# Influence of Human Umbilical Cord Blood Mesenchymal Stem Cells on Cerebrolysin Amelioration of Neurological Deficit Following Cerebral Ischemia-Reperfusion Injury in Rats

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## Abstract

*Background:* Stroke results in disability and rapid brain damage. Many studies reported that stem cells may be considered as a potential therapeutic strategy for treatment of strokes.

*Aim of Study:* In this study, we aimed to investigate the effects of adding human Umbilical Cord Blood Mesenchymal Stem Cells (hUCB-MSCs) to cerebrolysin in improving the neurological function after stroke in rats.

*Material and Methods:* Rats randomly divided into four groups: Group 1: Control group, Group 2: Ischemia-reperfusion (I/R) untreated group, Group 3: (I/R) cerebrolysin-treated group, and Group 4: (I/R) both cerebrolysin and stem cell treated group. After 4 weeks' animals were sacrificed, Malondialdehyde (MDA), Superoxide Dismutase (SOD), Tumor Necrosis Factor-α (TNF-α), nuclear factor κB (NF-κB), ACH E, and P-AKT levels were measured. Also brain sections were examined histopathologically for GFAP and caspase-3 activities.

*Results:* hUCB-MSCs functioned as an effective stimulator for enhancing the neuroprotective effect of cerebrolysin through activation of the pro-survival protein Akt with subsequent inhibition of the apoptotic signaling pathways. In addition, this combination can effectively inhibit intracellular ROS generation, inhibit neuro-inflammatory cytokines, reduce the infarct volume and improve the neurological deficits in the motor function.

*Conclusion:* This combination also showed improvement in histological and immunohistochemical pictures of the brain tissue.

*Key Words: Stroke – Malondialdehyde – Superoxide dismutase – Tumor necrosis factor-α – Nuclear factor κB – Cerebrolysin.*

## Introduction

**STROKE** results in long-term functional disability and rapid brain damage [1]. Pharmacological treatment for this serious condition depends mainly on recombinant tissue plasminogen activator (rtPA) which has limited success rate because of the increased incidence of subarachnoid hemorrhage as well as the narrow therapeutic window of time between infarction and treatment (4-5 hours) [2]. Many other pharmacological agents which classified as neuroprotective drugs aiming to stop or decrease the brain damage induced by the stroke but with limited efficacy in clinical trials [3].

Cerebrolysin is a brain-derived neuropeptide anti-inflammatory mixture isolated from the pig. It has been tried in the treatment of many neurological disorders including stroke, Alzheimer's disease, vascular dementia and other neurodegenerative diseases [4,5].

Several clinical trials have shown that treatment with cerebrolysin promotes neurogenesis, reduces cerebral infarction and enhance brain functional recovery in rat cerebral infarction after transient ischemia [6].

Recently, many studies have reported that stem cells have many beneficial outcomes in modulating apoptosis, inflammation and have a regenerative effect on damaged tissues, so considered as a potential therapeutic strategy to treat strokes [7,8].

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*Abbreviations:*

| | |
|---|---|
| AchE | : Acetyl Choline Esterase Enzyme. |
| GFAP | : Glial Fibrillary Acidic Protein. |
| hUCB-MSCs | : Human Umbilical Cord Blood Mesenchymal Stem Cells. |
| MCAO | : Middle Cerebral Artery Occlusion. |
| MNCs | : Mononuclear Cells. |
| rtPA | : Recombinant Tissue Plasminogen Activator. |
| MDA | : Malondialdehyde. |
| SOD | : Superoxide Dismutase. |

*Aim of the work:* Here we tried to demonstrate the effects of administration of human umbilical cord blood mesenchymal stem cells (hUCB-MSCs) with cerebrolysin in improving the neurological function after stroke in Wistar rats.

## Material and Methods

*Animal welfare & ethical statements:*

Adult male Wistar rats, weighing 200-250gm, were obtained from National Research Laboratory, Cairo, Egypt. At September 2017 the rats were housed under controlled environmental conditions, and kept on a 12-hour light-dark/cycle, fed standard pellet chow and allowed free access to tap water. All experimental protocols were approved by the Ethics Committee of Zagazig University. All efforts were made to minimize the number of animals used and their suffering. All animals were kept in the Animal House for five days before the experiment. Animals were cared for in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication No.80-23) revised 1996.

*Experimental design:*

32 male rats randomly divided into four groups, each group contain 8 rats: Group I the control group, Group II Ischemia-reperfusion (I/R) untreated group, Group III (I/R) cerebrolysin-treated group, and Group IV (I/R) both cerebrolysin and stem cell treated group. Rats of the control group were subjected to the surgical procedures described below, except for bilateral common carotid artery ligation. In the (I/R) untreated group, rats were subjected to 30 minutes of ischemia via bilateral common carotid artery ligation followed by reperfusion. Rats of (I/R) cerebrolysin-treated group were subjected to 30 minutes of ischemia then reperfusion. One hour after reperfusion this group received 5ml/kg/day cerebrolysin intraperitoneally (i.p.) for 4 weeks. This dose of cerebrolysin has been examined as a neuroprotective dose [9], whereas in (I/R) group received cerebrolysin and hUCB-MSCs, rats were subjected to 30 minutes of ischemia then reperfusion. One hour after reperfusion this group received 5ml/kg/day cerebrolysin (i.p.) for 4 weeks and also injected with $1.5 \times 10^6$ cells of hUCB-MSCs intravenously single dose. All efforts were made to minimize the number of animals used and their suffering.

*Surgical procedure:*

Rats were anesthetized with urethane (1.3g/kg, i.p.) a ventral midline incision was made to expose both common carotid arteries then gently freed from the carotid sheath and vagus nerve [10].

For achieving ischemia each common carotid artery was clamped for 30 minutes using a non-traumatic artery clamps. Then, the carotid arteries were released by removing the clamps allowing reperfusion, restoration of the blood flow in the carotid arteries was observed carefully to be confirmed. Animals were returned back to their cages following the surgical procedure and kept for 4 weeks. Then, they were sacrificed. Both cerebral hemispheres of each rat were dissected, ice cooled, homogenized and centrifuged to obtain cerebrum extract according to Wong et al., [11].

*Preparation of hUCB -MS Cs:*
*Collection of the cord blood:*

After informed consent of the parents, the cord blood samples were collected under complete aseptic conditions from full-term placentae of healthy women (n=8, 24-35 years old) who had undergone full-term cesarean section.

