Efficacy of cerebrolysin on dentate gyrus of hippocampus after experimentally induced acute ischemic stroke in adult albino rats (Histological, immunohistochemical and biochemical study)

Heba M. Abdel-Aziz¹, Samah M. Ahmed¹, Maha Z. Mohammed¹ and Hanim M. Abdel-Nour²

¹Department of Histology and Cell Biology, ²Department of Medical Biochemistry, Faculty of Medicine, Zagazig University

ABSTRACT

Background: Acute ischemic stroke (AIS) is a brain medical disorder characterized by the sudden loss of blood circulation to an area of the brain, resulting in a loss of its neurologic function. Cerebrolysin is a mixture of neuropeptides and free amino acids.

Aim of the work: This study aimed to evaluate the role of cerebrolysin in ameliorating the histological, immunohistochemical and biochemical harm in post ischemic stroke and also to assess its dose dependent effect.

Materials and Methods: Thirty adult male rats were divided into 3 equal groups; control, ischemic and post ischemic treated groups. After dissecting hippocampal dentate gyrus prepared sections were stained with hematoxylin and eosin, Erβ and calretinin proteins. Oxidative stress parameters, TNF α, and HSP-70 assay. Also, Gh receptor gene expression and DNA fragmentation test were measured. Immunoperoxidase reactions for GFAP in astrocyte was estimated. Statistical analysis was conducted.

Results: Ischemia group showed decrease number of granule cells with small dark stained nuclei, areas of cell loss and numerous spindle shaped cells in the sub-granular zone. A faint positive immunoreaction for ERβ in nuclei of granular cells and negative reaction for calretinin was detected in granule cells. Antioxidant enzymes in the brain tissue as MDA, TNF α and HSP-70 were significantly elevated in ischemic group compared to control and post ischemic treated groups. Gh receptor gene were decreased, while Fragmentation index of DNA was significantly increased. Administration of 2.5 mg/kg cerebrolysin showed partial improvement, whereas, 5 mg/kg dose displayed more ameliorative effects. Increased GFAP immune-expression in the cytoplasm of astrocytes in ischemic group compared to control and post ischemic treated groups.

Conclusion: Cerebrolysin was effective in experimentally induced AIS (Acute ischemic stroke) in a dose-dependent manner as proved by improving the histological structure, immunohistochemical reactions and biochemical parameters of the dentate gyrus of hippocampus in adult male albino rats.

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Key Words: Cerebrolysin, ghrelin, histology, ischemia, oxidative stress.

Corresponding Author: Heba M. Abdel-Aziz, Lecturer of Histology and Cell Biology, Faculty of Medicine, Zagazig University, Zagazig, Egypt, Tel.: +20 1281547665, E-mail: hebaeraky37@gmail.com

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INTRODUCTION

Acute ischaemic stroke (AIS) is a major cause of death and disability all over the world¹. It is the third cause of death, after heart diseases and cancer in major industrialized countries². The cerebral ischemia results from a temporary or permanent reduction of cerebral blood flow that may lead to both acute or chronic dysfunctions in the central nervous system (CNS)³. Acute cerebral ischaemia caused mostly by thrombosis in cerebral vessels or embolism from large blood vessels or even from systemic hypo perfusion⁴. Chronic cerebral hypoperfusion is a major cause of dementia and Alzheimer’s disease and can a result from hypertension, diabetes, generalized atherosclerosis and smoking⁴. Throughout cerebral ischemia, tissue damage results from diverse mechanisms with central involvement of inflammation, oxidative stress, free radicals overproduction, resulting in an activation of transcription factors and alteration in gene expression⁵,⁶. Additionally, a highly pleiotropic inflammatory cytokines as TNF-α expected to augment or discourage cellular survival through activation of receptor-mediated signal transduction⁷.

The hippocampal system consists of the dentate gyrus, cornu ammonis (CA) fields and the subiculum. Dentate gyrus has three layers; molecular, granule cell and polymorphic layers. The sub-granular zone (SGZ) is one of the stem-cell-containing niches in the adult mammalian brain and present at the interface between the hilus and the granular
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Cell layer. It is estimated that approximately 100 to 150 neurons are generated per day in SGZ of adult rodents. The milieu environment of it allows neuronal stem cell (NSC) proliferation and promoting the differentiation of dentate granule neurons. Adult-born dentate granule neurons pass through several consecutive developmental stages before they become functionally integrated into the hippocampal circuitry[11]. Radial glia-like cells (RGLs, Type 1 or B-cell) express nestin and GFAP and have prominent radial processes are thought to represent the NSC population and can generate intermediate progenitor cells (IPCs, type 2 cell or D- cells) that proliferate at a high rate[12]. These type 2 cells can give rise to neuroblasts (type 3) that subsequently differentiate into mature dentate granule neurons. Newly born neurons are located at the border of the subgranular zone–granular cell layer. When these neurons mature, they become positive for NeuN and calbindin[13].

Estrogen regulates the synaptic plasticity and physiology of the hippocampus. It is demonstrated that its on hippocampal are mediated through ERß[14]. Calretinin is a calcium-binding protein which was shown to be present in many brain regions[15]. Regulation of calcium homeostasis may be a key in the protection of the brain from injury[16].

