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Original Article

Ameliorative effects of Cerebrolysin on Traumatic Brain Injury in Adult male albino rats

Marwa M. El Sawy, Hany W. Abdel Malak, Mervat T. Naguib, Ahmed M. Desouky

Department of Anatomy and Embryology, Faculty of Medicine, Ain Shams University, Egypt,

ABSTRACT

Background: Traumatic brain injury is a community problem with high significance in illness or death. Moreover, recurrent mild traumatic injury, could resulted in increased risk of neurodegeneration as in Alzheimer. On the other hand, there were no effective treatment to overcome or improve TBI related cerebral damage. However, cerebrolysin was significantly enhanced mental functions in people with vascular dementia.

Aim: So, the current work was designed to simulate a repeated brain concussion model in adult male albino rats to test its effect on the structure of the ependyma and detect the possible ameliorative effects of cerebrolysin supplementation on the post traumatic changes in ependyma and subventricular zone.

Material and Methods: Twenty adult male albino rats were divided into four groups: Control, concussion, spontaneously recovered and cerebrolysin treated groups. Brain specimens were processed for light microscopy and immunohistochemistry followed by statistical analysis.

Results: The current study revealed that early cerebrolysin treatment induced neurogenesis and were associated with improved post traumatic ependymal changes.

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Key Words: Ependyma, Cerebrolysin, SVZ, TBI.

Corresponding Author: Hany W. Abdel Malak, Department of Anatomy & Embryology, Faculty of Medicine, Ain shams University, Cairo, Egypt. **Tel.:** 0122.293.1505, **E-mail:** hwabdelmalak@gmail.com

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INTRODUCTION

Traumatic brain injury (TBI) indicates a change in cerebral function or construction, caused by an external force. It signifies a community problem with high significance in illness or death[1]. TBI are responsible for 59,000 deaths and leave 90,000 disable persons[2&3]. Road traffic accidents (about 17%) are the major causes of TBI in the United States. Sport-associated concussions are about 130,000 / year at age group from 5 -18 years[4]. In dynamic military individuals, explosions are a leading cause of traumatic injury[5]. Now, TBI severity level is classified according to the Glasgow coma scale (GCS) in addition to lasting mental and social defects. Moreover, recurrent mild traumatic injury, could resulted in increased risk of neurodegeneration as in Alzheimer[6], chronic traumatic encephalopathy[7] and Parkinson's disease[8]. Experimental animal models of TBI revealed seizure susceptibility, neuronal damage, neurophysiological changes including physical, cognitive, communication, and behavioral disabilities at several levels[9]. On the other hand, most researches have investigated changes in neural function underling TBI-related cognitive deficits. Also, the brain ventricles together with cerebrospinal fluid dysfunction were contributed to cognitive difficulties[10]. In addition, the neuroepithelial injury of the ventricular zone (VZ) was characterized by alteration in monociliated neural stem cells, loss or detachment (VZ disruption), impaired cell junctions, and gliosis in the affected area[11].

On the other hand, there were no effective treatment to overcome or improve TBI related cerebral damage[12]. Cerebrolysin is a peptides

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assortment from pig brains, including brain-neurotrophic factor, and nerve growth factor[13]. During investigational ischemic stroke, cerebrolysin reduced the infarction volume and enhance function by inhibiting the formation of the free radicals[14], declining neuronal inflammation and cell death[15] and stimulating neuronal budding[16] enhancing survival of cells[14]. In addition, cerebrolysin was significantly enhanced mental functions in people with vascular dementia[17]. This inspiring efficacy and promoted therapy provided an opportunity for clinical studies.

So, the current work was designed to simulate a repeated brain concussion model in adult male albino rats to test its effect on the structure of the ependyma and detect the possible ameliorative effects of cerebrolysin supplementation on the post traumatic changes in ependyma and subventricular zone.

MATERIAL AND METHODS

Chemicals: Cerebrolysin (CBL). Ever Neuro Pharma, Austria (Imported by: United Company for Distribution) in a dose of 2.5 ml/kg (sterile isotonic saline solution).