*Isolation of mononuclear cells (MNCs):*

The MNCs isolation was processed as reported by [13]. In brief, in 50ml tubes containing 2mM EDTA (Lonza, Bioproducts) dissolved in 5ml PBS, eight blood samples (50ml each) were obtained. Then, the samples were diluted 3x with PBS buffer pH 7.2 containing 2mM EDTA. Over 15mL of Ficoll-PaqueTM ($\rho = 1.077$g/ml) Lymphocyte Separation Medium 1.077, Lonza Bioproducts), 35ml of diluted cell suspension was carefully layered in 50ml conical tubes and centrifuged at 400xg for 20 minutes at 20°C in a swinging bucket rotor without a break. Aspiration of the upper layer was done, leaving MNCs layer. The MNCs layer was carefully transferred into a new 50ml conical tube, filled with buffer, mixed and centrifuged at 300xg for 10 minutes at 20°C. The supernatant was carefully aspirated.

*Test of cell viability* [12]:

Cells were tested to detect their viability by trypan blue test (0.4% trypan blue/PBS). A 100uL sample was added to an equal volume of dye (Stem Cell Technologies, USA).

*Preparation of hUCB-MSCs* [13]:

The MNCs were sub-cultured in Dulbecco's Modified Eagle's Medium (1.0g/L glucose, 10% fetal bovine serum and 1% penicillin-Streptomycin-Amphotericin B Mixture as 10IU/10IU/25ug/100 ml) (LonzaBioproducts, Belgium) at a concentration of 5000/cm2/0.2-0.3ml media. The culture was incubated in a humidified incubator containing

5% Co2 at 37ºC (Heraeus, Germany). The medium was changed every 3 days (to remove non-adherent cells) for 10 days as a primary culture and the cellular growth was examined daily under the inverted microscope. Then, the whole adherent cells were detached with 0.25% trypsin/ EDTA 1mM (1:250) [13]. 50ml conical tube, filled with buffer, mixed and centrifuged at 300xg for 10 minutes at 20ºC. The supernatant was carefully aspirated.

*Immunophenotypic characterization of hUCB-MSCs* [12]*:*

The expression of surface antigen markers CD34 and CD 105 was determined using flowcytometry in the laboratories of Zagazig university hospitals. Antibody against human CD105 (Cat No: 323205) or human CD34 (Cat No: 4084644) were obtained from BD Bioscience. The hUCB-MSCs were incubated with these antibodies for 40min at 40ºC. After washing, the expression of surface antigens of cells was analyzed by FAC Scan flow cytometer (Becton Dickinson, Heidelberg, Germany) [12].

*Biochemical analysis:*

*Determination of lipid peroxidation:*

Oxiselect TABRS Assay Kit (MDA Quantitation) (Cell Biolabs, Inc. San Diego, USA) used for measuring MDA level. SOD activity was measured according to [14].

*Determination of brain NF-κB:*

NFκB level was determined in brain tissue homogenates using a double antibody sandwich Enzyme-Linked Immunosorbent Assay (ELISA) using commercially available ELISA kit (Glory Science Co., Ltd, De Rio, TX, USA).

*Determination of brain TNF-α:*

Brain homogenates were used to determine TNF-α by using commercially available ELISA kit based on the instructions of the manufacturer (R & D systems, USA).

*Determination of brain Ach E:*

Quantitative determination of rat acetyl cholinesterase enzyme (AchE) level in brain homogenates by using commercially available ELISA kit (CSB-E 11304r) according to the manufacturer's instructions.

*Determination of brain P-Akt:*

In the brain homogenates, phosphorylated-Akt (P-Akt) activities were determined using a solid phase sandwich (ELISA). (kit-3997, DRG International, Inc., USA) according to the manufacturer's instruction.

*Measurement of motor coordination:*

In our study Rotarod apparatus was used for assessing motor coordination (Ugo Basile, Italy), a rotating (18r.p.m) bar (2.5cm diameter). To remain on the apparatus, rats were first trained. On the day of testing motor coordination, the latency to fall from the apparatus was recorded (one trial of a maximum of 120 seconds) [15].

*Histological methods:*

After of excision of rat brains, they were fixed in 10% formalin saline. Then we processed to obtain 5-μm-thick paraffin sections, which were then stained with hematoxylin and eosin stain [16].

*Immunohistochemical methods:*

Immunostaining was performed using the avidin-biotin-peroxidase technique for detection of Glial Fibrillary Acidic Protein (GFAP) and caspase 3. Paraffin sections mounted on coated slides were deparaffinized and treated with 0.01M citrate buffer for 10 minutes to unmask antigens. Then sections were incubated in $H_2O_2$ for 10 minutes to abolish endogenous peroxidase activity before blocking with 5% horse serum for 2h at room temperature to inhibit non-specific immunoreaction. Primary monoclonal anti-GFAP serum (AM020-5M Bio-Genex) and rabbit-anti-cleaved caspase3 serum (Cat. No. PAI-29157, Thermo Fisher Scientific Co., USA) were applied at 1:5000 dilutions and 1:1000, respectively. Sections were incubated with both 1ry monoclonal antisera for 36h at 4ºC. After washing, they were incubated with biotinylatd secondary antibodies (REF85-9043-Zymed1:200) for 5h, then followed by avidin-biotin peroxidase complex. Finally, the immune reaction was visualized with 0.05% diaminobenzidine. Then slides were counterstained with Mayer's hematoxylin before mounting [17,18].

*Morphometric analysis:*

The area percentage of immune reaction to GFAP in astrocytes and area percentage of immune reaction to caspase 3 were measured by using the Fiji Image J (1.51n, NIH, USA) program.

*Statistical analysis:*

Data were tabulated as mean $\pm$ SEM. We used one-way analysis of variance (ANOVA test) for analysis of data, followed by LSD (least significance difference) test using SPSS software (SAS Institute Inc., Carg, NC, USA). Statistically, significant difference was considered when $p < 0.05$.

## Results

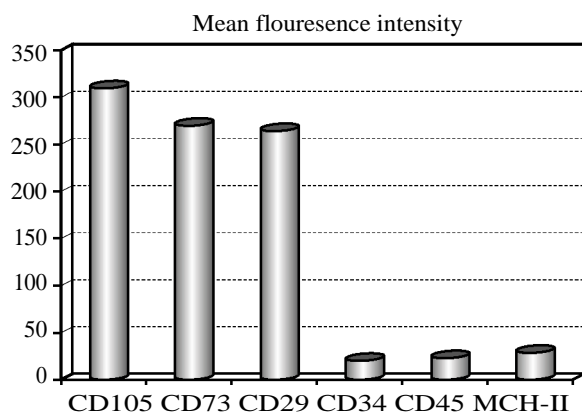*Results of flow cytometry:*

Mean flouresence intensity



Fig. (1): Shows Fluorescence-Activated Cell Sorting (FACS) analysis for characterization of hUCB-MSCs. Mesenchymal stem cell markers D29, CD73, and CD105 were expressed with high mean fluorescence intensity whereas CD34 and CD45 and MHC-II which are markers for hematopoietic stem cells were not expressed with the same intensity.