Ghrelin, a gastrointestinal peptide with a major role in regulating feeding and metabolism. It has recently been investigated for its neuroprotective effects and useful therapeutic role in protecting the brain against injury[17].

Amelioration of cerebral ischemia injury is one of the major problems of experimental medicine and biology[18]. Since approval by the FDA in 1996, intravenous administration of recombinant tissue plasminogen activator (rtPA) which dissolves the obstructive clot remains the only treatment for patients within 4.5 hours of stroke onset. However, only a small percentage of patients with ischaemic stroke are eligible for rtPA treatment, due to its narrow therapeutic window and the risk of brain hemorrhage[19,20].

Cerebrolysin is a mixture of an amino acid and peptide[21] that successfully has been used to support synaptic growth in the treatment of Alzheimer's disease[22], traumatic brain injury and neurodevelopmental disorders, including schizophrenia[23]. Recent studies report that it reduces the central and peripheral neuropathy secondary to chronic hyperglycemia induced by streptozotocin in mice and rats[24].

Recent data from a large scale randomized clinical trial showed that cerebrolysin can be safely administered to stroke patients with a trend towards improvement in the patients with severe stroke[25]. In another study[26], the authors mentioned contradictory results because they did not obtain a beneficial effect of the drug in similar cases. Therefore, the purpose of this study was to investigate the effectiveness of cerebrolysin improving the histological, immunohistochemical and the biochemical changes of the dentate gyrus of hippocampus in experimentally induced cerebral ischemia in adult male albino rats and to explore whether this effect is changed with varying doses (dose-dependent response) or not.

MATERIALS AND METHODS

Animals

Thirty healthy adult male albino rats (3-5 months) weighing 180-200 g were used in this study. They were purchased from the centre of experimental animals, Faculty of Veterinary medicine, Zagazig University. They were housed in the animal house of faculty of medicine, Zagazig University under controlled laboratory conditions at room temperature (20 ± 2°C). They were fed a standard balanced diet and allowed water ad libitum. They were adapted to laboratory environments one week before initiation of the experiments[27].

Chemicals

Cerebrolysin: was purchased from Sigma-Aldrich, Steinem, Germany Chemical Company. Cerebrolysin(vial) was given through dilution in a standard solution (physiological saline solution) infused intraperitonial slowly over approximately 20 to 60 minutes daily in the morning (between 10:00 and 12:00 am) for a period of 10 days.

Induction of acute cerebral ischemia

According to Carvalho et al.[28] after one week of acclimatization, the rats were anesthetized by halothane inhalation, fixed in a supine position and intubated with an oro-tracheal cannula. A midline incision was made in the neck, after that the incision was extended 1 cm to the right then both common carotid arteries and the right common jugular vein were exposed carefully by blunt dissection. The distal end of the common jugular vein was ligated cranially and sectioned for the retrograde introduction of a 2.5 cm long obstructive 4-0 mononylon suture with one end thickened with silicone over an extension of 5 mm. The suture was introduced until to reach the common carotid artery and then cranially progressed through the internal carotid artery until to reach and ligate the middle carotid artery to make acute cerebral ischaemia. Then the muscles and the skin of the neck were sutured separately.

To prevent wound damage from other animals, they were caged separately for post-surgical care. According to Al-Bazii[29], the surgical wound of the operated rats was cleaned with povidone iodine twice a day for 5 days. Rats in the sham group received the fake operation.

Experiment design

Three groups of albino rats were assigned

Group I (Control group): included ten rats, and further subdivided into two equal subgroups (5 rats each):

Subgroup Ia (Negative control group): rats were kept without any treatment.

Subgroup Ib (Positive control group; sham operated):

Subgroup Ib (Positive control group; sham operated):
Rats were submitted to the same surgical procedure (as group II) without ligation of middle carotid artery[30].

Group II (ischaemic group): ten rats were subjected to acute cerebral ischaemia through middle carotid artery occlusion[31].

Group III (post ischaemic treated group): ten animals were equally subdivided into two groups (5 rats each)

Subgroup IIIa: rats were administered 2.5 ml/kg of cerebrolysin infused intraperitoneally after 4h of onset of cerebral ischaemia daily for 10 days[31].

Subgroup IIIb: rats were administered 5 ml/kg of cerebrolysin infused intraperitoneally after 4h of onset of cerebral ischaemia daily for 10 days[31].

At the end of the experiment, all rats then were injected intraperitoneally with 25 mg/kg sodium thiopental. They were put in a supine position on a dissection table, then the chest wall was opened, they were perfused transcardially through the left ventricle with 10% formalin solution, when the left ventricle was not clear, the aorta was ligated before perfusion and the right atrium was opened once perfusion had started and when the venous return from the right atrium became clear, the perfusion was stopped[32,33]. Successfully perfused brains were dissected out through an incision made in the posterior of the neck. The skull was removed carefully to expose the brain. Left and right hemispheres of the brain were dissected out and kept in 10% formalin at room temperature[30]. Brain specimens were prepared for histological, immunohistochemical and biochemical studies.

