Original Article

Cerebrolysin attenuates diabetic – induced synopathy in hippocampus of albino rats

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ABSTRACT

Background: Cognitive dysfunction is one of the central nervous system complications of diabetes.
Aim of work: The aim of the present study is to investigate cerebrolysin (Cbl) therapy neuroprotective influence on synaptopathy that diabetes causes in the hippocampus.
Material and Methods: Twenty four male albino rats were randomly distributed among four groups (each group 6 rats); control, diabetic, cerebrolysin (Cbl) and diabetic administered cerebrolysin (diabetes+Cbl). Part of cerebrum of rats were taken after 8 weeks from all groups and the samples were collected for histological and histochemical evaluation using cresyl violet, hematoxylin and eosin and immunohistochemical staining with glial fibillary acidic protein (GFAP) for fibrosis, synaptophysin for synaptogenesis evaluation.
Results: Treatment of streptozotocin-induced diabetic rats with cerebrolysin showed minimal pathological changes than that reported in the diabetic group as detected by mild neuronal injury in the granular layer of the Cornu Ammonis and normal molecular layer. Cresyl violet stain showed mild increase in Nissl granules but less than in the control group. Moreover, diabetic rats receiving Cbl showed significant decrease in the area fraction of GFA immunoreactivity but have significant increase in Synaptophysin manifestation.
Conclusion: Cerebrolysin has the ability to protect against changes caused by diabetes in the hippocampus through reducing gliosis but significantly improving cognitive dysfunction by improving synopathy.

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Key Words: Cerebrolysin, Diabetes, GFAP, hippocampus, synaptophysin.

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INTRODUCTION

Diabetes mellitus (DM) is characterized by increase of the blood sugar level. Decrease of insulin secretion or failure to respond to the action of insulin or both is the cause of the disorder[1]. Variations in the proteins, lipid metabolism and carbohydrates are factors that distinguished diabetes[2]. Multiple organ systems are affected by diabetes, especially the central nervous system. Diabetic patients are more liable to cognitive dysfunction[3]. The mechanism by which DM deteriorates the function of the brain is still not well understood[4]. The cognitive dysfunction that occurs as a complication is still needed to be appropriately diagnosed & treated.

The hippocampal formation (consisted of the hippocampus proper, the dentate gyrus and the subiculum) is the brain area concerning with many forms of learning and memory[5] and is particularly sensitive to changes in glucose homeostasis[6]. Many experimental researches focused on neural loss in diabetic animals[7]. It has been found that there was dramatic reduction in the cell proliferation in the dentate gyrus in diabetic rat models[8]. Moreover, analysis of behavioral performance and hippocampal synaptic plasticity have informed variable results in the various experimental models of diabetes. While a number of studies stated that there was a reduction in the

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performance of water maze performance and tests of passive avoidance[9], others reported that these measures were unchanged[10].

The glial cell types, astrocyte and microglia, play different roles in the function of the CNS as scavenging dead cell debris, regulation of the innate immunity, maintenance and regulation of synaptic transportation and integration of formation of neurons[11,12]. These glial cells react to neuronal damage insult by over expression of the glial fibillary acidic protein (GFAP). This phenomenon protects the CNS cells through the uptake of oxcitotoxic glutamate, the production of the antioxidant glutathione and the neuroprotective adenosine.

Synaptic vesicles contain many proteins including synaptophysin[13]. Synaptophysin has four transmembrane domains including synaptoporin and synaptogyrin[14]. Synaptophysin is distributed in all neural synapse and has many roles including formation of synaptic vesicle membrane[15]. Diabetes mellitus causes synaptic loss and damage in the hippocampus by affecting the synaptic plasticity[16].

Cerebrolysin is a neuroprotective anti-inflammatory mixture isolated from pig brain tissue[17]. Cerebrolysin was first used in 1973[18] as a hydrostate in patients with cerebral arteriosclerosis. It has been recommended for several types of neuronal degeneration disorders[19,20] and organic mental disorders[21]. It was approved that the antioxidant properties of cerebrolysin are approximately 300 times than that of vitamin B[22].

The aim of the present research was to study the expression of synaptophysin and GFAP in the hippocampus of diabetic rat by immunohistochemical technique. The study also aimed to investigate the protective effect of cerebrolysin on the possible changes in the hippocampus of diabetic rat.