*Homing of hUCB -MSCs in the brain by detection of the human albumin gene:*

It includes two steps; DNA extraction from the rat brain using TIAN genomic DNA kit (Beijing, China) and polymerase chain reaction to detect presence of human albumin gene in Group IV rats.
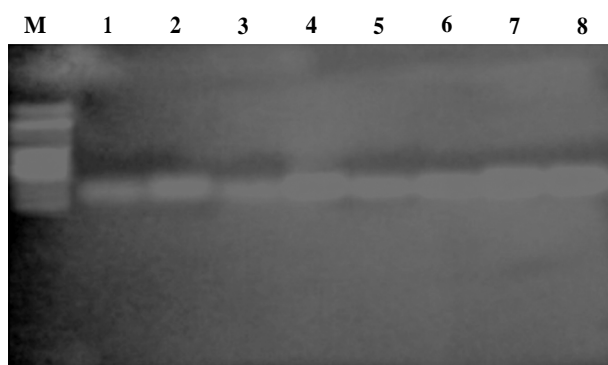


Fig. (2): Homing of hUCB-MSCs in the brain.

A photograph showing agarose gel electrophoresis of PCR product (329bp) of human albumin gene showing M; marker (100bp), positive results for rats received human umbilical cord blood MSCs (Lanes 1-8) Fig. (2).

*Effect of hUCB-MSCs and cerebrolysin on brain content of TNF α:*

In the (I/R) untreated group the level of TNF-± was significantly elevated to 15.4 ±0.58pg/mg of protein compared to the control group. This in-

creased level was significantly suppressed by treatment with cerebrolysin to 7.83±0.27pg/mg when compared to (I/R) untreated group. Adding hUCB-MSCs to cerebrolysin in the stem cell-treated group IV resulted in significant decreased TNF- α level to 5.57±0.43 pg/mg compared to both (I/R) untreated group and group III which treated with cerebrolysin alone Fig. (3).

*Effect of h UCB-MSCs and cerebrolysin on brain content of NF- κB:*

NF- κB level in the brain was significantly increased in the (I/R) untreated group to 3.7 ±0.26 U/L compared to the control non-ischemic group. Treatment with cerebrolysin resulted in a significant reduction in the brain NF- κB level to 1.91 ±0.19 U/L compared to the (I/R) untreated group. In the group IV treated with hUCB-MSCs plus cerebrolysin, brain NF- κB level was 1.06±0.11U/L which was significantly lower than both (I/R) untreated group and cerebrolysin treated group Fig. (3).

*Effect of hUCB-MSCs and cerebrolysin on brain-Reactive Oxygen Species (ROS) content:*

Regarding Malondialdehyde (MDA) levels, the cerebral (I/R) untreated group showed significant ($p<0.05$) increased MDA level to 13.4±0.58umol/g when compared to the control group. With cerebrolysin treated group, MDA level was significantly decreased to 7.33 ±0.41umol/g compared to the (I/R) untreated group. MDA level in the Group IV received hUCB-MSCs with cerebrolysin showed significantly decreased level to 4.5 ±0.55umol/gin comparison to the (I/R) untreated group and when also compared with the cerebrolysin treated group Fig. (3).

Regarding the SOD activity, the (I/R) untreated group showed significantly decreased to 1.13±0.15 u/g in relation to the control group. SOD activity was obviously increased in cerebrolysin treated group to 2.57±0.34 u/g compared to the (I/R) untreated group. SOD activity was more significantly increased to 4.6±0.15u/g in Group IV treated with both cerebrolysin and hUCB-MSCs when compared with the (I/R) untreated group and also when compared to cerebrolysin treated group Fig. (3).

*Effect of h UCB-MSCs and cerebrolysin on brain Ach E level:*

(I/R) untreated group induced significant ($p <0.05$) decrease in the level of Ach E from 11.83 ± 0.32u/gm in the control group to 3.13 ±0.15u/gm. Cerebrolysin treatment produced significant ($p<0.05$) increase in the level of ACH E to 5.8±0.26

u/gm compared with (I/R) untreated group adding hUCB-MSCs to cerebrolysin in Group IV produced significant ($p<0.05$) increase in the level of Ach E to 9.47±0.35u/gm compared to both (I/R) untreated group and cerebrolysin treated group Fig. (4).

### Effect of hUCB-MSCs and cerebrolysin on P-Akt:

The level of P-Akt showed significantly ($p <0.05$) decrease from 11.23±0.52u/g in the control group to 2.83±0.55u/g in the (I/R) untreated group, with cerebrolysin treatment the level of P-Akt significant increased ($p<0.05$) from 2.83±0.55u/g in the (I/R) untreated group to 6.83 ±0.67u/g. Adding hUCB-MSCs to cerebrolysin in Group IV resulted in a significant increase ($p<0.05$) the level of P-Akt to 9.67±0.64u/g compared to both (I/R) untreated group and cerebrolysin treated group Fig. (4).

### Locomotor activity and rotarod test:

Adding hUCB-MSCs to cerebrolysin in Group IV showed a significant increase in the motor coordination tested by the rotarod, indicated by significant increase in the duration of a sustained balance of rats on rotarod when compared to rats in both (I/R) untreated group and cerebrolysin treated group Fig. (5).
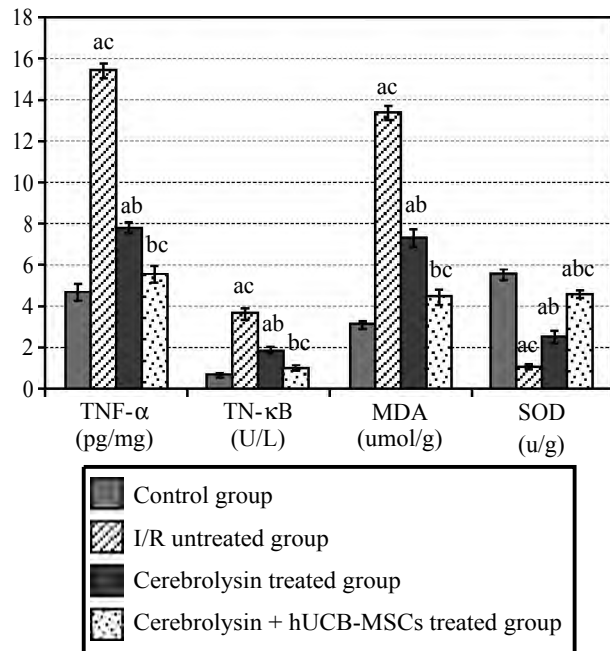


Fig. (3): The effect of hUCB-MSCs and cerebrolysin on levels of TNF-α NF-κB, MDA and SOD activity in the brain homogenates of rats.