Methods

Histological study

Brain tissue was placed in 10% formalin solution, then embedded in paraffin for histological analysis and. Paraffin sections of 4-5 µm thickness were prepared to be stained by Haematoxylin and Eosin (H&E)[35].

Immunohistochemical study

Immunohistochemical detection of GFAP, Erβ, and calretinin proteins was carried out using streptavidin–biotin complex immunoperoxidase system. Serial paraffin sections were deparaffinized on charged slides, then, incubated in 0.1% hydrogen peroxide for 30 min (to block the endogenous peroxidase) and finally incubated with the primary antibody[35].

Detection of GFAP

Sections were incubated with mouse anti-GFAP antibody (Cat. No. MS- 280- R7, Lab Vision Corporation, Fremont, USA) diluted in 1:100 in phosphate buffered saline in Lab Vision antibody diluents (Cat. TA- 125- UD). The primary antibody was incubated for 2 hours at room temperature, then washed with phosphate buffer solution (PBS). The primary antibodies were detected by incubation with biotinylated goat anti-mouse IgG in a concentration of 1:500 diluted in phosphate buffered saline for 1 hour (Zymed Laboratories; South San Francisco, CA, USA) for 30 min at room temperature[30].

ERβ expression

Sections were incubated rabbit polyclonal IgG anti-rat Erβ antibody (Cat. No. PA-1-311, Thermo Fisher Scientific, Rockford, USA) at 1-2 μg/mL diluted in PBS for 2 hours at room temperature. After several washes with PBS, primary antibodies were detected by incubation with biotinylated anti-rabbit antibodies diluted in phosphate buffered saline (versal kits, Zymed laboratories) for 1 hour at room temperature[37].

Calretinin protein expression

Sections were incubated with calretinin rabbit polyclonal antibody (Catalog ID: GTX103261; Lifespan Biosciences, Inc., Seattle, WA, USA; at a dilution of 1:100 MS- 280- R7, Lab Vision Corporation, Fremont, USA) diluted in phosphate buffered saline for 1 hour in Lab Vision antibody diluents (Cat. TA- 125- UD) for 2 hours at room temperature. After several washes with PBS, primary antibodies were detected by incubation with biotinylated goat anti-mouse IgG (Zymed Laboratories; South San Francisco, CA, USA) for 1 hour at room temperature[37].

All sections were incubated with the streptavidin–biotin peroxidase complex for 30 min at room temperature. After washing with PBS, reactions were visualized with 3′, diaminobenzidinetetrahydrochloride (DAB - Sigma-Aldrich Chemical Co., St. Louis, USA) used as chromogen to visualize antibody binding. The sections were counterstained with Mayer’s hematoxylin, dehydrated and mounted by DPX. Negative controls were processed according to the same protocol, except for the use of the primary antibody[37].

Biochemical study

For biochemical determinations a supernatant obtained from brain tissues were performed as the following. The tissue sample (one gram of tissue) was rinsed in cold physiological saline and homogenized with tissue homogenizer. The homogenate was centrifuged at 4000 RPM for 15 min at 4°C for 30 min, and the supernatant was stored on ice was kept at -2°C for analysis[35].

Oxidative stress parameters

These parameters include malondialdehyde (MDA) as an index for lipid peroxidation (LP), antioxidant enzymes as Catalase (CAT) and reduced glutathione (GSH). The amount of malondialdehyde (MDA) in tissue homogenate was determined according to Ohkawa et al.[38] based on the reaction with thiobarbituric acid. SOD activity was estimated based on inhibiting pyrogallol auto-oxidation by it as described by Marklund[39]. The inhibition rate is directly proportional to the activity of SOD in tissue. Catalase (CAT) activity was determined according to Clairborne[40] based on the decomposition of hydrogen peroxide by catalase enzyme. Reduced glutathione (GSH) concentration was assayed according to Ellman[41].
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The spectrophotometric detection at O=532 and 600 nm follows a reaction in an acid environment and incubation at 95°C. All metabolites were purchased from (Biodiagnostic, Giza, Egypt).

**Tumor necrosis factor alpha (TNF-α) assay**

TNF-α levels were determined by Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) using rat TNF-α kits were procured from Sigma-Aldrich Saint Louis, MO, USA. Homogenates of brain tissue groups were made and centrifuged at 4°C. The supernatants were collected, and used to detect the levels of TNF-α(43).

**Measurement of HSP-70**

HSP-70 protein expression in brain tissue was detected using a commercial HSP70 ELISA kit (StressgenBireagents, Ann Arbor, MI). The protein was isolated from brain tissue homogenate by using protein extraction reagent. According to the assay proceduresupplemented with the kit, HSP-70 standards and samples were added to the HSP-70 immunoassay plate, incubated and washed, then anti-HSP-70 biotin conjugate was added, incubated and washed, finally avidin-HRP conjugate was added, incubated and washed. The colour was developed with 3, 3’, 5, 5’ tetramethylbenzidine. We measured its absorbance at 450nm. HSP70 in the samples was calculated. Tissue total protein concentration was also measured with Coomassie blue method (assay kit was purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China)(43).

