group I: Controls
Group II: Diabetic
Group III: Diabetic + *Cichorium intybus* treated

500 mg of shade-dried, *Cichorium intybus* leaf powder mixed with restricted pellet diet was given to each animal in group III for 30 days and were then rendered diabetic and decapitated 48 h after alloxan administration.
The brains were quickly removed and washed in ice-cold saline. Cerebral hemispheres were dissected out and carefully separated at 0^0C with bent forceps and scalpel, weighed in an electric balance in mammalian Ringer solution and immediately used for biochemical analyses.
Glucose levels were measured in blood and cerebral hemispheres by colorimetric method using standard analytical kit. Lipid peroxidation[2] was determined by

measuring malondialdehyde (MDA) formed by thiobarbituric acid (TBA) reaction. Catalase (CAT) activity was estimated by measuring the rate of decomposition of H_2O_2. Ten percent (W/V) tissue homogenate was prepared, centrifuged for 90 min and the resulting supernatant was used for determining CAT activity.[3] Agar gel electrophoresis was employed to study isoforms of CAT and the relative activity measured. Ten percent (W/V) brain tissue homogenate in 0.1M phosphate buffer pH 7.0 was prepared and centrifuged at 2000 rpm for 30 min. 20 µl of the clear supernatant was spotted on the filter paper strip embedded in centrally made slots of the 2.5% solidified agar gel spread over 245x70mm glass plate with 3 mm height frame. Constant current of 10mA (Electroselenium Ltd, Essex, England) was employed**.** Electrophoresis was carried out at 7^0 C for 16 hours. After the run, the gels were removed washed in phosphate buffer and soaked in 0.6M H_2O_2 in 0.2M phosphate buffer (pH 7). In few minutes at the region where CAT has migrated, O_2 bubbles were liberated due to enzymatic hydrolysis of H_2O_2. As the bubbles accumulated a pearly granular region became visible on the gel representing activity bands. Cerebral protein carbonyl content (PrC) and glutathione (GSH) levels were determined according to previously reported methods.[4, 5]

AChE activity was determined colorimetrically.[6] Isoenzymatic specturm of AChE was resolved by polyacrylamide gel electrophoresis, as develop by Davis[7] and Ornstein [8]. One hundred percent (w/v) homogenates of brain tissue were prepared in deionized distilled water and centrifuged at 7000 rpm for 1 h and the supernatants were used for electrophoresis.

Gels were prepared by polymerization of acrylamide monomers. Raymond's buffer system (pH 8.5) was used.[9] Ten micro litres of the test samples were spotted on the gel for electrophoresis. The tubes were run at 6 million amps per tube for 2 h using Raymond's buffer system. After the electrophoretic run the gels were incubated for AChE activity and stained.

Data were statistically analyzed by student's '*t*' test. $p<0.05$ was considered significant.

Results

Blood sugar levels in alloxan diabetic aged rats increased to 220mg/dl. Feeding with dried powder of *Cihorium intybus* (Chicory) leaves along with standard pellet diet decreased ($p<0.01$) the blood glucose level and reversed it to near normal level (85-100mg/dl). Brain glucose also enhanced during diabetes and in chicory-fed animals, a decline in glucose content was observed (Table 1).

Ageing diabetic rats exhibited increased MDA levels ($p<0.001$) compared to control rats (Table 2). Administration of *Cichorium intybus* reduced MDA levels ($p<0.001$) and increased glutathione content ($p<0.01$) which otherwise had decreased in comparison to control rats (Table 2). *Cichorium intybus* enhanced CAT activity ($p<0.01$) in the brain of diabetic rats (Table 2). AChE activity of ageing rat brains declined as a function of diabetes (Table 2). The isozyme profile of diabetic animals exhibited qualitatively different 2 isoforms in controls, a single form in diabetics and restoration to near normal pattern on treatment with *Cichorium intybus* (Fig. 3).

Table 2 shows the alterations occurring in LPO, CAT and AChE in different groups of rats. The brain LPO increased significantly in diabetic rats (Group 2). The anti-diabetic treatment with *Cichorium intybus* (chicory) decreased LPO and significantly enhanced CAT activity compared to diabetic aged diabetic rats (Group 2, Table2). Glutathione and sulphydyl (-SH) levels were significantly depleted in diabetes (Fig.1 and Table 3).