MATERIAL AND METHODS

Experimental animals:

A total of 24 male albino rats 5-6 months old rats, collected from MERC (Mansoura Experimental Research Centre, Egypt), weighting 200-250 g were used in this study. Rats were housed in special wire cage, in a constant temperature (22-24 C), relative humidity and light controlled room on an alternating 12:12 h light-dark cycle. The rats were fed with water and standard diet ad libitum. All protocols of the current study were approved by the Institutional Review Board (IRB) of Mansoura Faculty of Medicine.

Diabetic model induction:

Rats were randomly subdivided into four equal groups (each with six rats) as follow: control, cerebrolysin (Cbl), diabetic, and diabetic treated with cerebrolysin (diabetes+Cbl).

The animal model of diabetes was created via single intraperitoneal injection of 60 mg/kg streptozotocin (STZ, Sigma, St Louis, MO, USA) dissolved in 0.1 citrate buffer (pH. 4.5). A sample of rat tail vein was checked for blood glucose from diabetic and diabetic treated with Cbl groups, 72 hours after STZ injection using Accuchek sensor comfort (Roche Diagnostics, Berlin, Germany). Rats were confirmed to be diabetic when fasting blood glucose (FBG) was > (250 mg/dl) for 2 consecutive days[23]. For continuous assessment of blood glucose level, blood samples were taken every week[24]. After eight weeks from induction of diabetes, all animals were weighed.

Cerebrolysin (Ever Pharma, Germany) was administrated in a dose of 5mg/kg/day to the cerebrolysin group and the 'diabetes + cerebrolysin' groups for eight weeks[25]. This chosen dose of cerbrolysin corresponds to the neuroprotective dose in nerve injuries[26].

Control rats were injected with citrate buffer. At the assigned time, the animals were sacrificed under deep anesthesia using xylazine (15mg/kg) and ketamine (90mg/kg). The cerebrum was removed, fixed in 10% formalin, prepared for paraffin section and cut at 5 microns for histopathological and immunohistochemical examination.

Histological and histochemical stains:

Specimens of the cerebrum were taken, prepared for paraffin section and cut at 5 micron and stained with hematoxylin and eosin stains (Hx & E) for general morphological changes of the Cornu Ammonis of the hippocampus of the rat cerebrum and cresyl violet stain for Nissl's granules.

Immunohistochemical techniques:

Glial fibrillary acidic protein (GFAP) primary antibody was used to identify astrocytes (1:400 lab vision)[4], and synaptophysin (1:800 sigma) primary antibody was used to detect the level of synaptogenesis[27]. Brain sections (5 μm) were deparaffinized and hydrated in alcohol solution of descending concentrations. Then, the slides were rinsed with phosphate buffered saline (PBS) and incubated for 15 minutes in H2O2 solution (3%). Slides were washed with PBS twice and incubated in 10% normal goat serum at room temperature. Slides were incubated overnight with primary antibodies. After that the slides were rinsed with PBS, then secondary biotinylated antibody was used to incubate them for 20 min. Then, the enzyme conjugate (Streptavidin-Horseradish Peroxidase) and 3,3, -aminobenzoic acid were applied. Slides were counterstained with hematoxylin and positive staining appeared as brown color.

Quantification of immunohistochemical staining

Five non-overlying fields of the hippocampal regions (200x) for each brain section were captured using digital camera (Olympus). The stained area fraction of GFAP and synaptophysin was counted using ImageJ (NIH) software (version 1.33). All pixels of the grey regions selected were marked by the program. The optical density of these markers (GFAP and synaptophysin) was quantified and the mean grey area was calculated[28]. A measure of the optical density in three consecutive brain sections in every animal was taken and the data were shown as mean ± SD.

Statistical data:

Data processing and analysis was done by SPSS (statistical package of social science) version 17. One-way ANOVA with Tukey post-hoc test was used. $P < 0.05$ was considered significant.

RESULTS

The body weight and blood glucose in different groups:

Eight weeks after STZ injection, the diabetic rats showed highly significant reduction in the body weight as compared to the control. Diabetic rats treated with cerebrolysin showed reduction of body weight but less than that observed in the diabetic rats.