**Gene expression of Gh-R "Ghrelin receptor gene":(Assayed in brain tissue by Real-time RT-PCR)**

Samples of brain tissue were dissected immediately and frozen in liquid nitrogen. Total-RNA was extracted from tissue samples using Trizol reagent (Invitrogen) according to standard protocol as described by Brinkhof et al. The standard amount of total RNA (500 ng) was used to synthesize the first strand cDNA (High Capacity RNA-to-cDNA kit, Applied Biosystems, Foster City, CA). RT-PCR amplification mixtures (25µl) contained 1µl template cDNA, SYBER Green master mix buffer (Quanti-Teest, Qiagen, Hilden) and 400nM (10 pmol/reaction) forward and reverse primer. Reactions were run on step one plus Real-Time PCR detector (Applied Biosystems). The results were analyzed by SDS software vs. 2.3 (Applied Biosysytems). The expression of genes of interest was normalized to the housekeeper gene beta-actin and calculated using AA Ct method and calculated using AA Ct method.

**Histo-morphometric analysis**

The image analyzer computer system Leica Qwin 500 (Leica Ltd, Cambridge, UK) at the Image Analyzing Unit of Pathology Department, Faculty of Dentistry, Cairo University, Egypt, was used to evaluate the astrocyte number and the area percent of GFAP, Erβ and calretinin protein by the interactive measure menu. The measuring frame of a standard area equal to 118476.6 mm² was chosen so that the brown positive immune reaction could be seen and masked to be measured. Ten readings from five non-overlapping sections from each rat of all groups were examined.

**Statistical analysis**

All data were expressed as mean ± SD. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software, version 13.00 (Chicago, Illinois, USA). Statistical significance was determined by one-way analysis of variance for differences between the means of different groups. Further analysis was carried out using the post-hoc test to compare the parameters between the different groups with each other. Probability of P less than 0.05 was considered statistically significant.

**RESULTS**

**Histological results**

H&E stained sections of the control hippocampus showed parts of the CornuAmmonis (CA) as CA1, CA2, CA3& CA4 regions. Dentate gyrus was a C shaped structure enclosing CA4 (Fig. 1a). Dentate gyrus was formed of three layers; molecular, granule cell and polymorphic layers. The molecular layer was a relative cell-free layer containing neural cells and blood vessels. The granule cell layer contained rounded granule cells with vesicular nuclei. Spindleshaped cells were detected in the sub-granular zone(SGZ). Different types of cells as astrocytes were noticed in the polymorphic layer (Fig. 1b). Examination of H&E stained sections of the ischaemia group showed small dark stained nuclei and areas of cell loss in the granule cell layer. Numerous spindle shaped cells were noticed in SGZ. The molecular and polymorphic layers revealed numerous astrocytes (Fig. 1c). Sections in the dentate gyrus of subgroup IIIa revealed some granule cells with rounded vesicular nuclei, however others had small dark nuclei. The molecular layer contained astrocytes and blood capillaries. The polymorphic layer showed some vacuolations in the neuropil (Fig. 1d).

Using DNA extraction kit and gel electrophoresis:

DNA was extracted using genomic DNA extraction kit (TIANGEN, Beijing, china). Qualitative analysis of DNA fragmentation was done by submersive Gel Electrophoresis system (Pharmacia Biotech by SEMKO AB, Sweden) and submersive chamber (Maxicell, EC360, M-E-C apparatus co. StPetersburg, FL). The gel image was photographed under ultraviolet trans-illumination (Heralab GmbH laborgerate trans-illuminator, Germany)(44).

**DNA fragmentation test for apoptosis**
vesicular nuclei. Small spindle and rounded shaped cells were detected in SGZ. Molecular and polymorphic layers contained astrocytes (Fig. 1e).

**Immunohistochemical results**

Immunohistochemical reaction for GFAP in sections of the dentate gyrus of the hippocampus of the control group showed positive cytoplasmic reaction in the body and processes of the astrocytes that was less noticed in the granule cell layer (Fig. 2a). In the ischaemia group, strong positive cytoplasmic reaction for GFAP in the body and processes of astrocytes in the three layers of dentate gyrus (Fig. 2b). Immunohistochemical results for GFAP-stained sections of subgroup IIIa revealed positive cytoplasmic reaction in astrocytes that was moderate in the granule cell layer (Fig. 2c). GFAP-stained sections for subgroup IIIb revealed positive expression for GFAP in astrocytes that was less noticed in the granule cell layer (Fig. 2d).

Immunohistochemical reaction for ERβ in sections of the dentate gyrus of the control group revealed a strong positive nuclear reaction of granule cells (Fig. 3a). Faint positive immunoreaction for ERβ in some nuclei of granular cells of the ischaemia group (Fig. 3b). Immunohistochemical reaction for ERβ stained sections of subgroup IIIa revealed a strong positive nuclear reaction in most granule cells (Fig. 3c). ERβ immunoreaction in sections of the subgroup IIIb revealed a strong positive nuclear expression of granular cells (Fig. 3d).