Animals:

After obtaining an approval from ethical committee for animal research at Ain Shams faculty of medicine, Twenty adult male albino rats, weighing 150-250 gm, were used (Got from MASRI research center). Rats were placed in good ventilation and suitable temperature under a normal light & dark cycle. They had a standard balanced diet and water. Rats were then divided randomly into four equal groups:

Group I: control group consisted of 5 adult albino rats. The rats were not exposed to concussion.

Groups (II, III and IV): Consisted of 15 adult male rats. Rats were exposed to recurrent concussive hits using weight-drop TBI model[1]. A free-falling steel ball (500 g) guided in a tube, falling from a height of 25 cm to hit the front of the skulls[18]. Rat's heads were braced on a thick foam block to allow partial head rushing, causing TBI[1]. After concussion, the animals suffered temporary blackout, tremors, and convulsions for seconds to minutes then left to rest. The traumatic model has repeated for 3 days. Dead rats from the process were replaced.

- Rats exposed to weight-drop were divided into three groups, each group formed of 5 animals:

Group II (Concussion group): Rat brains were dissected within 24 hrs. following 3rd concussion.

Group III (Spontaneously recovered group): Rats were left untreated for 10 days after 3rd concussion and then dissected.

Group IV (Cerebrolysin treated group): Rats received cerebrolysin (CBL) injection, 2.5 ml/kg (sterile isotonic saline solution) / day for a period of 28 days divided into four treatment cycles of five days and a pause of two days in between.

- Then the rats were sacrificed following the protocol of ethical committee. Brains were dissected out, collected, processed and prepared for examination using the following techniques:

1- Light microscopic study:

The brain specimens were cut and fixed in 10% buffered neutral formalin for a week. Brains were dehydrated in ascending grades of ethanol and cleared in xylol. Paraffin blocks were prepared and serial sections of 5 µm were prepared and stained with H & E[19]. The slides were examined and photographed using Olympus 330 E microscope in Anatomy & Embryology department, Ain Shams faculty of medicine.

2- Immunohistochemical study:

A. Glial fibrillary acidic protein (GFAP) immunostaining:

GFAP is the commonest method used to examine the distribution and reaction of astrocytes to neural degeneration or injury. Goat polyclonal anti-rat IgG (produced by Dako Cytomation) in a dilution of 1: 1000 was used as a primary GFAP antibody. Immunohistochemical staining was performed by avidin-biotin immunoperoxidase

complex, then incubation with 1/100 normal rabbit serum for 20 minutes to exclude non-specific background[19]. GFAP +ve astrocytes appeared brown. Protoplasmic astrocytes presents mainly in the gray matter of the CNS while fibrous astrocytes aggregates in white matter[20].

B. Synaptophysin (SYN) immunostaining:

SYN is a glycoprotein present in synaptic vesicles. It is involved in synaptic transmission within the brain and spinal cord. Synaptophysin immunostaining is used for quantifying the synapses[21]. Mouse SYN monoclonal antibody (1:200; Sigma-Aldrich Chemicals) was used. Specimens were incubated for 2 hours in the antibody at room temperature followed by avidin-biotin immunoperoxidase complex for 90 min. 90 minutes. Sections were then mounted on super-frost slides, dehydrated and cover slipped[19].

3- Computer image analysis:

Image analysis (for SVZ cell quantification and mean area percentage of GFAP & SYN Immunoreactivity) were done by using the image analyzer (T S View® program) in Anatomy and Embryology department, Ain Shams faculty of medicine. The computer image analyzer was connected to Olympus CH-2 microscope. First, the image analyzer was calibrated to convert the measurement units from pixels to micrometer units. Examined fields were enclosed in the standard measuring frame. In immunohistochemical stained sections, +ve GFAP immuno-stained areas were masked by a blue binary color and then measured. The mean area percentage of +ve immunostaining was measured in high power fields (x400) in five fields from five serial sections in each group[22].

4- Statistical data analysis:

Statistical data analysis were represented as mean ± standard deviation (SD) for each group. Analysis of variance using T-test was used. Results were considered statistically significant when *P value* is ≤ 0.05 and highly significant when it is ≤ 0.001[23].