- Data represent mean ± SE. $p<0.05$ was considered significant.
- MDA malondialdehyde, SOD superoxide dismutase, TNF-α tumour necrosis factor alpha, NF-κB nuclear factor kappa B. n=8.
a: Significant difference from the control group.
b: Significant difference from the I/R untreated group.
c: Significant difference from the Cerebrolysin treated group.



Fig. (4): The effect of hUCB-MSCs and cerebrolysin on levels of AchE and P-Akt in the brain homogenates of rats.

- Data represent mean ± SE. $p<0.05$ was considered significant. n=8.
- AchE acetylcholine esterase enzyme, P-Akt phosphorylated Akt.
a: Significant difference from the control group.
b: Significant difference from the I/R untreated group.
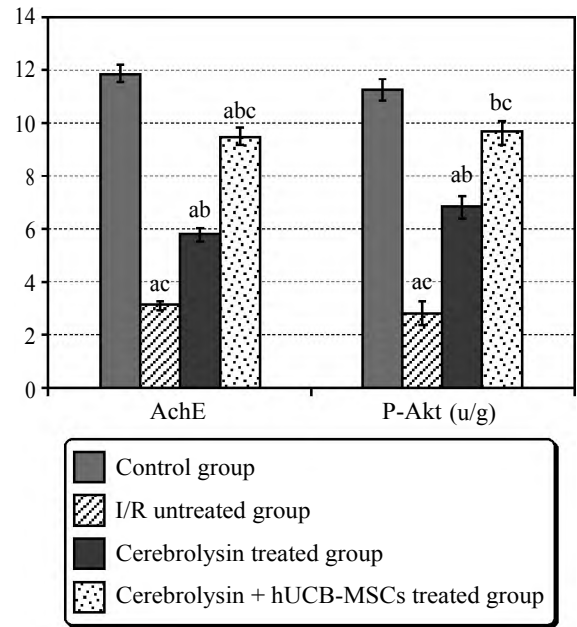c: Significant difference from the Cerebrolysin treated group.



Fig. (5): Effect of hUCB-MSCs and Cerebrolysin on the time spent on the rotarod test in rats.

- Data represent mean ± SE.
- $p<0.05$ was considered significant. n=8.
a: Significant difference from the control group.
b: Significant difference from the I/R untreated group.
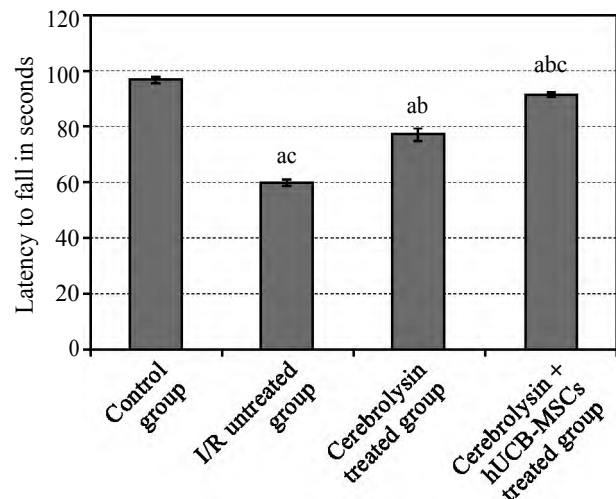c: Significant difference from the Cerebrolysin treated group.

### Histological results:

H & E stained sections of left frontal cerebral cortex control group showed the normal histological appearance of granular cells, pyramidal cells with vesicular nuclei, basophilic cytoplasm and processes and blood vessels, which were surrounded by normally visualized neuropil Fig. (6A).

Sections in the left frontal cerebral cortex of (I/R) untreated group showed that most cells were distorted with deeply stained shrunken nuclei and cytoplasm, unstained areas were surrounding the cells and areas of neuropil vacuolations and cellular degeneration was also seen Fig. (6B). Examination of the left frontal cerebral cortex of cerebrolysin treated group showed that some of the nerve cells in the cortical layers appeared normally with large vesicular nuclei, while other cells appeared distorted with deeply stained nuclei and cytoplasm with unstained areas were surrounding the cells. Some normal granular cells were seen Fig. (6C). Sections of the left frontal cerebral cortex of stem cell and cerebrolysin treated group showed that most of the nerve cells in the cortical layers appeared normal with large vesicular nuclei and also normal histological appearance of granular cells, neuropil and blood vessels were observed in this group Fig. (6D).
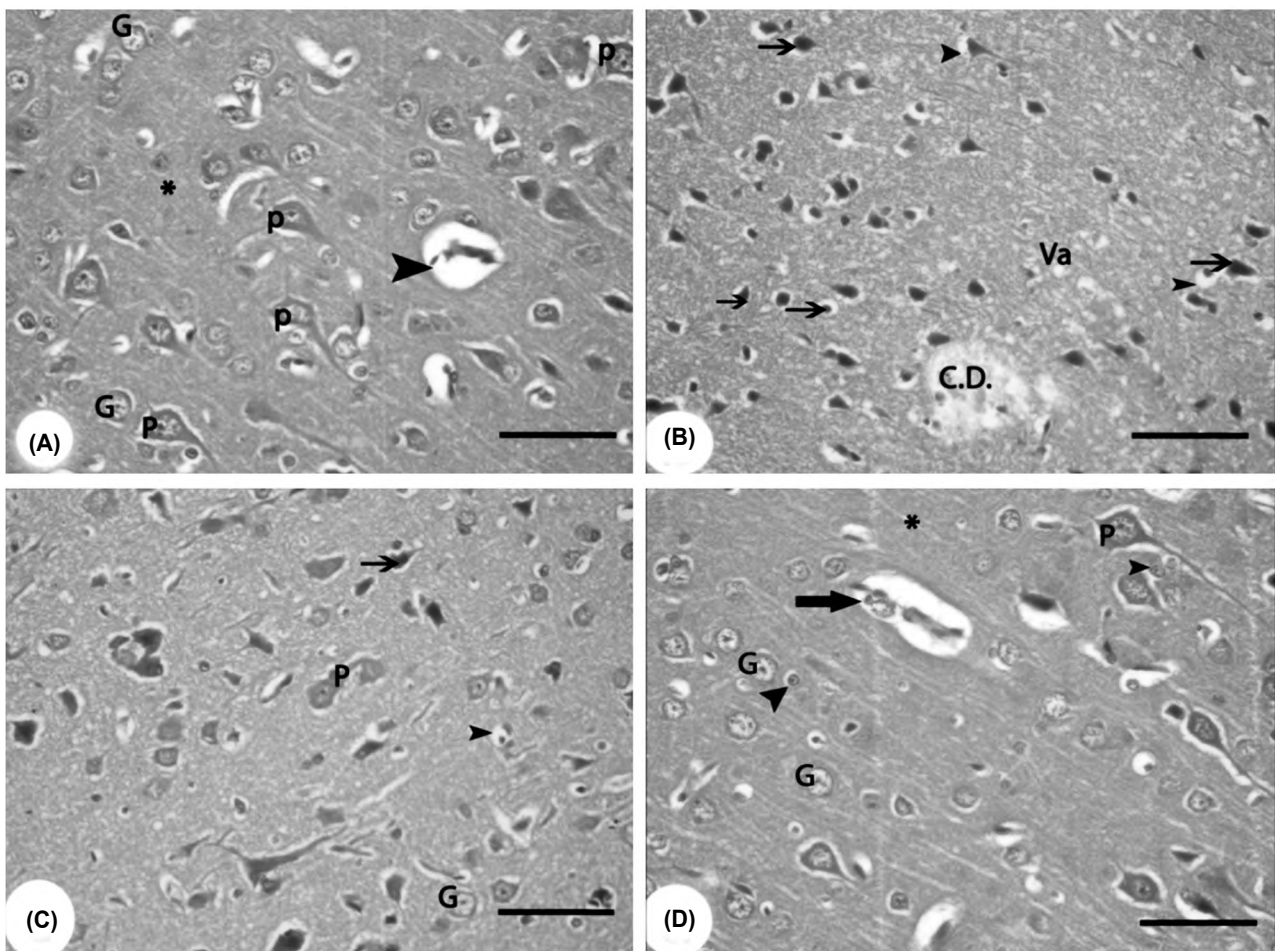


