

Alteration of Glial Fibrillary Acidic Protein Immunoreactivity in Astrocytes of the Cerebellum of Diabetic Rats and Potential Effect of Insulin and Ginger

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Abstract

Background: Diabetes causes increased oxidative stress and changes in vascular reactivity in the brain. Ginger has a wide range of pharmacological properties including anti-inflammatory, anti-microbial, antioxidant, and anti-tumour effects.

Aim of the work: The purpose of this study was to clarify the effect of induced type I diabetes on the cerebellum of albino rat and whether these changes could be prevented by insulin and ginger.

Material and methods: Forty albino rats were divided randomly into 4 groups; control non-diabetic rats; untreated diabetic rats; insulin-treated diabetic rats and combined insulin-ginger treated diabetic rats. After 8 weeks of treatment, animals were sacrificed; cerebellum was dissected, processed and stained with H&E and immunohistochemistry for Glial Fibrillary Acidic Protein (GFAP).

Results: Diabetes caused decrease in the body and cerebellum weights, degeneration of Purkinje cells and decreased expression of GFAP in the astrocytes. Treatment with insulin and ginger decreased the blood glucose level, prevented the loss of body and cerebellar weights and protected the cerebellum against diabetic induced changes. Insulin-ginger treatment prevented the changes of Purkinje cells and increased the expression of GFAP. The combined effect of both insulin and ginger increased the GFAP expression more than each of them separately.

Conclusion: The current results suggested that consumption of ginger together with insulin protected purkinji and glial cells against diabetic induced changes and improved GFAP expression. Thus, ginger could attenuate the neurodegeneration observed in the cerebellum of diabetic rats.

Keywords: Diabetes; Cerebellum; Ginger; GFA

Introduction

Diabetes mellitus (DM) is a chronic, systemic disease that, according to WHO, affects about 135 million individuals worldwide [1]. DM is a common metabolic disorder with well-known serious complications. Diabetic peripheral neuropathy was considered the major complication involving the nervous system, whereas the central nervous system (CNS) was believed to be spared from the direct effects of DM [2]. Lately, it has become obvious that DM may be responsible for CNS complications, with functional and cognitive defects [3].

Diabetes mellitus is a disease characterized by chronic hyperglycaemia which can alter brain glucose metabolism in rodents [4]. In addition, diabetes increases the oxidative stress in the brain [5]. These changes may explain the high incidence of seizure, stroke, and cognitive impairment in case of diabetes [3].

Astrocytes are the stellate-shaped glial cells in the central nervous system, have a major role in supporting neurons, scar formation, development and maintenance of the blood-brain barrier [6]. Astrocytes play a role in regulation of extracellular glutamate levels and production of antioxidant compounds [7]. These functions may hypothesize that altered astrocyte activity may cause CNS pathophysiology associated with diabetes [8].

Glial Fibrillary Acidic Protein (GFAP) is an intermediate cytoskeletal filament protein specific for astrocytes [9]. Glial fibrillary acidic protein immunoreactivity depends on the maturation of astrocytes, growth factors, hormones, disease and genetic disorders [10].

The streptozotocin (STZ)-induced diabetic rat is as an excellent

model to study the cellular and morphological changes in the brain induced by DM. STZ is used to induce DM in experimental animals because of its toxic effects on pancreatic β -cells [11].

Ginger (*zingiber officinale*) is a known species of ginger family (Zingiberaceae). It has been used as herbal medicine in India and China to treat headache and cold. Many studies have shown that long term dietary intake of ginger in human diet as a spice may cause hypoglycemic and hypolipidemic effects [12]. The high content of potassium in ginger protects the body against bone damage, paralysis, may regulate blood pressure and heart beats [13]. All Ginger's major active ingredients, such as zingerone, gingerdiol, zingibrene, gingerols and shogaols, are known to possess anti-oxidant activities [14].

The purpose of this study was to clarify the effect of induced Type I diabetes on the cerebellum of albino rat and whether these changes could be prevented by insulin and ginger. The expression of GFAP in the cerebellum has been analysed to describe the diabetic induced alteration in the morphology and reactivity of radial glial fibres.