The mean blood glucose levels in the different groups were as follows: 97.4 ± 9.9 in the control group, 326 ± 48.6 in the diabetic group and 286 ± 32.6 in the diabetic + cerebrolysin group. Treatment of diabetic animals with cerebrolysin failed to restore blood glucose level to the control value (Table 1).

The histological and histochemical changes of the hippocampus of different groups:

The hippocampus is formed of hippocampus proper, dentate gyrus and subiculum. The hippocampus proper is formed of Cornu Ammonis.

Hematoxylin & Eosin stained hippocampal section of the control and cerebrolysin groups showed normal granular cells of Cornu Ammonis. The molecular layer was present in the area between compact zones of the cells consists of glial cells, neuronal processes (axon, dendrites), and scattered nerve cells (Fig. 1A, B). Cresyl violet stain revealed cytoplasmic staining of Nissl's granules with normal density (Fig. 2A, B).

In the diabetic group, marked neural injury within the granular layer of the Cornu Ammonis was observed, represented by nuclear pyknosis and disorganization, associated with increase microglia within the molecular and granular cell layers (Fig. 1C). Cresyl violet staining revealed loss of Nissl substance with marked cellular degeneration. (Fig. 2C).

In the diabetic group treated with cerebrolysin, minimal pathological changes were observed as compared to those seen in the diabetic group, in the form of mild neuronal injury in the granular layer of the Cornu Ammonis and normal molecular layer in diabetic rats treated with cerebrolysin (Fig. 1D). Cresyl violet stain showed mild increase in Nissl's granules but were present less than those in the control (Fig. 2D).

Immunohistological changes of GFAP expression in rats of different groups.

Immunohistochemical staining of GFAP in the control and cerebrolysin groups showed mild

positive reaction in the glial cells of molecular layer (Fig. 3A, B). The protoplasmic astrocyte showed moderately stained cell bodies with short process. (Fig. 3A, B)

In hippocampi of diabetic rats, the astrocytes exhibited high positive level of reaction with thick wavy processes (Fig. 3C). The glial fibers increased in number and exhibited intense positive reaction (Fig. 3C).

In hippocampi of diabetic rats treated with cerebrolysin, the granular layer expression of GFAP showed astroglia with thin processes and moderate stained cell bodies (Fig. 3D). The glial fibers in the molecular layer increased in number and have moderate positive immune expression (Fig. 3D).

There was a significant increase (4.6±0.26) in the GFAP positive immunoreactivity area fraction in the diabetic group hippocampi as compared to the control group (0.9±0.1) (Table 2). For the Cbl-treated diabetic group, the GFAP positive immunoreactivity area fraction was decreased (4.3±0.3) when compared to diabetic group. These changes were not statistically significant.

Immunohistological expression of Synaptophysin of rats' hippocampi of different groups.

Synaptophysin expression in the groups of Cbl and control showed homogenous staining of neuronal processes in the molecular layer. The axonal region bodies were always negative to reaction, while in granular layer, the reactivity appeared as clusters (Fig. 4A, B).

Synaptophysin expression was decreased in the diabetic group compared to the control group (Fig. 4C).

In the diabetic + Cbl group, there was marked expression of Synaptophysin immunostaining within the granular and molecular layers compared to diabetic group (Fig. 4D).

The area fraction of synaptophysin immune expression of the hippocampus of diabetic group was significantly decreased (1.367±0.35) compared to the control group (5.467±0.404) (Table 2). In the diabetes+Cbl group the synaptophysin fraction area in the molecular and granular layers are significantly different than that of diabetic group.

Table 1: Means of blood glucose (mg/dL) and body weight (g) in different studied groups

	Control	Cerebrolysin	Diabetic	Diabetic + Cerebrolysin	**P value*
Body weight (g)	296.3 ± 4.6	285.8 ± 1.7	171.6 ± 6.7$^{\#\$}$	195.32 ± 5.4$^{\#\$\dagger}$	*<0.001*
Blood glucose (mg/dL)	97.4 ± 9.9	96.6 ± 8.2	326 ± 48.6$^{\#\$}$	286 ± 32.6$^{\#\$}$	*<0.001*

All data are expressed as mean ± SD. One way ANOVA with Scheffe posthoc test. **p value* of one way ANOVA, # significant vs control group, $ significant vs cerebrolysin group and †significant vs diabetic group. *P ≤ 0.05* is considered significant