Immunohistochemical reaction for calretinin in the dentate gyrus of the control group revealed strong positive reaction in the cytoplasm and processes of granule cells (Fig. 4a). Negative reaction for calretinin immunoreaction was detected in granule cells of the ischaemia group (Fig. 4b). Immunohistochemical results for calretinin stained sections of subgroup IIIa revealed strong positive reaction in most granule cells (Fig. 4c). Calretinin immunoreaction in sections of subgroup IIIb revealed strong positive expression in granule cells (Fig. 4d).

**Biochemical results**

**Oxidative stress parameters**

Assessment of the activities of lipid peroxidation revealed a significant increase of MDA in the ischaemic group as compared to the control group and post ischemic treated group IIIa and IIIb. There was a non-significant difference between the control group and post ischaemic treated group IIIb (Table 1).

**TNF-α and HSP-70 assay and Ghrelin receptors mRNA (Gh receptor) expression**

Assessment of brain tissue TNF-α and HSP-70 revealed a significant increase in the ischaemic group when compared with the control group, post ischemic treated group IIIa and IIIb. There was a non-significant difference between the control group and subgroup IIIb (Table 2). Ghrelin receptor gene expression showed a significant decrease in the ischaemic group when compared with the control, post ischemic treated group IIIa and IIIb. Furthermore, there was a non-significant difference between the control group and post ischemic treated group IIIb (Table 2).

**DNA fragmentation test for apoptosis**

DNA fragmentation was significantly observed in Lane 1 which represented the ischemic group, Lane 2 represented the control group, Lane 3, 4 represent post ischemic treated groups (IIIa) and (IIIb) respectively, showed regression of DNA shearing especially with post ischemic treated group (IIIb) that was treated with high dose of cerebrolysin (Fig. A).

**Histomorphometric and statistical results**

Statistically significant increase in the mean number of astrocytes was noticed in the ischaemia group as compared to the control group and subgroup IIIa and IIIb. No statistically significant difference was seen in the control group as compared to subgroups IIIa and IIIb (Table 3).

Statistically significant increase in the mean area percent of GFAP immunoreaction was noticed in Group II as compared to the control group and subgroups IIIa and IIIb. No statistically significant difference was seen in the control group as compared to subgroup IIIb (Table 3).

Statistically significant decrease in the mean area percent of ERβ immunoreaction was noticed in Group II as compared to Group I, subgroup IIIa, and IIIb. No statistically significant difference was detected between Group I as compared to subgroup IIIb (Table 3).

Statistically significant decrease in the mean area percent of calretinin immunoreaction was noticed in Group II as compared to Group I, subgroups IIIa and IIIb. No statistically significant difference was seen in Group I as compared to subgroup IIIb (Table 3).
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Fig. A: DNA fragmentation is significantly observed in Lane 1 which represents the ischemic group, Lane 2 represents the control group, Lane 3,4 represent post ischemic treated groups (IIIa) and (IIIb) respectively, showing regression of DNA shearing especially with post ischemic treated group (IIIb) that is treated with high dose of cerebrolysin.

Fig. 1a: A photomicrograph of H&E stained sections of the rat control hippocampus showing the Corna Ammonis (CA) parts as CA1, CA2, CA3 & CA4 regions. Dentate gyrus (DG) is a C shaped structure enclosing CA4.

1b: Dentate gyrus of the control group is formed of three layers; molecular (ML), granule cell (GL) and polymorphic layers (P). Granule cells (arrow) have rounded vesicular nuclei. Small spindle shaped stem cells (red arrow) are noticed in the sub-granular zone. The molecular layer was a relative cell-free layer containing astrocytes (arrow head) and blood vessels (c). The polymorphic layer (P) contained astrocytes (arrow head). (H&E ×40, scale bar 100 µm)

1c: A photomicrograph of H&E stained sections of the ischemia group showing small dark stained nuclei (arrow) and areas of cell loss (*) in the granule cell layer (GL). Numerous spindle shaped cells (red arrow) are noticed in the sub-granular zone. Moreover, molecular (ML) and polymorphic (P) layers reveal numerous astrocytes (arrow head). (H&E ×400, scale bar 20 µm)

1d: Examination of H&E stained sections of the dentate gyrus of subgroup IIIa showing granule cells with rounded vesicular nuclei (arrow), others have small dark nuclei (double arrow) in the granule cell layer (GL). The molecular layer (ML) contains astrocytes (arrow head) and blood capillaries (c). The polymorphic layer (P) shows some vacuolations (v) in the neuropil. (H&E ×400, scale bar 20 µm)

1e: H&E stained sections of the dentate gyrus of subgroup IIIb showing granule cells (arrow) with rounded vesicular nuclei in the granule cell layer (GL). Small spindle shaped cells (red arrow) are detected in the subgranular zone. The molecular layer (ML) and the polymorphic layer (P) contain astrocytes (arrow head). (H&E ×400, scale bar 20 µm)
Fig. 2a: A photomicrograph of immunohistochemical reaction for GFAP in sections of the control dentate gyrus showing strong positive cytoplasmic reaction in the body and processes of astrocytes (arrow) that is less noticed in the granule cell layer.