RESULTS

1- Histological results:

The ventricular system of the brain in the control group is lined with ciliated ependymal cells. Ependymal cells are cuboidal to columnar epithelioid glial cells (Figs. 1A & B). Cells have microscopically visible cilia at their apical or ventricular aspect (Fig. 1C). They have pale eosinophilic cytoplasm with oval hyperchromatic nuclei (Figs. 1B & C). The neurogenic niche of subventricular zone (SVZ) is homogeneously distributed in the anterior part of the anterior horn of the lateral ventricle next to the ependymal layer (Fig. 1A). The SVZ consists of darkly and lightly stained cells of undifferentiated morphology with blood capillaries scattered in between (Fig. 2A).

On the other hand, sections of the concussion group (group II) had dilatation in the lateral ventricle with stretching and flattening of the ependymal cells. The ependymal disruption was associated with ventricular hemorrhage (Fig. 1D). The SVZ niche showed an apparent reduction in the main cellular components and the remaining cells of SVZ have irregular outlines and pale cytoplasm (Fig. 2B).

The ependymal layer of the spontaneously recovered group (3rd group) appeared similar to the control group. Most of the ependymal cells were well aligned compared with the concussion group. However, focal loss and separation of the ependymal cells were observable. Numerous small vacuoles were detected in the neuropil close to the ependymal layer with loss of typical neuronal appearance (Fig. 1E). Although the SVZ cell population apparently regained its normal appearance, there were numerous vacuoles, located mainly between the ependymal layer and SVZ (Fig. 2C).

Moreover, sections of cerebrolysin treated group revealed marked apparent improvement. The ependyma was restored its architecture as in the control group (Fig. 1F). Also, SVZ was well defined with rich vascularity (Fig. 2D).

SVZ cell quantification:

Significant difference in SVZ cell number was observed between the experimental groups. The SVZ niche of rats exposed to concussion showed statistically significant reduction in the main cellular components compared with the control group. After 10 days of recovery period, Subventricular zone showed statistically significant increase compared with the concussion group. The injection of cerebrolysin led to statistically significant increase in SVZ niche more than the recovery group (Table1).

2- Immunohistochemistry results:

A. GFAP immunostaining:

Glial fibrillary acidic protein (GFAP, astrocytes marker) was performed to detect astrocytes with their processes. Astrocytes were interdigitated with ependymal cells and GFAP positive radial glial fibers appeared very thin and lightly stained perpendicular to the ependymal layer in the control group (Fig. 3A). Also, GFAP +ve star-like astrocytes extended among SVZ cells (Fig. 3B). But, in 2nd (concussion) group, both the ependyma and SVZ expressed a marked reduction in astrocyte GFAP-immunoreactivity. The astrocytes were rare and dispersed in the SVZ (Fig. 3C), with statistically significant low GFAP-immunoreactivity, as compared to the control group (Table 1). After 10 days of recovery period, an increase in GFAP immunoreactivity was observed compared to concussion group (Table 1) but GFAP positive radial glial processes are thinner, and interrupted (Fig. 3D). In addition, the cells expressing GFAP were dispersed in SVZ of the recovery group (Fig. 3E).

The immunoreaction was diffuse and evenly distributed in the treated group. Morphological changes were also observed in GFAP-expressing cells; GFAP +ve radial glial cells exhibited elongated, thick, branch-like projections that were extending to the nearby cerebral cortex (Figs. 3F & G) This increase in positive GFAP immunostaining observed by light microscopic examination was further confirmed when compared with the control and spontaneously recovered groups with statistically highly significant difference in the mean area percentage of GFAP-immunoreactivity ($p < 0.00$) (Table 1).

B. Synaptophysin (SYN) immunostaining:

In control group, SYN-immunoreactivity appeared uniformly distributed and was demarcating the neurons in neuropil (Fig. 4 A). But, in concussion group, low reactivity to SYN appeared within the neuropil (Fig. 4 B) with significant reduction in immunoreactivity compared to the control rats (Table 1). In spontaneously recovered group, synaptophysin immunoreactivity appeared very similar to concussion group (Fig. 4 C). While, in cerebrolysin treated group, SYN-immunoreactivity was comparable to the control group (Fig. 4 D). Also the mean area percentage of positive SYN-immunoreactivity was statistically significantly higher compared to the recovery group (Table 1).