Table 2: Means of ROI of GFAP and Snaptophysin in different studied groups

	Control	Cerebrolysin	Diabetic	Diabetic + Cerebrolysin	**P value*
ROI of GFAP	0.9 ± 0.1	2.267 ± 0.31$^{\#}$	4.6 ± 0.26$^{\#\$}$	4.3 ± 0.3$^{\#\$}$	*<0.001*
ROI of synpatophysin	5.467 ± 0.404	6.467 ± 0.31$^{\#}$	1.367 ± 0.35$^{\#\$}$	5.467 ± 0.47$^{\$\dagger}$	*<0.001*

All data are expressed as mean ± SD. One way ANOVA with Scheffe posthoc test. **p value* of one way ANOVA, # significant vs control group, $ significant vs cerebrolysin group and †significant vs diabetic group. *P ≤ 0.05* is considered significant

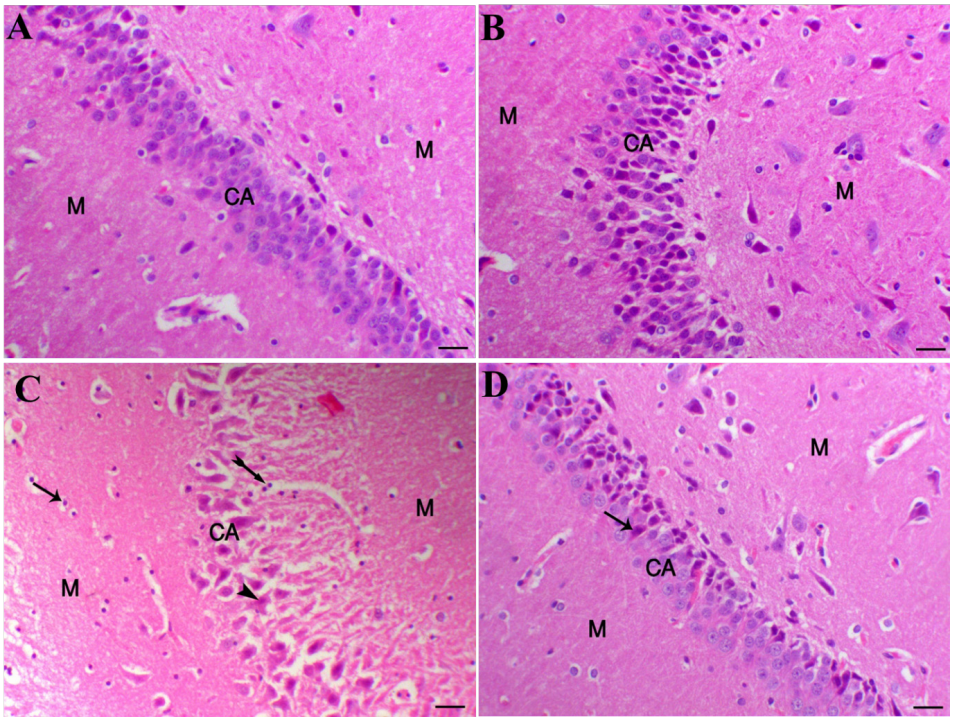

Fig. 1: Histopathological analysis of the hippocampus of control (A), cerebrolysin (B), diabetic (C) and Diabetes + cerebrolysin (D)groups; Hippocampal section of control and cerebrolysin treated animals showing normal granular cells of Cornu Ammonis (CA) and molecular layers (M) (A, B). In diabetic rats (C), there is marked degree of neuronal injury within the granular layer of the Cornu Ammonis (CA) represented by nuclear pyknosis and disorganization (arrowhead) and associated with increase microglia population within the molecular (M) (arrow) and granular cell layers (tailed-arrow). In diabetic rats treated with cerebrolysin (D), mild degree of neuronal injury within the granular layer of the Cornu Ammonis (CA) and normal molecular layer was observed. Haematoxylin and Eosin stain Scale Bar=50 µm.