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Materials and Methods

Animals

Forty adult male albino rats (13-18 weeks) old weighting 200-250 g were obtained from the Faculty of Pharmacy animal house, Mansoura University. The use of experimental animals was prospectively approved by the Committee at Mansoura University, Faculty of medicine. The animals were housed two or three in a cage at a constant temperature 18°C and humidity 45% on a 12-h light/dark cycle. They had free access to standard diet and drinking water. All the experiments were carried out according to the rules and regulations laid down by the committee on animals' experimentation of Mansoura University. Ginger (zingiber officinale) was obtained from Sigma Aldrich Company.

Experimental protocol

The animals were divided randomly into 4 groups.

Group 1: control non-diabetic (n=10) received vehicle only.

Group 2: STZ-induced, untreated diabetic rats (n=10).

Group 3: STZ-induced diabetic, insulin-treated rats (n=10) which received a single daily subcutaneous injection of protamine zinc insulin, a long-acting insulin, the dose of insulin was carefully adjusted to maintain serum blood glucose levels close to normal levels, approximately 100–200 mg/dl.

Group 4: STZ-induced diabetic, combined insulin and ginger treated rats (n=10), received daily dose of both insulin and ginger. Ginger powder was given in dose of 300 mg/kg every day, added to the food.

Induction of diabetes

Diabetes was induced by a single intraperitoneal (i.p.) injection of STZ (50 mg/ kg, freshly dissolved in 5 mmol/L citrate buffer, pH 4.5) [15]. Two days after STZ treatment, development of diabetes was confirmed by measuring blood glucose levels in tail vein blood samples. The drop of blood was immediately placed onto an ACCU-CHEK glucose test strip and evaluated with the ACCU-CHEK glucose meter (Roche Diagnostic Corporation, Indianapolis, IN). Rats with blood glucose levels of 250 mg/dL or higher were considered to be diabetic.

Processing of the specimen

After 8-week treatment, all animals were weighed. Rats were anesthetized with Ketamine (60 mg/kg i.p.) and perfused intracardially with 0.9% NaCl, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.2. The brain was removed; the cerebellum was separated and weighed. Cerebellum was fixed in the same fixative for 4-6 h and processed for paraffin blocks. Sagittal sections (4-7 µm) were cut and used for H& E and immunohistochemistry stains.

GFAP immunohistochemistry

The tissue sections were deparaffinized and rehydrated. The sections were incubated with GFAP, mouse monoclonal antibody (1:500) (Dako, N-series Ready- to use primary antibody). The immunostaining was amplified and completed by Hoarseradish Peroxidase complex (Dako, REALTM EnVision TM / HRP, Mouse ENV). Sections were developed and visualized using 3,3-diaminobenzidine (Dako, REALTM DAB+Chromogen). The substrate system produced a crisp brown end product at the site of the target antigen. Sections were counterstained with haematoxylin. The sections were then dehydrated in alcohol, cleared in xylene and coverslipped with permount [16].

Quantitative analysis

Slides were digitized using Olympus digital camera installed on Olympus microscope with 1/2X photo adaptor, using 40X objective. The result images were analyzed on Intel Core I3 based computer using Video Test Morphology software (Russia) with a specific built-in routine for immune-histostaining analysis and stain quantification. The system measured the area percentage of GFAP positive expression.

The software routine of quantification includes:

Step 1: Live Images were transferred from the camera to the computer using a u-tech frame grabber. The required images were captured as a snapshot and saved as 1024×768 dpi TIFF format.

Step 2: Automatic image adjustment (including automatic colour balance and contrast) was done to obtain a high contrasted image and a well-defined range of the target stain. That also reduces the background interference with the target stain which may affect the result.

Step 3: The enhanced image with high image details was then subjected to automatic thresholding using the target stained area to adjust the threshold level. That converts the target stained area to a binary red mask that overlay the original image. This area is defined as (ROI) region of interest. The system uses this red mask to calculate the area percentage of the target stain.

Statistical analysis

Statistical analysis was done using computer software SPSS program (statistical package for social science) version 10. All data were expressed as the mean ± SD. All the data obtained were subjected to statistical analysis using independent samples t-test for parametric values and Mann-Whitney test for nonparametric values. The significance level considered was $P \leq 0.05$.

Results

Body weight of the rats

Diabetic animals that experienced 8 weeks duration of the diabetic state showed significant decrease in their weight compared with 8 weeks normal group ($P < 0.05$). Treatment with insulin, combined insulin and ginger caused significant weight gain as compared with the diabetic rats (Graph 1).