Fig. (6): (A) A photomicrograph of a section of left frontal cerebral cortex from control group showing a normal histological appearance of granular cells (G), pyramidal cells (P) with vesicular nuclei, basophilic cytoplasm and processes and blood vessels (arrowhead), that are surrounded by normally visualized neuropil (asterisk). (B) A photomicrograph of a section of left frontal cerebral cortex from (I/R) untreated group showing that most cells are distorted with deeply stained shrunken nuclei and cytoplasm (arrow). Unstained areas are surrounding the cells (arrowhead). Areas of neuropil vacuolations (Va) and cellular degeneration (C.D.) are also seen. (C) A photomicrograph of a section of left frontal cerebral cortex from cerebrolysin treated group showing that some of the nerve cells in the cortical layers appear normally with large vesicular nuclei (P). Other cells appear distorted with deeply stained nuclei and cytoplasm (arrow) with unstained areas are surrounding the cells (arrowhead). Some normally appeared granular cells (G) are seen. (D) A photomicrograph of left frontal cerebral cortex from stem cell and cerebrolysin treated group showing that most of nerve cells in the cortical layers appear normally with large vesicular nuclei (P), normal histological appearance of granular cells (G), neuropil (asterisk) and blood vessels (thick arrow) is observed. Neuroglial cells (arrowhead) are seen. (H & E X400, Scale bar; 40 μm).

*Immunohistochemical results:*

Examination of left frontal cerebral cortex sections of the control group stained with anti-GFAP antibody revealed weak positive immunoreaction in the cytoplasm of few astrocytes Fig. (7A), while sections of (I/R) untreated group revealed

strong positive immunoreaction in the cytoplasm of many astrocytes Fig. (7B). Left frontal cerebral cortex sections of cerebrolysin treated group revealed moderately positive immunoreaction in the cytoplasm of some astrocytes Fig. (7C). Cerebrolysin and stem cell treated group sections showed weak positive immunoreaction in the cytoplasm of few astrocytes Fig. (7D).

Concerning reaction to caspase 3, left frontal cerebral cortex sections of the control group stained with anti-caspase 3 antibody revealed weak positive immunoreaction in the cytoplasm of few neurons Fig. (7A), whereas sections of I/R untreated group revealed strong positive immunoreaction in the cytoplasm of many neurons Fig. (7B). Left frontal cerebral cortex sections of cerebrolysin treated group revealed moderately positive immunoreaction in the cytoplasm of the neurons Fig. (7C). Sections of cerebrolysin and stem cell treated group showed weak positive immunoreaction in the cytoplasm of few neurons Fig. (7D).
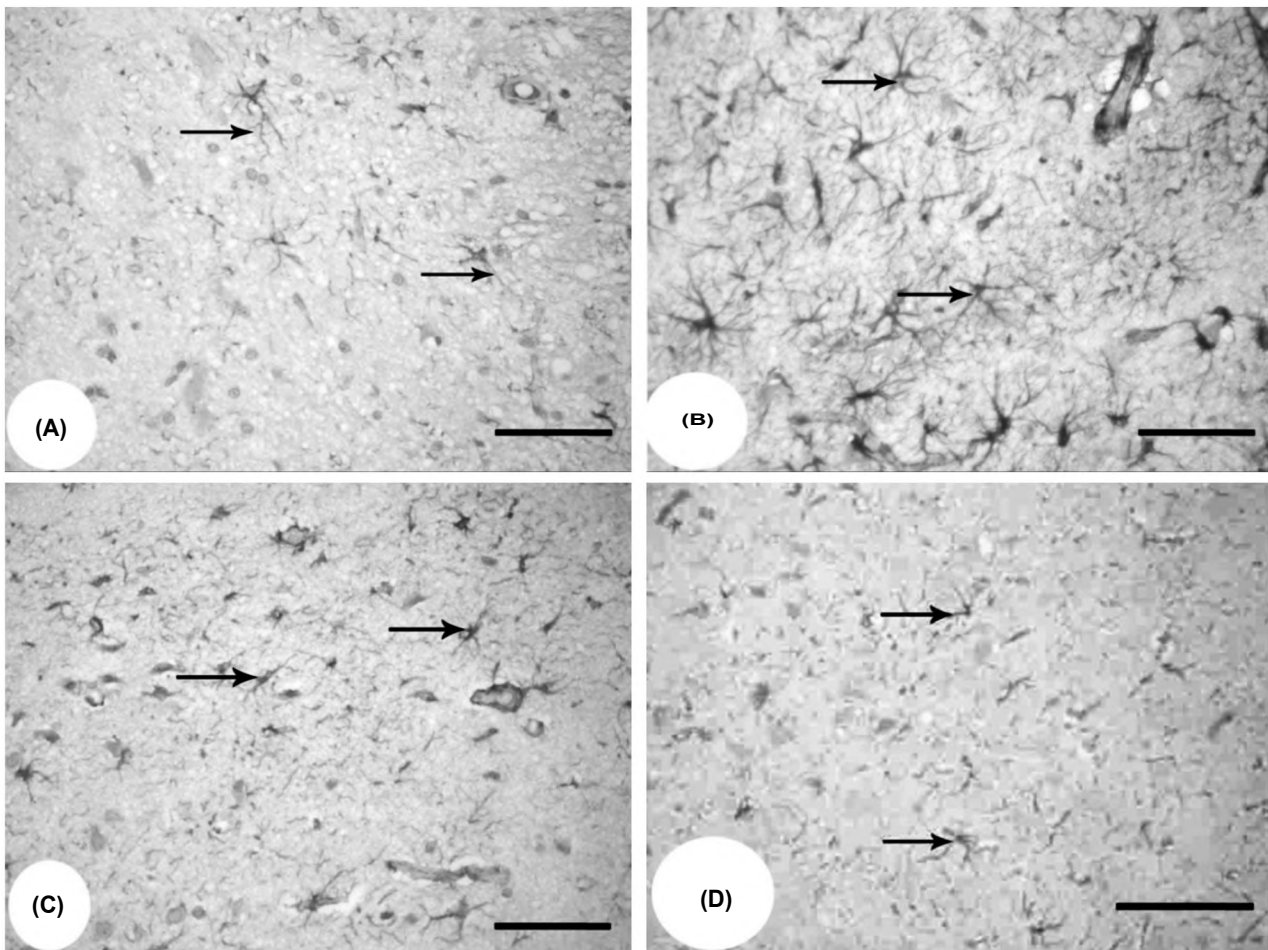