Table 1: Changes in brain glucose levels in diabetic rats

Brain Region	Control	diabetic	Percentage change
Cerebrum	2.02 ± 0.02	2.70 ± 0.05	35.0*
Cerebellum	1.49 ± 0.04	0.90 ± 0.03	-40.0**

Values (µM of glucose /gm) are mean ± S.D of 5 observations.
** $p<0.01$ * $p<0.05$ (control versus diabetic)

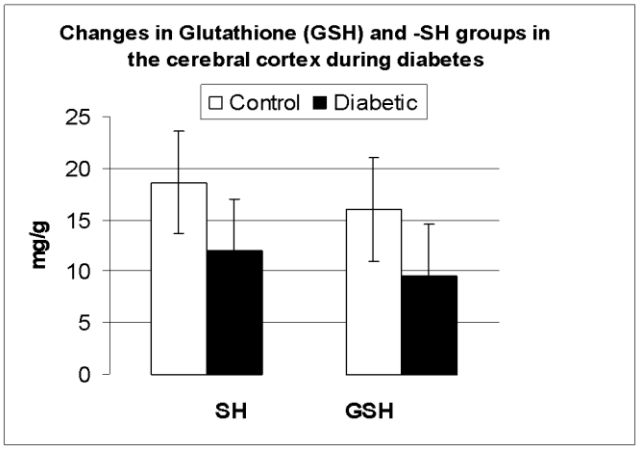


Figure 1 shows a reduced GSH and –SH on the cerebral cortex of diabetic rats. Values are mean ± S.D. of 9 individual observations (n=7).

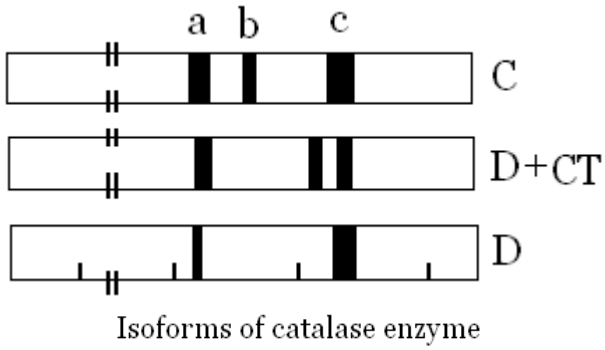


Figure 2: Isoforms of catalase of cerebral cortex resolved on agar gel plates (245 x 70 mm glass plates; 3 mm height frame) at constant current of 10mA for 16 h. The pattern was resolved from 4 runs. C= Control; D = Diabetic; D+CT=Diabetic fed on Chicory.

Table 2: Effect of different treatments on lipid peroxidation (LPO), catalase (CAT) activity and acetylcholinesterase (AChE) activity in the brain of aged rats with diabetes

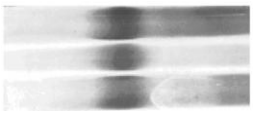
Groups	LPO CBrH 6 months	LPO CBrH 22 months	AChE CBrH 6 months	AChE CBrH 22 months	Catalase CBrH 6 months	Catalase CBrH 22 months
Control	180.4 +/- 8.7	261.5 +/- 7.0	5.9 +/-0.4	3.8 +/- 0.3	4.4 +/- 0.4	3.7 +/- 0.2
Diabetic***	476.2 +/- 12.0	598.2*** +/- 11.5	3.9 +/- 0.4	2.9*** +/- 0.2	7.3 +/- 0.6	5.9*** +/- 0.5
(Diabetic+ Chicory treated)***	333.5 +/- 11.7	301.1*** +/- 9.2	4.5 +/- 0.6	5.6*** +/- 0.7	15.8 +/- 0.2	15.0*** +/- 1.1

Values are mean ± S.D. of 9 animals per group. LPO expressed as nM of MDA formed/g. CAT activity expressed as nmol H_2O_2 decomposed/min/mg protein. Group 2 was compared with group 1 while group 3 was compared with group 2. ***P<0.001 CBrH=Cerebral hemisphere.

Table 3: Effect of different treatments on lipid peroxidation (LPO), catalase (CAT) activity and acetylcholinesterase (AChE) activity in the brain of aged rats with short- and long-term diabetes.

Groups	PrC mg/g n=7 6 months	PrC mg/g n=6 22 months	ACh mg/g n=5 6 months	ACh mg/g n=5 22 months	GSH mg/g n=9 6 months	GSH Mg/g n=7 22 months
Control	1.8 +/- 0.7	2.1 +/- 0.4	5.9 +/-0.4	3.8 +/- 0.3	16.4 +/- 1.4	10.7 +/- 2.2
Diabetic***	4.6 +/- 0.05	5.8*** +/- 0.1	3.9 +/- 0.4	2.9 +/- 0.2	9.2 +/- 0.6	6.9*** +/- 0.7
(Diabetic + Chicory treated)***	3.2 +/- 0.01	3.0*** +/- 0.02	4.5 +/- 0.6	3.6*** +/- 0.7	11.2 +/- 0.2	9.9*** +/- 1.1

LPO expressed as nM MDA formed/g. CAT activity expressed as m mol H_2O_2 decomposed/g/hr. Group 2 was compared with group 1 while group 3 was compared with group 2. ***P<0.001 CBrH=Cerebral hemisphere. PrC= protein carbonyl content; ACh= Acetylcholine



Iso-1 Iso-2

Figure 3: Photograph of brain AChE isozymes on polyacrylamide gel electrophoresis. Note that 2 isozymes were observed in control (lower lane), one in diabetic (middle lane) and 2 in diabetic rats fed on *Cichorium intybus* (upper lane). Iso-1 = Isozyme-1; Iso-2 =Isozyme-2.