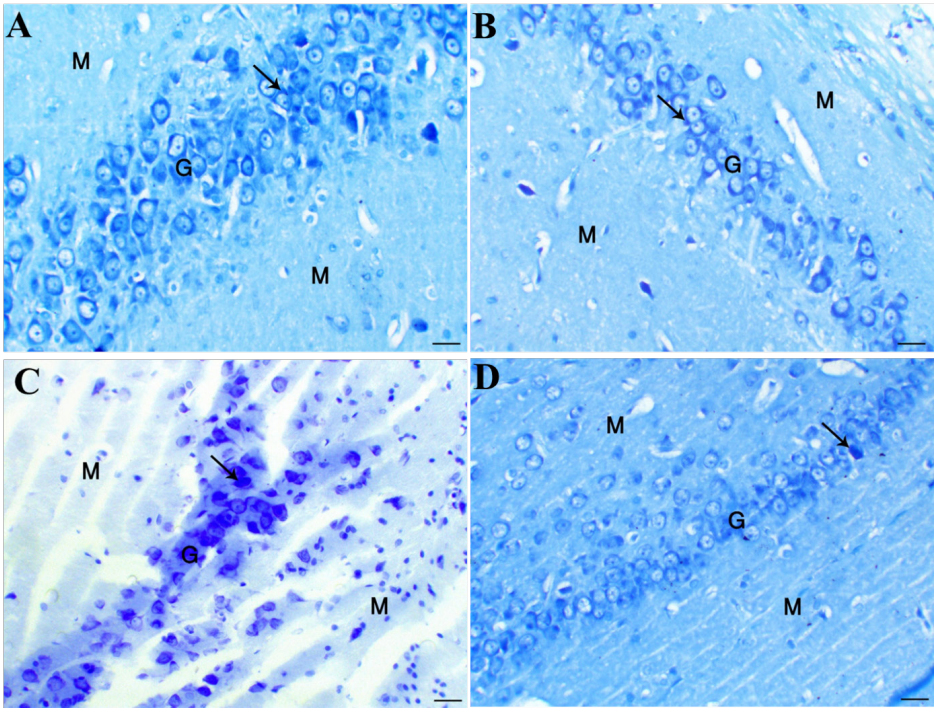

Fig. 2: Cresyl violet stained sections of control (A) and Cbl (B) groups showing Nissl's granules (arrow) (G indicates granular layer, M indicates molecular layer). In diabetic group (C). loss of Nissl's substance with marked cellular degeneration (arrow). In diabetic+Cbl group (D), mild neuronal damage within granular layer (G) and normal molecular layer (M). Cresyl violet stain scale Bar=50 µm