Fig. (7): (A) A photomicrograph of a section of left frontal cerebral cortex from control group showing weak immunostaining reaction for GFAP in the cytoplasm of some astrocytes (arrow). (B) A photomicrograph of a section of left frontal cerebral cortex from (I/R) untreated group showing strong immunostaining reaction for GFAP in the cytoplasm of many astrocytes (arrows). (C) A photomicrograph of a section of left frontal cerebral cortex from cerebrolysin treated group showing moderate immunostaining reaction for GFAP in the cytoplasm of many astrocytes (arrows). (D) A photomicrograph of left frontal cerebral cortex from stem cell and cerebrolysin treated group showing weak immunostaining reaction for GFAP in the cytoplasm of few astrocytes (arrows). (Immunoperoxidase technique for GFAP X400, Scale bar; $40\mu$m).

*Results of Immunohistochemical stains:*

*Effect of hUCB -MSCs and Cerebrolysin on area percentage of immune reaction to GFAP:*

Adding hUCB-MSCs to Cerebrolysin in Group IV showed a significant decrease in the area percentage of immune reaction to GFAP when compared to rats of (I/R) untreated group. Cerebrolysin treated group also showed a significant decrease in the area percentage of immune reaction to GFAP when compared to rats of(I/R) untreated group (Table 1).

*Effect of hUCB-MSCs and Cerebrolysin on area percentage of immune reaction to caspase 3:*

Adding hUCB-MSCs to Cerebrolysin in Group – showed a significant decrease in the area percentage of immune reaction to caspase 3 when compared to rats of (I/R) untreated group. Cerebrolysin treated group also showed a significant decrease in the area percentage of immune reaction to caspase 3 when compared to rats of (I/R) untreated group (Table 2).
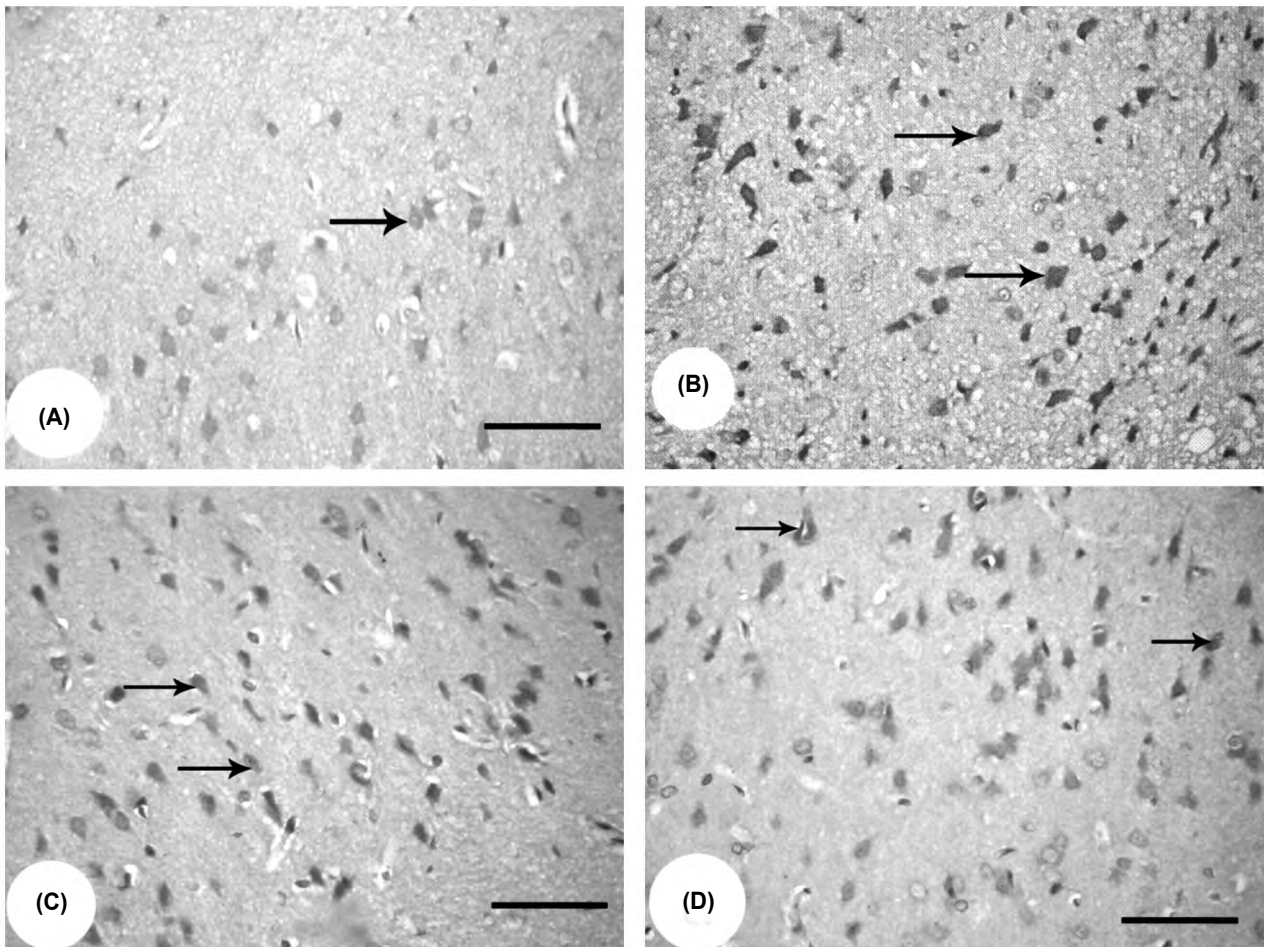
Fig. (8): (A) A photomicrograph of a section of left frontal cerebral cortex from control group showing weak immunostaining reaction for caspase 3 in the cytoplasm of some neurons (arrow). (b)A photomicrograph of a section of left frontal cerebral cortex from (I/R) untreated group showing strong immunostaining reaction for caspase 3 in the cytoplasm of many neurons (arrows). (C)A photomicrograph of a section of left frontal cerebral cortex from cerebrolysin treated group showing moderate immunostaining reaction for caspase 3 in the cytoplasm of some neurons (arrows). (D)A photomicrograph of left frontal cerebral cortex from stem cell and cerebrolysin treated group showing weak positive immunostaining reaction for caspase 3 in the cytoplasm of few neurons (arrows). (Immunoperoxidase technique for caspase 3 X400, Scale bar; 40 μm.