Table 1: SVZ cell quantification and mean area % of GFAP & SYN Immunoreactivity per Microscopic field

	SVZ cell quantification	Percentage of area for GEAP/Microscopic field	Percentage of area for SYN/Microscopic field
Groups	Mean ± SD	Mean ± SD	Mean ± SD
Group I	36.6±0.8	9.2±0.6	20±0.9
Group II	19.8±0.7	3.2±0.9	11±0.9
Group III	27.6±1.3	13.2±0.7	16.6±3.3
Group IV	32.2±0.7	22.8±0.8	22.8±0.7
Significant difference between	Group II and I Group III and I, II Group IV and I,II, III	Group II and I Group III and I, II Group IV and I[H]*, II, III	Group II and I Group III and I, II Group IV and I,II, III

[H]* High significant increase

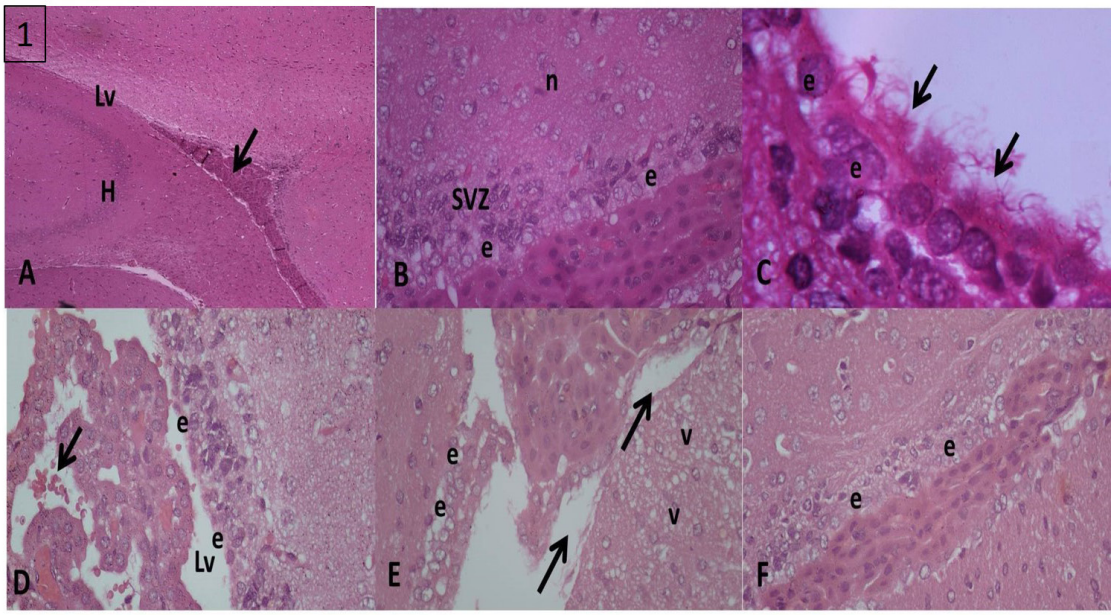

Fig. 1: Photomicrographs of parasagittal sections in rat's brain showing (A-C) control group with typical organization of lateral ventricle (Lv) and the choroid plexuses (arrow) near to the hippocampus (H). (B) A neurogenic niche of subventricular zone (SVZ) is homogeneously distributed in the anterior part of the anterior horn of the lateral ventricle next to the ependymal layer (e). Note the presence of typical neurons (n) in the neuropil close to the SVZ. (C) ependyma (e) have microscopically visible cilia (arrow). (D) concussion brain show collection of red blood cells (arrow) in dilated lateral ventricle (Lv) and flattening in the ependymal layer (e). (E) in spontaneous recovery group, well arranged ependymal layer (e) is present but disrupted in some areas (arrows). Note numerous small vacuoles (v) in the neuropil close to the ependymal layer. (F) in treated group, well arranged ependymal layer (e) nearby choroid plexuses is well observed. H & E - Scale bar: A x100 , B x400, Cx1000, D-F x 400