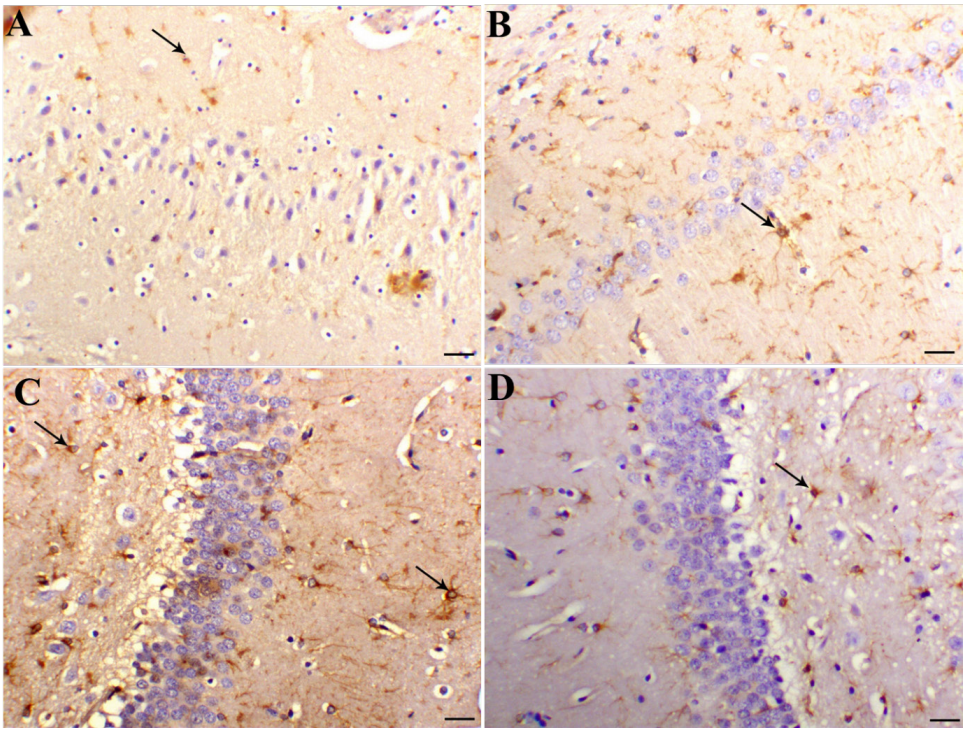

Fig. 3: Immunohistological staining of GFAP in control (A) and Cbl (B) groups showing mild positive reaction in the glial cells of molecular layer (arrow) of the hippocampus. In diabetic group (C) showing astrocytic glial cells with marked immunoreaction of their fibers, cells have thick wavy process: while in diabetic +Cbl group (D) GFAP positive reaction downregulated in comparison to diabetic group. GFAP immunstainig Scale Bar=50 µm

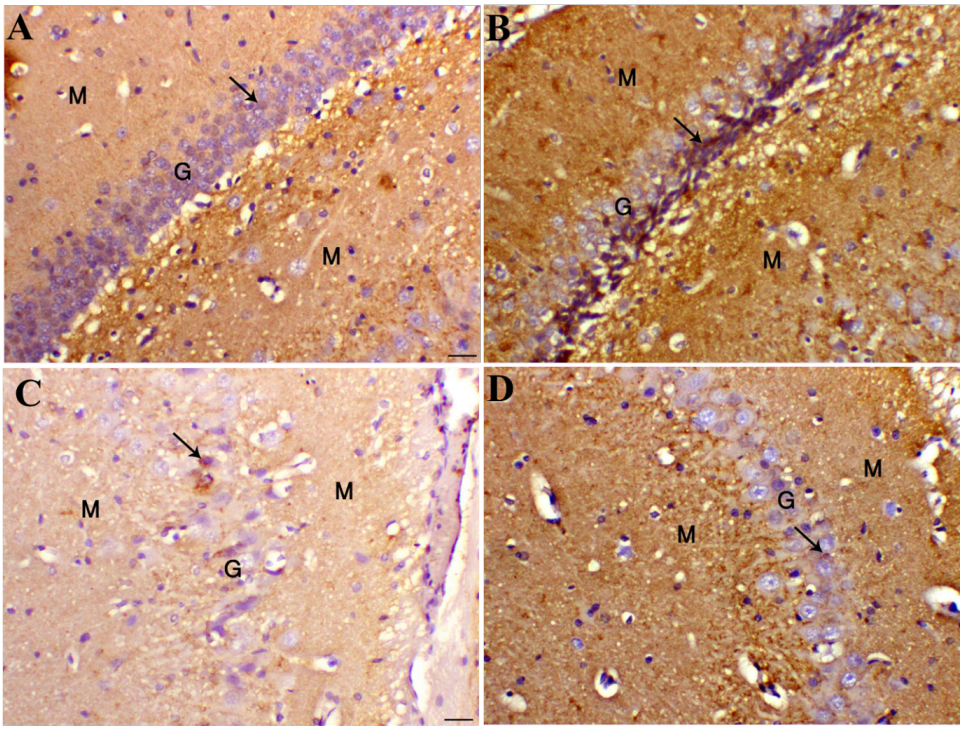

Fig. 4: Immunohistological expression of Synaptophysin of the hippocampus of controls (A), cerebrolysin (B), diabetic (C) and Diabetes + cerebrolysin groups (D). Synaptophysin positive stain observed in cluster (arrow) within the granular layer (G), M indicates molecular layer of hippocampus of control and cerebrolysin groups (A, B). In diabetic group (C) there is marked decrease of synaptophysin expression within the granular (G) and molecular (M) layers (arrow), hippocampus of diabetic+cerebrolysin (D) showing marked reappearance of synaptophysin immunostaining within the granular (G) and molecular (M) layers (arrow) synaptophysin immunstainig Scale Bar=50 µm Scale Bar=50 µm

DISCUSSION

Diabetes mellitus, a disease attributed to be the direct consequences of chronic hyperglycemia, is associated with several detrimental effects on the CNS[29].