2b: A photomicrograph of immunohistochemical reaction for GFAP in sections of the dentate gyrus of the ischemia group showing strong positive cytoplasmic reaction in the body and processes of astrocytes (arrow) in the three layers of dentate gyrus.

2c: Immunohistochemical results for GFAP stained sections of subgroup IIIa reveals strong positive cytoplasmic reaction in astrocytes (arrow) that is moderately detected in the granule cell layer.

2d: GFAP stained sections for subgroup IIIb reveals strong positive expression of GFAP in astrocytes (arrow) that is less noticed in the granule cell layer.

(Immunoperoxidase technique for GFAP×400, scale bar 20 µm)

Fig. 3a: Immunohistochemical reaction for ERβ in sections of the dentate gyrus of the control group reveals strong positive nuclear reaction of granule cells (arrow).

3b: Faint positive immunoreaction for ERβ in some nuclei of granule cells (arrow) of the ischemia group.

3c: Immunohistochemical reaction for ERβ stained sections of subgroup IIIa reveals strong positive nuclear reaction (arrow) in most of granule cells.

3d: ERβ immunoreaction in sections of the subgroup IIIb reveals strong positive nuclear expression (arrow) of granule cells.

(Immunoperoxidase technique for ERβ, ×400, scale bar 20 µm)
Fig. 4a: Immunohistochemical reaction for calretinin in the dentate gyrus of the control group showing strong positive reaction (arrow) in the cytoplasm and processes of neuronal cells.

4b: Negative reaction for calretinin immunoreaction is detected in neuronal cells (arrow) of the ischemia group.

4c: Immunohistochemical results for calretinin stained sections of subgroup IIIa showing strong positive reaction in most neuronal cells (arrow).

4d: Calretinin immunoreaction in sections of subgroup IIIb showing strong positive expression in neuronal cells (arrow). (Immunoperoxidase technique for calretinin×400, scale bar 20 µm)

Table 1: Oxidative and antioxidant biomarkers levels in brain tissue values expressed as mean ±SD

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (I)</th>
<th>Ischemia (II)</th>
<th>Post ischemic IIIa</th>
<th>Post ischemic IIIb</th>
<th>F</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td></td>
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</tr>
<tr>
<td>MDA (nmol/g tissue)</td>
<td>45.3±1.3</td>
<td>81.4±2.5</td>
<td>49.3±1.3</td>
<td>42.5±4.5</td>
<td>238.372</td>
<td>&lt;0.001**</td>
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<td>GSH (nmol/g tissue)</td>
<td>2.1±0.1</td>
<td>0.9±0.02</td>
<td>2.6±0.1</td>
<td>2.2±0.1</td>
<td>213.706</td>
<td>&lt;0.001**</td>
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<tr>
<td>CAT (u/g tissue)</td>
<td>8.7±0.6</td>
<td>5.4±0.8</td>
<td>10.7±0.5</td>
<td>9.3±0.9</td>
<td>48.961</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>

** Highly significant p value <0.001

By LSD for comparison in-between groups (significant p.value < 0.05)

*significant with control.

#significant with ischemia.

$ Significant with Post ischemic IIIa.

@ Significant with Post ischemic IIIb.
DISCUSSION

Acute ischemic stroke (AIS) is one of the major leading causes of death and common causes of adult disability worldwide\(^{[47,48]}\). Considering poor prognosis of AIS, it is crucial to develop effective therapies to improve neurological manifestations of its patients. Cerebrolysin, a neuroprotective compound, was tested in several clinical studies with uneven conclusions\(^{[19]}\). Therefore, the aim of this study was to examine the efficacy, safety and dose-dependent effects of cerebrolysin administration on dentate gyrus of hippocampus in AIS.

Examination of H&E-stained sections of the ischaemia group showed small dark stained nuclei in the granule cell layer and areas of cell loss. The same results obtained by Cao et al. and Irmak et al.\(^{[46,48]}\) who stated that cerebral ischemia increased the expression of transcription factor that plays a pivotal role in mediating inflammatory response and reactive oxygen species (ROS) protein. This overproduction of ROS, inflammation and oxidative stress lead to subsequent neuronal injury and damage\(^{[49,50]}\) and increases the occurrence of apoptotic cell death in the brain\(^{[51,52]}\). Increase ROS causing release of free calcium that causes deterioration of membrane structure and enzyme activities. Consequently, nitric oxide radical formation is increased, resulting in cell damage and apoptosis. Cells are characterized by a decrease volume, deterioration in membrane integrity and nuclear fragmentation\(^{[53,54]}\). Additionally, the same group showed that the fragmentation index of genomic DNA from brain tissue was significantly higher as compared to control. Similar results were obtained by Liang et al.\(^{[5]}\), who noticed that karyopyknosis was evident and the number of neurons was reduced. The ischemic group showed significantly increased MDA with significantly decreased the activity of antioxidant enzymes CAT and GSH when compared to the control group. The same results obtained by Liang et al.\(^{[5]}\) who found that cerebral ischemia increased the content of MDA and NF-kBp65; an important transcription factor that plays a pivotal role in mediating inflammatory response and reactive oxygen species (ROS) protein expression.