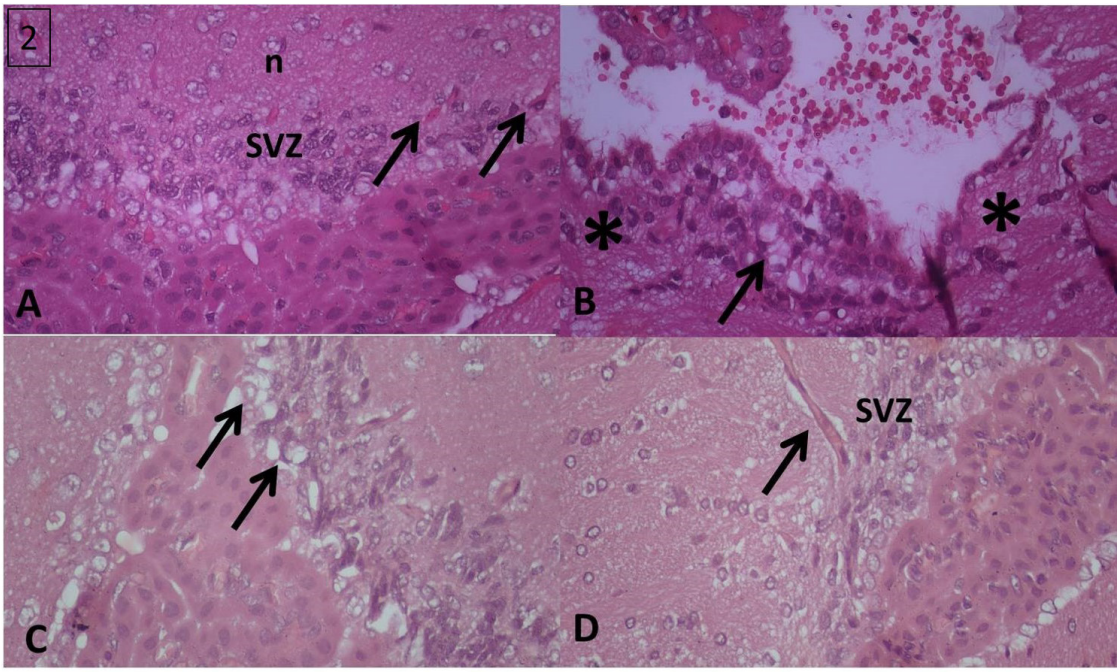

Fig. 2: Photomicrographs of parasagittal sections in rat's brain showing (A) subventricular zone (SVZ) of control rats formed of a collection of darkly and lightly stained cells of undifferentiated morphology with blood vessels (arrows) in between. Note the presence typically presented neurons (n) in the neuropil close to the SVZ (B) in concussion group, the section shows reduction in the main cellular components of the SVZ niche (*). Remaining cells appeared irregular with pale cytoplasm (arrow). (C) spontaneous recovery group shows restoration of SVZ cell population but numerous vacuoles forming groups (arrows) are mainly located between the ependymal layer and SVZ (D) in treated group, a well-defined SVZ with rich vascularity (arrow) is present. H & E - Scale bar: A-D x 400