Weight of the cerebellum

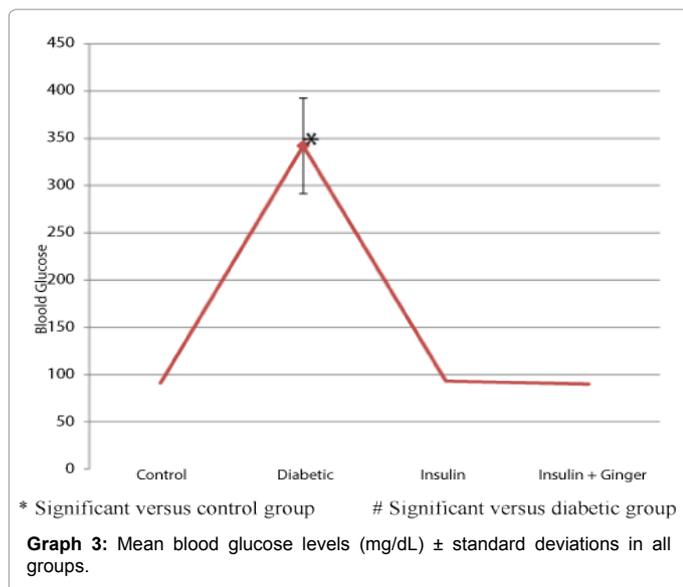
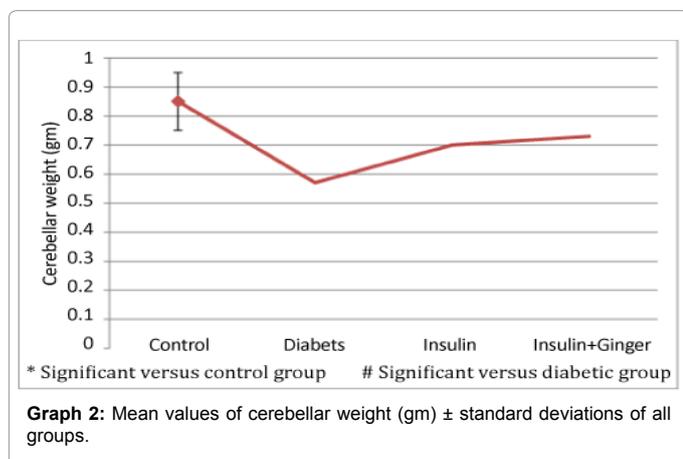
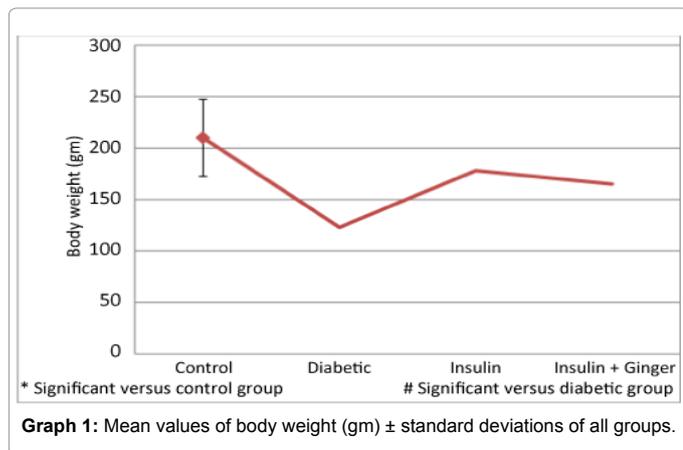
Diabetic animals that experienced 8 weeks duration of the diabetic state showed significant reduction in cerebellar weight compared with the control group. Rats treated with insulin, combined insulin-ginger showed significant increase in cerebellar weight as compared with the diabetic rats (Graph 2).

Blood glucose level

Eight weeks diabetes caused significant increase in blood glucose level as compared with control level. Treatment with insulin, combined insulin-ginger caused significant decrease in blood glucose level as compared with the diabetic rats (Graph 3).

Histological and immunohistochemical findings

Group 1 (control group): In H& E-stained sections, the cerebellar cortex was built up, from outside inside, of the molecular layer, the Purkinje cell layer, the granular layer and the white matter. The Purkinje



cell layer was arranged in one row with typical round or fusiform in shape with prominent nucleolus (Figures 1 and 2).