Typically, four weeks post STZ administration to rats, diabetes is confirmed by elevation in blood glucose level and elevation of HbA1c%[30]. The brain-derived neurotropic factor which is abundant in the hippocampus plays an important role in hippocampal neurogenesis and consequently cognitive functions[31]. It is known that the brain-derived neurotropic factor is a component of CBL[32], and its administration in obese diabetic mice succeeded to reduce HbA1c intermittently[33]. In the present study, cerebrolysin did not significantly reduce blood glucose in relation to the diabetic group. Moreover, reduction in body weight and retardation of animal growth was observed in the present study. In STZ induced diabetes, the serum level of TNF was reported to decrease, causing inhibition of circulating free fatty acids uptake and accelerating the lipolysis in adipose tissue[34],. This could give reasonable explanation for the reduction in body weight; both former events were impeded by CBL administration in the present work.

In hyperglycemia, the neuronal damage in the brain could be attributed to oxidative stress[3,35] resulting from generation of excess free radicals, via autoxidation of elevated intracellular glucose[36]. The increase in brain glucose level resulted from the increase in the blood glucose level[37]. Singh *et al*.[38] found that glial activation in the hippocampus was due to increase in glycogenolysis which affected the membrane glucose transporters. The pathophysiological mechanisms of hyperglycemia include generation of free radicals[39] which are responsible for the astrocytic reaction in diabetes[40, 41].

The hippocampus forms part of the limbic system and is involved in short-term memory. Damage to the hippocampus can cause a major effect on the learning process of the individual. Astrocytes are the most abundant glial cells in the brain, and they help to fill the spaces, forming scars caused by neuronal damage and also help in repair of damaged cells that can't be regenerated and keep neuronal connection. Therefore, reduction in the number of these glial cells will cause defect in cell to cell connection.

Duarte *et al*.[42] examined the hippocampus of type 2 diabetic mouse model and noticed the increase in glial fibrillary acidic protein (GFAP) immunoreaction (astrogliosis) and the decrease in density of Synaptophysin in the nerve terminal. The present study, discussed the role of hyperglycemia on the GFAP expression as astrogliosis marker and Synaptophysin as synaptogenesis marker. The hippocampus of the diabetic animals showed marked degree of neuronal injury within the granular layer of the Cornu Ammonis represented by nuclear pyknosis, disorganization and was associated with increase microglia population within the molecular and granular cell layers. That result was in agreement with Pamidi and Satheesha Nayak[43]. There was glial activation in the formed astrocytes, hypertrophy with GFAP dense stains in the thick processes in both layers, the granular and molecular. The positive GFAP immunoreactivity area fraction was also increased significantly. Astrocyts play an essential role in the regulation of the barrier of blood brain and as an antioxidant shield. Therefore, destruction of the processes of astrocytes caused oxidative stress[44] and disturbance in the barrier of blood brain function[45]. The reactive gliosis resulted from the effect of diabetes which was in agreement with[2,40,46,47] and may be due to the oxidative stress and glucose level variations since the only brain cell that have receptors of insulin are the astrocytes[2,47]. The present observation disagrees with[48] who reported the decrease in astrocyte GFAP expression. This contradiction in the GFAP expression might be due to the different brain areas and models of the studied animals.

Cerebrolysin produced neuroprotection through its nerve growth factors and polypeptides which intersect the blood brain barrier[49]. Cerebrolysin causes in numerous rat models a reduction neuronal degeneration, as in cases of damage in spinal cord[50,51], Alzheimer's disease in human[52], atrophy of optic nerve[53] and aging[54]. The present study, using cerebrolysin as treatment for diabetic rats insignificantly decreased astrogliosis which resulted from elevated blood sugar level. In contrast,[55] stated that using cerebrolysin for treatment, improve the histopathological hippocampus variations that was caused by elevated blood glucose level. Until now the confirmed mechanism of action of cerebrolysin on CNS is not known.

It may be through minimizing gliosis, oxidative stress and apoptosis[56].

Synaptophysin is a presynaptic protein[57], its immune-expression elevation is a sensitive measurement of synaptogenesis[58]. In the present study, it was found in the hippocampus granular layer a decrease in Synaptophysin immune reaction caused by diabetes. This was confirmed by the Synaptophysin OD which decreased significantly and was consistent with[59] who found that there was decrease in Synaptophysin expression in numerous neurodegenerative diseases. Such changes are responsible for learning and memory changes[60]. The release of neurotransmitters may be the reason for the observed synaptophysin expression decrease in the molecular layer of that study[61,62]. In our study, cerebrolysin treatment significantly increases Synaptophysin OD expression in comparison to that diabetic rats. This increase may be related to reactivation of silent connections, dendritic arborization, synaptogenesis and nerve cell regeneration[63]. Thored *et al*.[64] reported the enhancement role of neurotropic factors on neuroplasticity. Cerebrolysin contains active fragments of neurotropic factors that can protect neurons, stimulate neuroplasticity and enhance neurorecovery process[65,66].