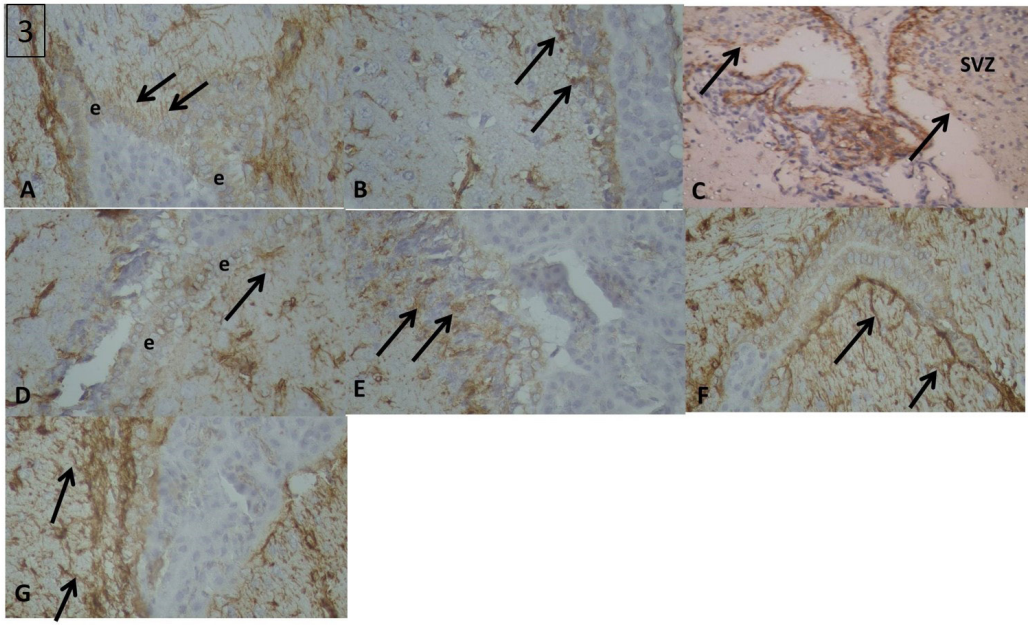

Fig. 3: Photomicrograph showing the distribution of the immuno positive cells for GFAP in the ependymal layer and the subventricular zone of parasagittal sections in rat's brain. (A) control group showing the distribution of astrocytes that interdigitate with ependymal cells (e). GFAP positive radial glial fibers appear very thin and lightly stained (arrows). (B) control group showed the astrocytes as dark brown star-shaped granules with their processes in the SVZ (arrows). (C) in concussion group, loss of astrocytes in some regions (arrows) is observed and GFAP +ve astrocytes are rarely seen in SVZ. (D) in recovery group, GFAP positive radial glial processes are thinner, interrupted and not as evident as control group. (E) in spontaneous recovery group, GFAP +ve astrocytes (arrows) are detected within SVZ cell population. (F) in treated group, most GFAP-positive radial glial cells exhibited numerous long, thick projections (arrows). (G) Treated group shows an apparently increased positive GFAP immunostaining in SVZ with numerous long, thick projections of radial glial cells (arrows) extending to the nearby cerebral cortex. GFAP - Scale bar: A-G x 400