Protein carbonyl content

A significant increase in protein carbonyl content was found in the cerebrum of rats in diabetes compared to controls (Table 3). Figures 2 and 3 show isozyme profile of AChE. It is clear from the figure that diabetes caused an abolition of one form of isozyme. Characteristically, an induction of an isozyme, which had lost its expression due to diabetes was traced in the isoform profile of AChE on administration of *Cichorium intybus* leaves for 10 days (Fig 2). Brain choline acetyltransferase showed significant increase (50.0%) with a concomitant elevation in the content of acetylcholine (59.7%) (Table 2).

Analysis of catalse isoforms revealed an over-expression of CAT activity in diabetic rats treated with *Cichorium intybus* (Fig. 3).

Discussion

The central nervous system (CNS) is highly susceptible to oxidative stress. Most of the reactive oxygen species (ROS)-dependent central nervous disorders have been observed to be actually triggered by the presence of free radicals. Free radical generation during brief periods of cerebral ischaemia has been suggested to induce delayed neuronal death.[7] Antioxidant therapy has proved to be remarkably beneficial to combat ROS-induced injury in the CNS.

Diabetes is associated with a higher oxidative stress. ROS-induced damage to the insulin-producing pancreatic beta-cells induces type 1 diabetes. Antioxidant therapy involving the use of enzymes and metal chelators has been shown to protect against such damage. It has been shown that under physiological conditions glucose may undergo auto-oxidation and contribute to ROS formation.[11] Diabetes-induced oxidative damage is responsible for the changes occurring in the activities of membrane-bound enzymes of significance leading to impaired neuronal activity.

In the present study, diabetes caused significant increase in the level of blood glucose. Cerebral glucose level also exhibited a significant increase after the onset of diabetes. The impaired cerebral glucose uptake and glucose oxidation may account for the failure of cerebral glucose homeostasis.

Our result showed that lipid peroxidative damage of the brain is increased in diabetes. This is associated with a significant increase in protein oxidative injury, as reflected by the significant increase in the PrC content in diabetic brain. Accumulation of oxidized proteins is damaging to cell as protein carbonylation is one of the indices of tissue oxidative stress.[13,14]

In diabetic rats, an elevated lipid peroxidation and protein oxidation is due to enhanced oxidative stress. Treatment with anti-diabetic agent namely, *Cichorium intybus* decreased LPO level, indicating lowered oxidative stress in the brain of diabetic rats fed on *Cichorium intybus*. It also reduced the deleterious PrC content thus safe-guarding the brain from oxidative injury.

The increased lipid peroxidation during diabetes, as found in the present study may be due to the inefficient anti-oxidant system prevalent in diabetes. The elevated lipid peroxidation is responsible for the formation of lipid hydroperoxides in membrane and would result in damage of the membrane structure and inactivation of membrane-bound enzymes. The accumulation of lipid peroxides adds hydrophilic moieties into the hydrophobic phase and thereby brings about changes in the membrane permeability and cell functions.[14] The significant elevation found in protein carbonyl content (PrC), and substantial decline seen in GSH and –SH levels in the cerebrum of the diabetic rats corroborate this reports.

Diabetes-induced oxidative damage[11] is responsible for the changes occurring in activities of membrane-bound enzymes of significance in neuronal activity. Earlier studies have also reported an increase in lipid peroxidation leading to impaired neuronal activity in diabetes mellitus.[12] Treatment with anti-diabetic agent such as *Cichorium intybus* decreased LPO level, indicating lowered oxidative stress in the brain of diabetic rats fed with the plant. Chicory treatment significantly lowered LPO in the brain of diabetic rats and simulated the anti-diabetic response of insulin treatments.