Table (1): Effect of hUCB-MSCs and Cerebrolysin on area percentage of immune reaction to GFAP.

| Parameter | Control Group Mean ± SEM | I/R untreated group Mean ± SEM | Cerebrolysin treated group Mean ± SEM | Cerebrolysin + hUCB-MSCs treated group Mean ± SEM |
|---|---|---|---|---|
| • Area percentage of immune reaction to GFAP | 3.03± 0.58 | 14.3 [a]± 3.31 | 5.6[b]± 0.996 | 4.6[b]± 0.86 |

a: Means significant with the control group.
b: Means significant with (I/R) untreated group.

Table (2): Effect of hUCB-MSCs and Cerebrolysin on area percentage of immune reaction to caspase 3.

| Parameter | Control Group Mean ± SEM | I/R untreated group Mean ± SEM | Cerebrolysin treated group Mean ± SEM | Cerebrolysin + hUCB-MSCs treated group Mean ± SEM |
|---|---|---|---|---|
| • Area percentage of immune reaction to caspase 3 | 4.61 ± 0.6 | 12.51 [a]± 0.96 | 5.35 [a]± 0.6 | 4.6[a]± 0.61 |

a: Means significant with the control group.
b: Means significant with (I/R) untreated group.

## Discussion

Recently stem cell therapy has emerged as a promising alternative therapy for many disorders, based on its ability to promote tissue repair and angiogenesis [19]. Indeed, hUCB-MSCs show higher proliferation rate and expansion potential compared with adult bone marrow stem cells [20]. hUCB-MSCs have many advantages as easily and less invasively obtained after birth from cord blood, and also they don't induce immunological problems in the body of the host as they don't express T-cell co-stimulatory factor or major histocompatibility complex class II molecules [21,22].

In this study, we examined the effect of intravenous administration of hUCB-MSCs with cerebrolysin in rats after brain ischemic insult.

Experimental cerebral ischemia in rats induced oxidative stress which is an imbalance between oxidants and anti-oxidants, with increasing oxidants, leading to molecular and cellular damage, apoptosis, and neuroinflammation. Accumulation of lipid peroxides change the cell membrane permeability and damage many cell functions. MDA is an end product of lipid peroxidation which produced by ROS [23]. Another response to oxidative stress is the depletion of the antioxidant protective enzymes such as SOD and GSH which could prevent the deleterious effects of stroke-induced ROS generation [24]. So the levels of these enzymes in the brain tissue can estimate the amount of oxidative stress. In this study, the levels of MDA and SOD activity showed that the cerebral ischemia increased ROS and decreased the activity of endogenous antioxidant enzymes.

Treatment with both cerebrolysin and hUCB-MSCs resulted in significantly lower levels of MDA and increased the activity of SOD in the brain tissue, indicating that they exert an antioxidant as well as neuroprotective role against cerebral ischemia via alleviating oxidative stress.

ROS activates inflammatory cellular signaling pathways, as increasing the expression of TNF-α and promote the expression of NF-κB. TNF-α, is an important pro-inflammatory cytokine, has a great role in stimulating inflammation, participating in many deleterious cellular responses including proliferation up to cellular apoptosis and death [25].

NF-κB is another important pro-inflammatory cytokine involved in the inflammatory responses. It is sensitive to oxidative stress and is thought to play a major role in the development of many cellular effects as included in fibrosis, inflammation, cell proliferation, tumour development, and apoptosis [26].

In the present study we found that, the administration of hUCB-MSCs with cerebrolysin in the Group IV treated rats resulted in a reduction of the neuroinflammatory response and neuronal damage induced by cerebral I/R injury indicated by a significant decreased TNF-α and NF-κB levels in the brain tissue [27] found that NF-κB is linked with neuronal cell death in focal cerebral ischemia.

PI3k/Akt pathway is a major intracellular signaling pathway in promoting and maintaining cell survival [28]. Numerous studies have shown that the activation of PI3k/Akt can regulate the expression of BCl-2 level and inhibit ROS generation, in addition, increased BCl-2 level can scavenge free radicals and effectively inhibit the formation of superoxides which consequently ameliorates the oxidative damage in ischemic neurons caused by ROS over-expression [29,30].

Our results reported that, treatment with hUCB-MSCs plus cerebrolysin significantly increased the level of phosphorylated Akt (P-Akt) compared with cerebrolysin without stem cell administration. These results suggested that hUCB-MSCs enhance neuronal cell survival and blocking apoptotic cell death through P-Akt up-regulation. These results are in accordance with Crowder & Freeman [31] who reported that, the over expression of activated Akt promoted the survival of cervical neurons in cell culture and also in agreement with Pugazhenthi et al., [32] who demonstrated that, activated Akt stimulated the anti-apoptotic effects in several different cell types including hippocampal neurons.

Chen et al., [33] also demonstrated that activation of PI3k/Akt pathway is included in stimulating dendrite branching and regulating neurogenesis.

AchE is an enzyme that hydrolyzes the neurotransmitter acetylcholine (Ach) at the post-synaptic membrane. The cholinergic system has an important modulatory role in the brain. It regulates high cognitive functions such as learning, memory and neuronal development [34].

In this study, cerebral I/R injury resulted in decreased Ach E level in the brain tissue suggesting neurotoxicity. This decline in the level of AchE was effectively reversed by treatment with cerebrolysin plus hUCB-MSCs suggesting the efficient neuroprotective effect of this combination.

In the present work, motor coordination was assessed by using accelerated rotarod. Treatment of rats with cerebrolysin and hUCB-MSCs resulted in a significant increase in the activity on the rotarod after 4 weeks of treatment as indicated by the significant increase in the duration of rotation on rotarod.

There was no significant difference observed between the treated groups regarding spontaneous alteration performance on the T-maze test.