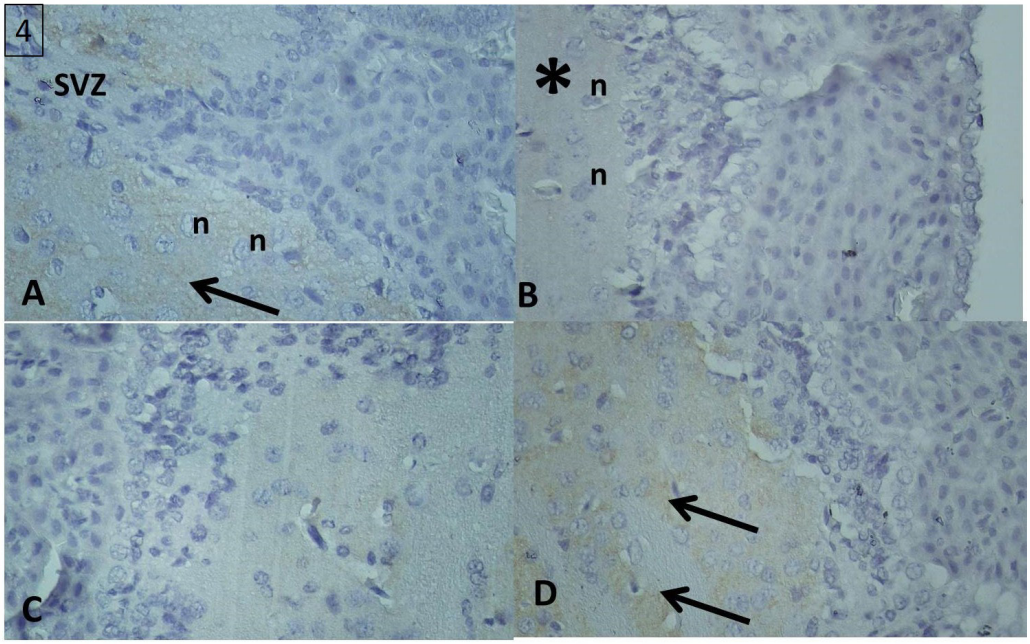

Fig. 4: Photomicrograph showing the distribution of the positive immunoreactivity for synaptophysin within the neuropil in parasagittal sections in rat's brain. (A) control group showing uniform distribution of positive SYN-immunoreactivity in the neuropil (arrow). It demarcates the neurons (n) and SVZ (B) In concussion group, apparent reduction in SYN-immunoreactivity in the neuropil (*) is noticed. Also, note the degenerated neurons (n). (C) in spontaneous recovery group, decreased synaptophysin immunoreactivity appears very similar to the concussion group. (D) treated group, showing apparently increased positive synaptophysin immunoreactivity (arrows) distributed within the neuropil. SYN - Scale bar: A-D x 400

DISCUSSION

The brain response to TBI is a complex process resulting in a cascade of various neurochemical and metabolic effects. Brief cerebral edema, memory debits and depression are symptoms of concussive like TBI model[24].

Recurrent concussive hits used in the present study resulted in dilatation of the lateral ventricle with stretching and flattening of the ependymal cells. The ependymal disruption was associated with ventricular hemorrhage 24 hour after trauma. Ependymal pathological changes in the form of loss of cilia then microvilli, might cause hydrocephalus. These changes were attributed to extensive ependymal stretching[10].

Few data are present in the literature regarding the effects of TBI on cell proliferation of neural stem and precursor cells, with particular reference to SVZ. The SVZ niche in the current work showed marked reduction in the main cellular components that was confirmed by cell count. The remaining cells of SVZ were irregular with light cytoplasm, 24 hour after the trauma suggesting that apoptosis was more pronounced in the first period of experimental exposure. Previously, it was reported that, accumulation of Ca within the mitochondria could compromise its membrane-potential, generating undue free radicals leading to cell death[25,26]. Increased concentrations of Ca and free radicals following TBI in experimental models, might impair mitochondrial function with reduction of energy supply resulting in cell death[24]. Also, the present study revealed an increase of the total cell number of subventricular zone after 10 days of recovery period. It was possibly due to the niche of neurogenesis and gliogenesis that is unable to migrate. This is consistent with the findings reported by[27,28] where periventricular heterotopia formed by neurons unable to migrate with loss of scaffold characteristic of the neural stem cells and lose the capacity to attach to one another.

Neuronal degeneration with reduction of synaptophysin immunoreactivity within the neuropil in the concussion group of the present study were indicative signs of severe acute neuronal death. Decreased SYN-immunoreaction was associated with the severity of neurodegeneration or neuronal loss. These results could be referred to TBI-induced decrease in ATP with lowered potential and increased permeability of mitochondrial membrane in cortical synapses[29,30].

On the other hand, in the spontaneously recovered group of the present experiment, some ependymal cells endured focal loss and separation, possibly due to impairment of cell junction. cell junctions appeared to play an essential role in cytopathology of ventricular zone disruption with significant decreased expression of N-cadherin after 48 hours of ventricular zone disruption[11]. N-cadherin was distributed within the cytoplasm rather than bound to the cell membrane[27].

Moreover, neurotrophins are structurally similar growth factors that control cell survival, adjust neural functions and are critically involved in neuroplasticity in adults[31]. They are considered to be part of the therapeutic strategy for neurological disorders. CBL is a neuropeptide preparation having pro-neuroplastic pharmacological effects with respect to neuroprotection and repair[32, 17, 33,34].

In the present study, CBL induced two significant effects which could be of functional importance. A significant CBL-induced increase in SVZ cell count and mean area percentage of GFAP in cerebrolysin treated animals. CBL-mediated increase of in SVZ cell count is consistent with the data of[33,35]. The Cerebrolysin supports neuronal cell survival, stimulates neuronal cell differentiation, growth and sprouting, and promotes the formation of synaptic contacts in cell cultures and in animal models. Furthermore, Muresanu *et al*., 2015 reported that cerebrolysin enhances neurogenesis in different experimental conditions including closed head injury. It stimulates the restorative capacity of the brain after injury. This might be explained by Stepanichev *et al*., 2017[36], who observed that, neurotrophin receptors are involved in regulation of neuronal plasticity in promotion of adult neurogenesis. These receptors are increasingly expressed in neurons and glial cells in response to injury increasing neurotrophin affinity to promote cell survival.