In immunohistochemistry-stained sections, strong GFAP positive immunostaining was detected in the astrocytes in granular layer and white matter. Deeply stained GFAP positive brown fibres appeared thin with a regular feature running in the granule cell layer (Figure 3).

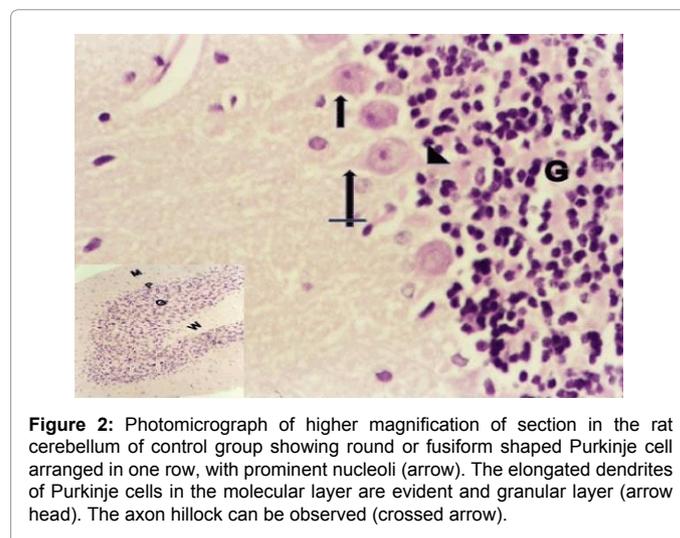
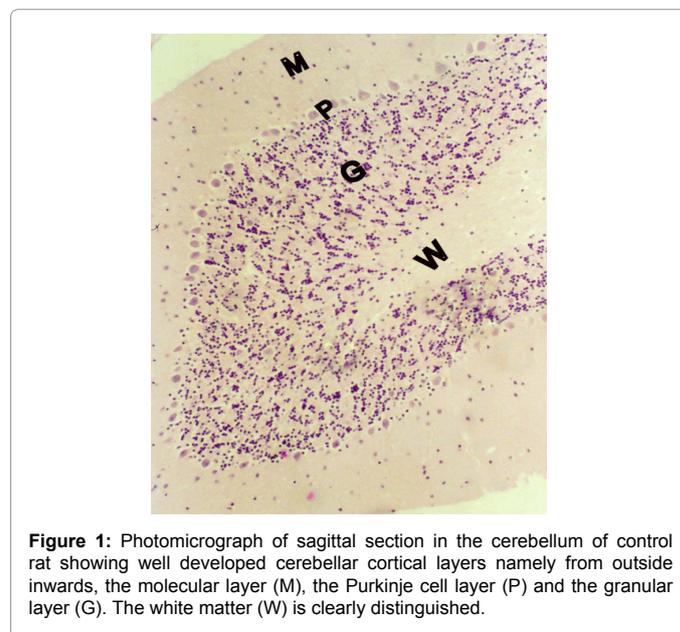
The area percent of GFAP-positive astrocytes was $18.36\% \pm 3$ (Histogram 1).

Group 2 (Uncontrolled diabetic group): In H&E-stained sections, the Purkinje cell was small in size with lightly stained cytoplasm and pyknotic nucleus. In addition, focal loss of Purkinje cells was observed. Dendritic arbors were absent. The granular layer appears disorganised (Figure 4).

Weak GFAP immunoreactivity was detected in astrocytes in the white matter with few, very thin and lightly stained GFAP-positive radial glial fibres (Figure 5).

Morphometric analysis of GFAP-immunoreactive astrocytes in different areas of the gray matter and white matter showed significant decrease in the area percent of GFAP-positive astrocytes in 8-weeks diabetic group ($2.16\% \pm 0.3$) compared with control group (Histogram 1).