Histological examination of H & E stained sections in (I/R) untreated group showed areas of neuronal death and cellular degeneration. This agrees Jung et al., [35], who reported that ischemia-reperfusion stroke causes marked production of ROS in mitochondria which leads to neurovascular injury, cerebral edema, neuronal death by either apoptosis or necrosis.

In cerebrolysin treated group, only some neurons were recovered. This agreed with Eapen et al., [36], who found that cerebrolysin decreased the size of infarction and ameliorated the neurologic insults that occurred following middle cerebral artery occlusion (MCAO). On the other hand, Zhang et al., [37] stated that administration of cerebrolysin 24 and 48h after stroke markedly improved cerebral functions but did not restore the normal histology of the cortex. In cerebrolysin and stem cell treated group, there was a significant improvement in H & E stained sections. This agrees with Jang et al., [38] who reported that intraperitoneal injection of hUCB-MSCs attenuated the renal injury after I/R.

Astrocytes are one of the most vital and numerous cells of the central nervous system that perform different functions [39]. They provide architectural structure, nutritional support, and homeostasis in the healthy and injured brain. Also, they regulate neuronal activity [40,41].

Injury to the brain, including ischemia, activates astrocytes causing increased expression of the Intermediate Filament (IF) such as proteins Glial Fibrillary Acidic Protein (GFAP), nestin and vimentin and altered expression of many other genes. This process is known as reactive gliosis. Reactive gliosis effectively restores the homeostasis and function of the brain [42]. This explains the increased expression of GFAP in (I/R) untreated group. After injecting cerebrolysin in group 3, the area percentage of immune reaction to GFAP is greatly diminished; this is in accordance with Muresanu, [43] who stated that cerebrolysin reduced gliosis in degenerative diseases of CNS. In cere-

brolysin and stem cell treated group, the area percentage of immune reaction to GFAP was non-significantly reduced when compared with that of cerebrolysin treated group. This agrees with Pavlichenkoa et al., [44] who found that MSCs injection in a rat model of stroke significantly reduces the area of gliosis.

Caspase-3 is one of the executioner caspases, that has an essential role in apoptosis of brain cells [45]. In (I/R) model of stroke, the damage resulting from ischemia and oxidative stress causes neuronal death and apoptosis. This explains the highly significant increase in area percentage of immune reaction to caspase 3 in (I/R) untreated group when compared with the control group [46].

In the cerebrolysin treated group, there was a highly significant reduction of immune reaction to caspase 3 in comparison to (I/R) untreated group due to the effect of cerebrolysin in enhancing the recovery of neurons and decreasing apoptosis [47]. In cerebrolysin and stem cell treated group, the expression of caspase 3 was markedly reduced toward the normal due to the additional effect of MSCs in reducing apoptosis [48]. This also agrees with Suda et al., [49] who reported that Bone Marrow Mononuclear Cells (BMMNCs) injection in the early phases of stroke diminished oxidative damage.

In summary, the use of hUCB-MSCs as a therapeutic agent for stroke is attractive. The study was designed to compare the therapeutic efficacy of combined hUCB-MSCs with cerebrolysin against treatment with cerebrolysin alone.

The results of this study showed that treatment with hUCB-MSCs significantly attenuated ROS contents with restoration of normal SOD activity as an antioxidant enzyme, modulated the PI3k/Akt pathway that regulates cell proliferation, cell survival and migration moreover down-regulated TNF-α that causing neuronal cell death via induction of free radicals in glial cells and apoptosis [50] as well as decreased the pro-inflammatory NF-κB. In addition, intravenous injection of hUCB-MSCs reduced apoptosis and promoting cellular proliferation after stroke with improved motor function compared to the rats of group who received cerebrolysin alone.

With considering these findings in total, we suggest that hUCB-MSCs will constitute an efficient source of stem cell therapy against focal cerebral ischemia. Further clinical studies on human are needed to determine its efficacy, long-term prognosis and optimal dosage of MSCs.

*Conclusion:*

We reported here that hUCB-MSCs functioned as an effective stimulator for enhancing the neuro-protective effect of cerebrolysin through activation of the pro-survival protein Akt with subsequent inhibition of the apoptotic signaling pathways. In addition, this combination can effectively inhibit intracellular ROS generation, inhibit neuro-inflammatory cytokines, reduce the infarct volume and improve the neurological deficits in the motor function and showed marked improvement in the histological and immunohistochemical picture of the cerebral cortex.

*Author contributions:*

Noha A.T. Abbas designed experiments. Noha A.T. Abbas performed surgeries. Noha A.T. Abbas, Sara M. Abdel Aal and Hanaa S.E. Mousa performed experiments. Sara M. Abdel Aal and Hanaa S.E. Mousa performed histological verification. Noha A.T. Abbas, Sara M. Abdel Aal and Hanaa S.E. Mousa wrote the paper.

*Conflicts of interest:*

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# النخاع الشوكى للعجز العصبى
## بعد إصابات نقص التروية الدماغية فى الجرذان

المقدمة: السكتة الدماغية تؤدى إلى الإعاقة وتلف سريع فى الدماغ. ذكرت العديد من الدراسات آن الخلايا الجذعية يمكن إعتبارها إستراتيجية علاجية محتملة لعلاج السكتات الدماغية.

الهدف من الدراسة: فى هذه الدراسة، نحن نهدف إلى التحقيق فى آثار إضافة الخلايا الجذعية من دم الحبل السرى البشرى إلى عقار سيريبروليسين فى تحسين وظيفة الجهاز العصبى بعد السكتة الدماغية فى الفئران.

المواد والطرق: الفئران مقسمة بشكل عشوائى إلى أربع مجموعات: المجموعة ١: مجحموعة التحكم، المجموعة ٢: السكتة الدماغية (المجموعة غير المعالجة)، المجموعة ٣: المجموعة المعالجة بالسيبروليسين، ومجموعة ٤: المجموعة المعالجة بالسيبروليسين مع الخلايا الجذعية. بعد ٤ آسابيع تم التضحية بالحيوانات، وإجراء بعض التحاليل المعملية للمخ، تم فحص الآنسجة بالميكروسكوب.

النتائج: الخلايا الجذعية من دم الحبل السرى البشرى تعمل كمحفز فعال لتعزيز تأثير عقار السيريبروليسين على السكتة الدماغية من خلال تفعيل البروتين المؤيد للبقاء على قيد الحياة Akt بالإضافة إلى ذلك، يمكن لهذا المزيج آن يمنع بفعالية توليد العوامل المؤكسدة داخل خلايا المخ، ويثبط السيتوكينات العصبية الإلتهابية، ويقلل من حجم الإحتشاء وآظهرت هذه التركيبة آيضا تحسين فى الصور النسيجية والمناعية الكيميائية لآنسجة المخ.