Furthermore, reactive astrocytic scars form one of the major barriers preventing axon regeneration after CNS injuries. A various inhibitory substrates are released by oligodendrocytes, which together with fibrotic tissues and inflammatory cells were

thought to highly suppress regeneration of injured CNS axons[37]. However, it was observed that, injured axons regenerated along the processes of reactive astrocytes[38]. Reactive astrocytes support growth of regenerating axons probably by guiding their elongation and bridging the stumps. Also, reactive astrocytes provide a beneficial role by reducing infiltrating immunoreactive cells into adjacent domains, protecting bordering neural tissue from damage and generating numerous supportive extracellular matrix (ECM) to promote cell survival and growth[39]. In addition, In addition, these astrocytes produce inhibitory proteoglycans that hinder myelination of injured axons[40].

So in the current work, CBL-mediated increase in GFAP +ve radial glial cells with elongated projections that extend to the cerebral cortex is correlated with good prognosis. This might be explained by induction of radial glial cells (RGC) in ischemia with proliferation and transformation of the ependymal cells to RGC. In SVZ, these cells guide the migrating neuroblasts to the ischemic borderline[41]. Moreover, following brain injury, activated astrocytes and vascular endothelial cells produce neurotropic factors to the neuroblasts in SVZ. Migrating neuroblasts also use blood vessels and radial glial processes as supports[42].

In the current study, a significant restoration of SYN-immunoreactivity and well-defined SVZ with rich vascularity were more obvious in the cerebrolysin treated group compared to the spontaneously recovered group. This might be attributed to the astrocytes restorative capacity of the brain after injury. The astrocytes have many branching projections. These branches terminate at synapses to regulate its function or enclose blood vessels to maintain the blood–brain barrier[43]. Furthermore, astrocytes link neuronal organization to the blood flow and are actively involved in maintaining neuronal synaptic junctions[44]. In addition to the synthesis of glial derived growth factors, hippocampal radial-glial cells promote formation of the new neurons in the adult dentate gyrus[45].

CONFLICT OF INTERESTS

There are no conflicts of interest.

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التأثيرات المُحسّنة للسريبروليسين على إصابات الدماغ الجرحية في ذكور الجرذان البيضاء البالغة

مروة محمد الصاوي, هاني وهيب عبد الملاك, ميرفت ثابت نجيب, احمد محمد دسوقي

(قسم التشريح – كلية الطب – جامعة عين شمس)

ملخص البحث

خلفية البحث: تشكل إصابات الدماغ الجرحية مشكلة مجتمعية ذات أهمية كبيرة في كل من المرض والوفاة. علاوة علي ذلك قد تؤدي الإصابات الجرحية البسيطة المتكررة الي زيادة خطر الإصابة بالأمراض التنكسية العصبية مثل الزهايمر. من ناحية اخري لا يوجد علاج فعال للتغلب علي أو إصلاح تلف الدماغ الناجم عن إصابات الدماغ الجرحية. ومع ذلك، فقد تبين أن السريبروليسين يحسن بشكل ملحوظ الوظيفة الإدراكية لدى الأشخاص الذين يعانون من الخرف الوعائي.

الهدف: محاكاة نموذج ارتجاج الدماغ المتكرر في الفئران البيضاء البالغين لتوضيح تأثيره على بنية البطانة العصبية وتحديد التأثيرات المحسنة المحتملة لامدادات السريبروليسين على التغيرات اللاحقة للصدمات في البطانة العصبية ومنطقة تحت البطين.

الطريقة: تم تقسيم عشرون جرذا ابيض بالغ إلى أربع مجموعات على النحو التالي: مجموعة ضابطة، مجموعة الارتجاج، مجموعة التعافي التلقائي والمجموعة المعالجة بواسطة السريبروليسين. تمت معالجة عينات المخ للمجهر الضوئي والكيمياء المناعية يليها تحليل إحصائي.

النتائج: كشفت نتائج الدراسة الحالية أن العلاج المبكر بالسريبروليسين نتج عنه تكون الخلايا العصبية وصاحبه تحسن تغيرات البطانة العصبية ما بعد الصدمة.