Group 3 (Insulin-treated diabetic rats): Haematoxylin and



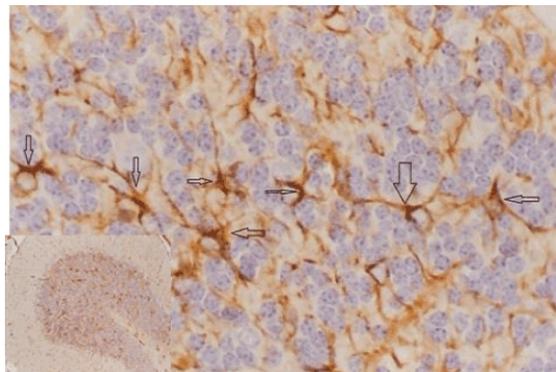
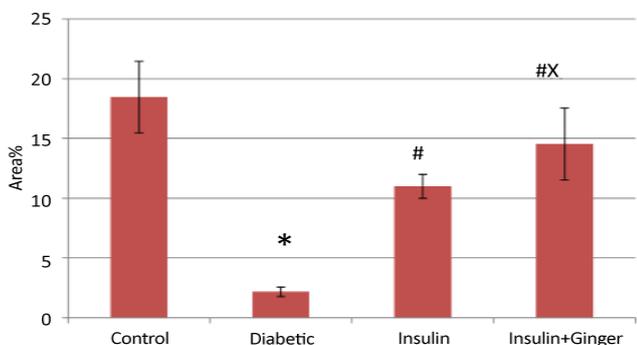


Figure 3: Photomicrograph of sagittal section in the rat cerebellum of control group showing strong GFAP positive stain in the astrocytes in white matter (arrows). GFAP positive radial glial fibres appear very thin and lightly stained.



* Significant versus control group
Significant versus diabetic group
X Significant versus diabetic and insulin group

Histogram 1: Mean area percentage of positive GFAP immunoreactivity (%) ± standard deviations in all groups.

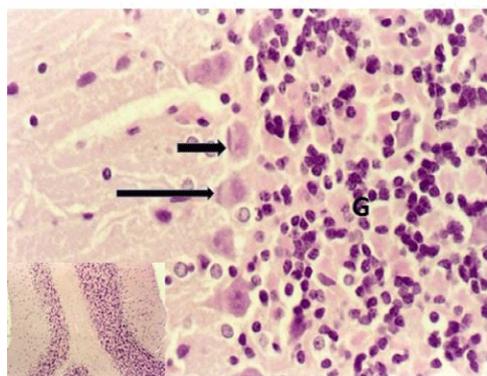


Figure 4: Photomicrograph of higher magnification of section in the rat cerebellum of untreated diabetic group showing small size Purkinje cells with lightly stained cytoplasm and pyknotic nucleus (arrows). The granular layer appears disorganised (G). The dendritic arbors of Purkinji cells are absent.

eosin-stained sections revealed normal Purkinje cell with prominent nucleolus and well developed processes (Figure 6).

In immunohistochemistry stained sections, strong positive GFAP immunoreactivity was detected in the astrocytes of white matter (Figure 7). There was a significant increase in the area percentage of GFAP

immunoreactive astrocyte ($11.86\% \pm 2.3$) especially in white matter compared with diabetic rats (Histogram 1).

Group 4 (Insulin and ginger treated rats): Insulin and ginger treated diabetic group showed normal Purkinje cell with prominent

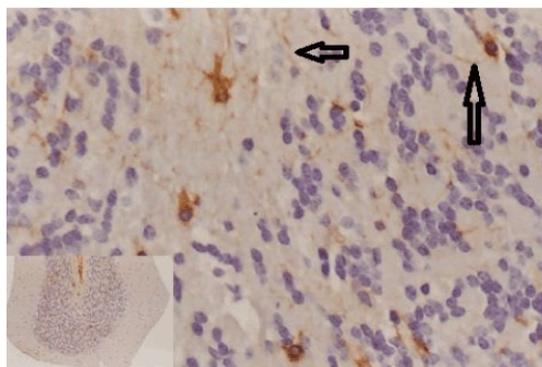


Figure 5: Photomicrograph of sagittal section in the rat cerebellum of untreated diabetic group showing weak GFAP positive stain in the astrocytes in white matter. Few GFAP positive radial glial fibres appear very thin, and lightly stained (arrows).

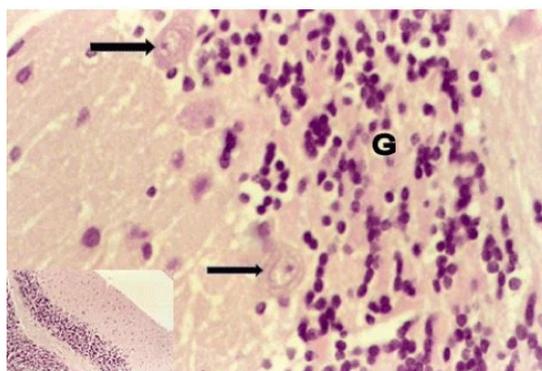


Figure 6: Photomicrograph of higher magnification of section in the rat cerebellum of insulin treated diabetic group showing normal Purkinje cell with prominent nucleoli and well developed processes (arrow heads).