Dong *et al*.[67] stated that the effects of cerebrolysin on the cells of the hippocampus which are affected by elevated blood sugar level depended on the dose, as the dose of the cerebrolysin is increased, the improvement in structural changes caused by diabetes would be better.

In the current study, treatment with cerebrolysin increased the OD of synaptophysin indicating increase in synaptogenesis and therefore improving cognitive function and spatial memory. Coleman *et al*.[68] found that there is a strong relationship between injection of nerve growth factors and synaptophysin synthesis, that fact agreed with[69] who confirmed that synaptogenesis improved after injection of nerve growth factor in the ventricles through increasing the synapses number in the hippocampus

Cerebrolysin proved to have a slight neuroprotective effect on the diabetic rat hippocampus. However, there are some limitations to such result. The dose dependent effect of this drug on the hippocampus of diabetic rats should be recorded by trying different cerebrolysin doses. The core finding of the present study is that CBL halts STZ hippocampal synaptopathy which affects cognitive functions, spatial memory and many shrunken pyramidal cells compared to the non-diabetic groups. This is in agreement with previous studies who reported the failure of cerebrolysin in reversion of hyperglycemia associated effects[43].

CONCLUSIONS

To our knowledge, the present study is pioneer in proving a neuroprotective effect of cerebrolysin on hippocampal synaptopathy caused by diabetes in the form of increase in synaptophysin expression. Cerebrolysin however, failed to decrease the histopathological changes and altered GFAP expression in the diabetic rats. The results provide theoretical support for the use of cerebrolysin in ameliorating the diabetic induced synaptopathy and neuronal changes. However, the therapeutic mechanisms of action of cerebrolysin will require further investigation.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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سيريبروليسين يخفف من الاعتلال العصبى الناجمة عن السكري في الحصين من الفئران البيضاء

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ملخص البحث

الخلفية: الخلل المعرفي هو أحد مضاعفات الجهاز العصبي المركزي لمرض السكري.

الهدف: الهدف من هذه الدراسة هو التحقيق في التأثير الوقائى العصبى للسريبروليسين على اعتلال التشابك العصبي الذي يسببه مرض السكري في الحصين.

الطرق: تم توزيع أربعة وعشرين من الذكور من الفئران البيضاء عشوائيا بين أربع مجموعات (كل مجموعة مكونة من ٦ فئران) مجموعة ضابطة، مجموعة مصابة بمرض السكرى, مجموعة تناولت سربروليسين, مجموعة مصابة بمرض السكري و تم اعطائها سربرولايسين.، جزء من دماغ الفئران اتخذت بعد٨ أسابيع، تم جمع عينات من أجل الفحص النسيجي و النسيجى الكيميائى باستخدام الهيماتوكسيلين وا يوزين وكريزلر البنفسجي و الصبغة المناعية باستخدام بروتين الدبقية الحمضى و سينابتوفيزين لتقييم تكوين التشابك العصبى.

النتائج: علاج الفئران المصابة بالسكرى بواسطة السربروليسين أدى الى ظهور الحد الأدنى من التغيرات المرضية من التي وجدت في المجموعة المصابة بالسكرى في شكل إصابة معتدلة للخلايا العصبية في الطبقة الحبيبية من كورنو أمونيس و طبيعية الطبقة الجزيئية.وقد أظهرت صبغة الكريزلر البنفسجية زيادة طفيفة فى حبيبات نيسل ولكن أقل من المجموعة الضابطة.و أيضا أظهرت الفئران المصابة بالسكرى وتم علاجها بالسربروليسين انخفاض فى منطقة التفاعل المناعى لبروتين الدبقية الحمضى و زيادة فى تفاعل السينابتوفيزين.

الخلاصة: سريبروليسين له القدرة على حماية الحصين من التغيرات الناجمة عن مرض السكرى و ذلك بالحد من الدباق و قد يحسن الخلل المعرفى بتحسين اعتلال التشابك العصبى.