In the current study, H&E-stained sections of ischemic group revealed numerous spindle shaped cells in the subgranular zone (SGZ). These finding were explained by Neuberger et al.\(^{[55]}\) who stated that hippocampal dentate gyrus is a focus of enhanced neurogenesis and excitability after traumatic brain injury. Increased neurogenesis has been proposed to help repair of the injured granular cells.

Immunohistochemical reaction for ERβ in sections of the dentate gyrus of the ischemia group showed faint positive reaction in some nuclei of granule cells as compared to the control group. The same results were reported by Altuna et al. and Liet al.\(^{[54,56]}\) who added that improvement from ischemia was slower and less complete in animals lacking ERβ.

As regard calretinin immunoreaction, negative reaction was detected in granule cells of the ischemic group as compared to the control group. Uniform findings were also

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**Table 2:** TNF-α, HSP-70 and Ghrelin receptor gene expression values expressed as means ± SD.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (I)</th>
<th>Ischemia (II)</th>
<th>Post ischemic IIIa</th>
<th>Post ischemic IIIb</th>
<th>F</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (Pg/ml)</td>
<td>18.4±0.07(^{[53]})</td>
<td>36.5±2.3(^{[53]})</td>
<td>20.6±2(^{[53]})</td>
<td>18.0±2(^{[53]})</td>
<td>165.52</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>HSP-70 (Pg/mg)</td>
<td>2.3±0.4(^{[54]})</td>
<td>12.6±1.4(^{[54]})</td>
<td>6.4±0.4(^{[54]})</td>
<td>2.2±1(^{[54]})</td>
<td>207.469</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Ghrelin receptor gene</td>
<td>1.73±0.007(^{[53]})</td>
<td>0.52±0.01(^{[53]})</td>
<td>0.74±0.02(^{[53]})</td>
<td>1.72±0.02(^{[53]})</td>
<td>8672.475</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>

* Highly significant p.value <0.001

** Significant with Post ischemic IIIb

$ Significant with Post ischemic IIIa.

# Significant with ischemia.

* Significant with control.

By LSD for comparison in-between groups (significant p.value <0.05)

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**Table 3:** The mean astrocyte number of GFAP, ERβ, and calretinin area % immunoreaction in the studied groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (I)</th>
<th>Ischemia (II)</th>
<th>Post ischemic IIIa</th>
<th>Post ischemic IIIb</th>
<th>F</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>The mean astrocyte number</td>
<td>0.33±0.01(^{[55]})</td>
<td>0.63±0.007(^{[55]})</td>
<td>0.48±0.01(^{[55]})</td>
<td>0.32±0.02(^{[55]})</td>
<td>695.086</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Area % of GFAP</td>
<td>5.1±0.007(^{[55]})</td>
<td>9.8±0.02(^{[55]})</td>
<td>4.8±0.01(^{[55]})</td>
<td>5.1±0.1(^{[55]})</td>
<td>101856.927</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Area % of ERβ</td>
<td>145.4±2.8(^{[55]})</td>
<td>99.2±1.2(^{[55]})</td>
<td>127.3±2.1(^{[55]})</td>
<td>144.3±2.8(^{[55]})</td>
<td>422.501</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Area % of calretinin</td>
<td>67±1.4(^{[55]})</td>
<td>39±0.7(^{[55]})</td>
<td>58±0.4(^{[55]})</td>
<td>66.8±1.1(^{[55]})</td>
<td>887.064</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>

* Highly significant P.value <0.001

** By LSD for comparison in-between groups (significant p.value <0.05)

* Significant with control.

$ Significant with ischemia.

* Significant with Post ischemic IIIa.

@ Significant with Post ischemic IIIb

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found by Asker et al. Decrease calretinin expression in granule cells as a result of calcium channel dysfunction was found to cause abnormal neuronal excitability, impairment in the motor control in mouse cerebellum. Also, calretinin immunoreactivity of the Purkinje cells in the treated group was decreased which may be due to the massive degeneration of Purkinje cells, as it was reported that the neuronal degeneration resulted in decrease calretinin expression in the hippocampus and cerebellum.

TNF-α and HSP-70 assay showed statistically significant increase in ischemic group. The expression of inflammatory factors was increased in the pathological process of cerebral disease. The release of inflammatory molecules, including TNF-α, IL-1β, IL-6, NF-κB, and TGF-β, aggravates cell injury during ischemia stroke. This was found to be consistent with previous studies in rat models. HSP-70 which is a 70-kDa heat-shock proteins are stress-induced molecules that expressed in response to various types of CNS injuries, including stroke, trauma or neurodegenerative disorders. Marked increase of HSP-70 in brain tissue of ischemic group induced by oxidative stress. While, Robinson et al., Zhenget al. and Bienemann et al. stated that HSP-70 may have anti-inflammatory, cytoprotective and anti-apoptotic actions.