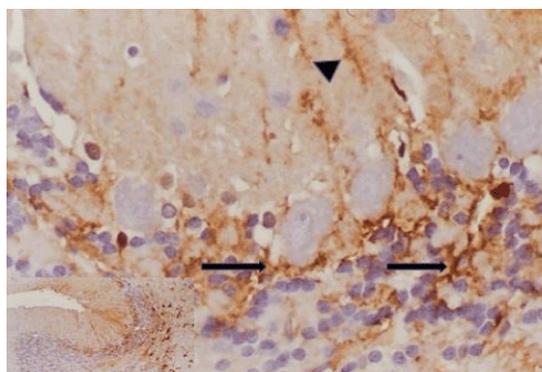


Figure 7: Photomicrograph of sagittal section in the rat cerebellum of insulin treated diabetic group showing strong GFAP positive stain in the astrocytes in granule cell and purkinji cell layers (arrows). Positive radial glial fibres appear in white matter (arrow head).

nucleoli and well developed processes. The granular cell layer appears organized, formed of small dense granule cells (G) (Figure 8).

In immunohistochemistry stained sections, positive GFAP immunoreactivity was detected in the astrocytes in white matter (Figure 9). The cerebellum showed a significant increase in the area percentage of GFAP immunoreactivity ($14.57\% \pm 3.7$) especially in white matter as compared with diabetic rats (Histogram 1).

Discussion

This is the first report, as far as I know, showing that ginger has beneficial effects on changes of GFAP cerebellar expression induced by diabetes. These results raise the possibility that ginger may help to reduce diabetic nervous changes.

In the current study, STZ injection caused significant increase in blood glucose levels in diabetic rats because STZ is specifically cytotoxic to insulin producing pancreatic beta cells. The current study showed that insulin and ginger treatment decreased blood glucose level of diabetic rats to near control. In consistent with the present result, it was documented reduction in the blood glucose levels with administration of ginger after one week of diabetes induction in rats. Maximum effect of ginger was after 42 days of treatment as a hypoglycaemic factor [17].

The diabetic rats showed significant reduction in the body weight

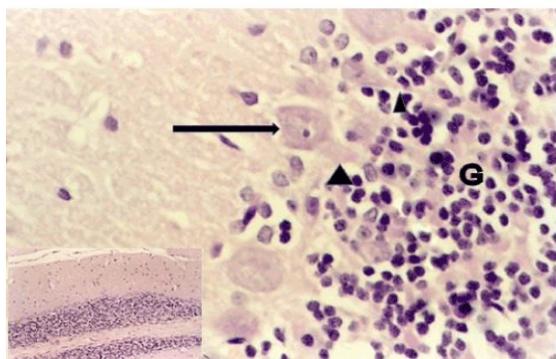


Figure 8: Photomicrograph of higher magnification of section in the rat cerebellum of insulin and ginger treated diabetic group showing normal Purkinje cell with prominent nucleoli (arrow) and well developed processes (arrowheads). The granular cell layer appears organized formed of small dense granule cells (G).

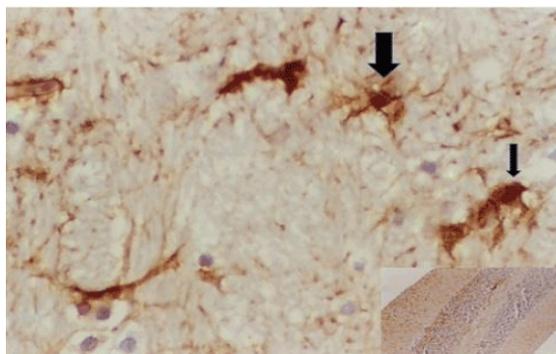


Figure 9: Photomicrograph of sagittal section in the rat cerebellum of insulin- ginger treated diabetic group showing strong GFAP positive stain in the astrocytes. Positive radial glial fibres appear deep brown thick fibres (arrows).

as compared with control rats. While the insulin and ginger treated rats showed significant increase in the body weight as compared with diabetic rats. It was reported that treatment of diabetic rats with insulin prevented diabetic induced reduction in body weight [18].