Oxidative stress-induced cytotoxic effects appear to be mediated by a perturbation of intracellular free calcium and thiol homeostasis.[13,14] An early response to oxidative stress is the depletion of cellular soluble and protein-bound thiols (glutathione-GSH) and vulnerable sulphydryl groups. In the present study, the reduction seen in the levels of GSH and -SH in the brain of diabetic rats indicates the toxic impact of oxidative stress in diabetes. *Cichorium intybus* (chicory) enhanced GSH and -SH levels in the brain, indicating that it has acted as an effective antioxidant and hence lowered LPO and enhanced GSH level thus safeguarding the brain diabetes-induced oxidative damage.

Studies on isozymes of CAT revealed two isozymes in diabetic rats (CATa and CATc). On feeding with chicory, 3 isozymal forms CATa, CATb and CATc were detected in the brain of diabetic rats indicating that CATb, which had lost its expression after the onset of diabetes was induced due to chicory treatment. This shows the profound capability of some factor/s in chicory which can induce gene expression leading to over expression of the anti oxidant enzyme CAT so needed to reduce LPO during diabetes.

Acetylcholinesterase (AChE) isoforms

The cholinergic neurotransmitter system analysis showed increased AChE activity in chicory-treated animals. It is clear that AChE activity declined due to abolition of an isozyme during diabetes. An induction of an isozyme which had lost its expression due to diabetes has been traced in the isozymic spectrum of AChE on administration of *Cichorium intybus* leaves for 30 days. This shows that feeding on chicory has enhanced the neuronal efficiency in the wake of attenuating the oxidative damage in the brain of ageing diabetic rats by over expression of an isozymal form of AChE. The cholinergic neurotransmitter system analysis therefore showed increased AChE activity.

Thus, *Cichorium intybus* has offered neuroprotection in diabetes by reducing the oxidative stress, restoring GSH levels and by causing over-expression of the activity of the violent antioxidant enzyme CAT thereby up-regulating the antoxidant defense.

In conclusion, diabetes-induced hyperglycaemic injury accounts for the oxidative damage and down-regulation of the activities of antioxidant and membrane-bound enzymes of functional significance leading to impaired neuronal function. Our study shows that *Cichorium intybus* intake is effective in up-regulating the antioxidant defense mechanism by attenuating LPO and PO. Changes in the cholinergic system, suggest that *Cichorium intybus* may have neuroprotective effect in alloxan-induced diabetes.

Acknowledgements

The author is thankful to All India Council of Technical Education, India for Emeritus Fellowship and to Prof. B.G. Shivananda for encouragement. Sincere interest of my granddaughter, Shaista Farheen in computerizing the figures and the help rendered by Dr. Nalini U.P. in resolving isozymes of catalase is gratefully acknowledged.

References

1. Nayeemunnisa M, Kumuda R. Cardioprotective effects of *Cichorium intybus* in ageing myocardium of albino rats Curr Sci 2003; 84: 941-943.
2. Buege JA, Aust SD, Microsomal lipid peroxidation Methods Enzymol 1978; 52: 302-310.
3. Aebi H.E. In: *Methods in Enzymatic Analysis*, New York, Academic press 1983; 273-302.
4. Levine RL, Garland D, Oliver CN, et al. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 1990; 186:464-478.
5. Tietze F. Enzymatic methods for quantitative determination of nanogram amount of total and oxidized glutathione. Application to mammalian blood and other tissues. Anal Biochem 1969; 27: 502-522.
6. Hestrin S. The reaction of Acetylcholine and others Carboxylic acid derivative with hydroxylamine and its

analytical application. J Biol Chem 1949; 180: 249-261.
7. Davis BJ. Disc Electrophoresis II. Methods and application to human serum proteins. Ann N Y Acad Sci 1964; 121:404-427.
8. Ornstein L. Disc electrophoresis I. Background and theory. Ann N Y Acad Sci 1964; 121:321-349.
9. Raymond S. Acrylamide gel electrophoresis. Ann N Y Acad Sci. 1964; 121:350-365.
10. Kitagawa K, Matsumoto M, Oda T, et al. Free radical generation during brief period of cerebral ischaemia may trigger delayed neuronal death. Neuroscience 1990; 35:551-558.
11. Sen CS. Oxygen toxicity and antioxidants: state of the art. Ind J Physiol Pharmaco 1995; 39: 177-196
12. Gupta A, Hussain M, Chandra R, Kapoor NK. Acetyl homo cysteine Thioactone (Citiolone) protects against restraint stress- induced adverse effects on lipid peroxidation products and catalase activity in the CNS of aged rats. Proc. National, Symposium on quality ageing October 29-31. Bangalore. India.
13. Reed DJ. Status of calcium and thiols in hepatocellular injury by oxidative stress. Semin Liver Dis 1990; 10: 285-292.
14. Pascoe GA, Redd DJ. Cell calcium, vitamin E and the thiol redox system in cystotoxicity. Free Radic Biol Med 1989; 6:209-224.