Astrocytes release a variety of trophic factors to influence neuronal survival and plasticity after brain injury. Reactive astrocytes also overexpress neuropilin-1 and vascular endothelial growth factor to promote angiogenesis after cerebral ischemia. This suggests that astrocytes may play an important role in functional recovery after stroke. Immunohistochemical results for GFAP-stained sections of the dentate gyrus of group III revealed strong positive expression in the hippocampus, hence undergoes changes throughout the lifespan and is also the most sensitive region of cerebral ischemic injury. Cerebrolysin augmented neurogenesis in the ischemic brain and improved functional outcomes. It attenuates neuroblast apoptosis and can enhance hippocampal neurogenesis by augmenting survival and proliferation of the hippocampal neural progenitor cells. Thus, it plays a major role in promoting neurogenesis and gliogenesis.
CONCLUSION AND RECOMMENDATION

Cerebrolysin effectively reversed ischemia-induced dentate gyrus injury in a dose-dependent manner. That is evidenced by the histological, immunohistochemical and biochemical improvement. However, further experimental and clinical studies are required on other CNS regions to clarify the therapeutic roles of cerebrolysin during acute ischemic stroke and its the molecular mechanisms.

CONFLICT OF INTEREST

There is no potential conflict of interest among the authors.

ABBREVIATIONS

AIS: Acute ischemic stroke
TNF-α: tumor necrosis factor-α
HSP-70: heat shock protein -70
Gh receptor: Ghrelin receptors
DNA: deoxyribonucleic acid
GFAP: glial fibrillary acidic protein
Erβ: estrogen receptor beta
MDA: Malondialdehyde
CAT: Catalase
GSH: Reduced glutathione
CNS: central nervous system
CA: cornu ammonis
FDA: food and drug administration
rtPA: recombinant tissue plasminogen activator
LP: lipid peroxidation
ELISA: Enzyme-Linked-Immuno-Sorbent-Assay
ROS: reactive oxygen species
SVG: sub-granular zone
NSC: neuronal stem cell
RGLs: Radial glia-like cells
IPCs: intermediate progenitor cells

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CEREBROLYSIN ON DENTATE GYRUS OF ALBINO RATS IN INDUCED ACUTE ISCHEMIC STROKE


فعالية سيريروليسين على بنية التلفيف المسنن من الحصين بعد السكتة الدماغية الإقفارية الحادة المستحثة تجريبيا في ذكور الجرذان البيضاء البالغة (دراسة هستولوجية وهستوكيميائية مناعية وبيوكيميائية) 

الملخص العربي

فعالية سيريروليسين على بنية التلفيف المسنن من الحصين بعد السكتة الدماغية الإقفارية الحادة المستحثة تجريبيا في ذكور الجرذان البيضاء البالغة (دراسة هستولوجية وهستوكيميائية مناعية وبيوكيميائية)

الخلفية: السكتة الإقفارية الحادة هي عبارة عن توقف دماغي مؤقت ينتج عن توقف الدورة الدموية المخية، مما يؤدي إلى فقدان وظائف مختلفة. يُعتبر سيريروليسين دواء للتغذية العصبية الحرة.

الهدف: تهدف هذه الدراسة إلى تقييم دورة سيريروليسين هستولوجيا وهستوكيميائي مناعية في السكتة الدماغية الإقفارية الحادة في ذكور الجرذان البيضاء البالغة وأيضًا تقييم تأثيرها على الجرعة.

المواد والطرق: استخدمت ثلاثون من ذكور الجرذان البيضاء البالغة، قسمتهم إلى ثلاث مجموعات متساوية: المجموعة الضابطة و مجموعة تسريروليسين و مجموعة ما بعد الاقفار. جُمعت المجموعة الثالثة إلى مجموعتين فرعيتين: مجموعة درجة أولى حيث عولجت الجرذان بسريروليسين 2.5 مجم / كجم، بينما عولجت جرذان المجموعة الفرعية الثانية سيريروليسين 5 مجم / كجم. صبغت مقاطع المخ بصبغة الهيماتوكسيلين والأيوسين و استخدمت صبغة هستوكيميائية مناعية لبروتين calretinin و مستقبلات الاستروجين ERβ و GFAP و HSP-70 و TNFα.

النتائج: لم تؤثر الجرعة على بنية التلفيف للحصين. تم الكشف عن تفاعلات مناعية إيجابية في الخلايا العصبية و انخفضت الإنزيمات المضادة في النواة الحبيبية. إذ علقت النتائج أن العلاج بسريروليسين 2.5 مجم / كجم أعطى نتائجًا أفضل. من ناحية المهاجمة، تجاوزت نتائج هذه الدراسة نتائج السكتة الإقفارية الحادة المستحثة تجريبيا في ذكور الجرذان البيضاء بطريقة توازية مع زيادة الجرعة و ظهر ذلك في تحسن البنية النسيجية والسيجي الكيميائية مناعية للتلقيح المسنن و كذلك تحسنت النتائج البيوكيميائية.

الخلاصة: نستخلص من هذه الدراسة أن عقار سيريروليسين كان فعالا في علاج السكتة الإقفارية الحادة المستحثة تجريبيا في ذكور الجرذان البيضاء بطريقة توازية مع زيادة الجرعة و ظهر ذلك في تحسن البنية النسيجية والسيجي الكيميائية مناعية للتلقيح المسنن و كذلك تحسنت النتائج البيوكيميائية.