In the present study, the cerebellar weight was significantly reduced in diabetic rats, while the insulin and combination of both insulin- ginger treatments produced significant increase in the cerebellar weight as compared with diabetic rats. These observations are consistent with the result that insulin therapy prevents diabetic induced reduction in the whole brain weight. No previous study commented on the effect of ginger on cerebellar weight of diabetic rats [19,20].

In the current study, the Purkinje cells of diabetic rats appeared small with lightly stained cytoplasm and absent processes which might indicate degeneration and apoptosis. It was found that two weeks after STZ induction of diabetes in rats, apoptosis of retinal neural cells occurred even in the early stages of diabetes [21]. Also, it was reported that cell death is increased and proliferation decreased in the cerebellum of streptozotocin-induced diabetic rats [22].

The current results showed that insulin treatment prevented Purkinje cell changes induced by diabetes as the cells were normal with prominent nucleolus and well developed processes. This could be explained that insulin is an antiapoptotic agent in diabetic cases through normalization of caspase-3 activity [23].

In the insulin- ginger treated rats, Purkinje cells appeared normal with prominent nucleoli and well developed processes. This finding could be explained by the antioxidant action of ginger which prevents apoptosis caused by oxidative radicles produced by elevated blood glucose level [24]. Similar results were observed in reduction of testicular apoptosis induced by metiram (fungicide) by administration of ginger [25].

The current study revealed a selective decrease in the area percentage of GFAP-immunoreactive astrocytes in the cerebellum of STZ-induced diabetic rats. This finding is in agreement with that of several studies which have shown a significant decrease in GFAP immunoblot signal levels in the hippocampus, cerebellum, corpus callosum and external capsule of STZ-induced diabetic rats [26-28]. GFAP content and immunoreactivity were also reduced in the olfactory bulb of 8-week STZ-diabetic animals as compared with the non-diabetic control [27]. Selective decrease in the number of GFAP-immunoreactive astrocytes in the gray matter of the spinal cords of STZ-induced diabetic rats was documented [28]. The reduction in protein (GFAP) expression in type 1 diabetic rats may contribute to oxidative damage associated with diabetes [29].

In contrast, other studies have shown an increase in the GFAP immunoreactivity in astrocytes of diabetic tissues. In humans, diabetic retinas showed GFAP induction in Muller cells and ganglion cell layers [30], GFAP immunostaining increased in the hippocampus of STZ treated mice [31]. These contradictory reports could result from differences in the immunohistochemical antibodies used by different investigators and STZ dosages used in diabetic models [26]. These discrepancies could be also due to differences in the time of diabetes evolution because GFAP levels in the cerebellum were found to increase at one week and decrease significantly thereafter [21].

Insulin treatment could reverse changes in glutamate uptake and prevent the diabetes-induced increase in sodium-independent glutamate uptake in the glial preparation [18]. This effect of insulin could improve the antioxidant status of the brain in diabetes. This is in

agreement with the current results as insulin treatment caused increase in the area percent of GFAP immunoreactivity. Rats treated with insulin for 48 hours had increased astrocytic GFAP immunoreactivity as compared with the diabetic rats [30].

Oxidative stress seems to play a major role in neuronal damage. Production of excessive free radical from the oxidation of elevated intracellular glucose levels is involved in the neuronal injury [32]. Ginger is known to be a powerful anti-oxidant agent [14], has hypoglycemic and hypolipidemic effects [12]. In the ginger- and insulin-treated group, there was an increase in expression of GFAP in the cerebellar white matter more than insulin therapy alone. This could be explained that ginger supplementation improve insulin sensitivity and lipid profile in diabetic patients, it may be effective in reduction of secondary complication of diabetes [33].

In conclusion, the current results suggest that diabetes caused glial injury. Consumption of ginger together with insulin protected glial cells and improved GFAP expression. Thus, ginger attenuated neurodegeneration observed in the cerebellum of diabetic rats. The decreased GFAP-positive astrocytes and positive GFAP area percentage of STZ-induced diabetic rats may be associated with altered functional properties of the astrocytes and reduced ability to maintain their role of neuronal support in the CNS. Further research is needed to be performed to elucidate the oxidative stress markers in the diabetic rat cerebellum and the exact mechanism of the increased GFAP expression caused by ginger treatment.

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