

Neuroprotection by Cerebrolysin and Citicoline Through the Upregulation of Brain-Derived Neurotrophic Factor (BDNF) Expression in the Affected Neural Cells: A Preliminary Clue Obtained Through an In Vitro Study

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Abstract

Objectives: Citicoline and cerebrolysin are two unique yet contentious medications because of inconsistencies in efficacy as well as the mystery surrounding their mode of action. The current study aimed to re-validate the neuroprotective benefits of these medications and investigate the possible molecular mechanism.

Methods: Neuro-2A cells were exposed to tert-butyl hydroperoxide, a consistent in vitro model of neuronal damage caused by oxidative stress. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, acridine orange/ethidium bromide (AO-EtBr) staining, and phase-view examinations were utilized to evaluate cell survival and cytotoxicity. Real-time reverse transcription-polymerase chain reaction (RT-PCR)-based gene expression studies were conducted.

Key finding: Observations revealed that these two medications had modest but considerable neuroprotective effects. While the majority of the genes' expressions remained unchanged, cerebrolysin upregulated Neuregulin 1, and both upregulated brain-derived neurotrophic factor (BDNF) expression.

Conclusion: The findings of the current study may be the first to suggest that citicoline and cerebrolysin may increase host cells' defense mechanisms (secretion neurotrophic factors) rather than carrying nutrients for cell survival. Because of its simplicity, the current study can readily be repeated to learn more about these two disputed medications for treating ischemic stroke.

Categories: Therapeutics

Keywords: neuroprotective agents, bdnf, ischemic neuronal injury, citicoline, cerebrolysin

Introduction

The effects of ischemia-induced pathological alterations in the central nervous system are widely documented. During the ischemia and reperfusion processes, free radicals are released, causing oxidative stress in neurons [1]. Despite numerous stroke therapy options, therapeutic care for cerebral ischemia is frequently limited to thrombolytic and anticoagulant medications [2,3].

Cerebrolysin and citicoline are two medications used to treat several neurological disorders, including stroke. Cerebrolysin, a peptidergic medication, is composed of 75% free amino acids and 25% low-molecular-weight peptides [4]. It was discovered to have positive benefits in both in vitro and in vivo experiments [5]. However, the likely mechanism underlying the purported positive effects remains unknown, and meta-analyses have not supported the use of cerebrolysin for stroke [6]. Similarly, citicoline (cytidine 5'-diphosphocholine) is a precursor of phosphatidylcholine, a component of the cell membrane [7]. Its ability to cure neurological problems is unclear. While citicoline was reported to be effective [8,9], some reports questioned its efficacy [10].

Despite these discrepancies, physicians frequently use these two drugs. In these conditions, the goal was to

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re-explore the potential of these two medications through experimental investigations. To achieve this goal, in the first phase, an in vitro study (cell culture method) was carried out to analyze the neuroprotective effect of these drugs as well as determine the likely mechanism in the event of any beneficial effects produced by them. The present study will provide an in vitro architecture with a consistent and reproducible platform for testing these medications.

Materials And Methods

Drugs were examined for their efficiency in recovering cultured neurons during the "reperfusion period" after oxidative stress. If neuroprotection occurred, it was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay. In addition to the phase-view analysis of the cells, they were stained with acridine orange/ethidium bromide (AO-EtBr) to detect cell death.

Cell culture conditions

It was decided to employ a well-known cell line that is available worldwide. The study used Neuro-2A (mouse neuroblastoma cell line). This cell line was obtained from the National Centre for Cell Science (NCCS), University of Pune Campus in Maharashtra, India. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g glucose/l, L-glutamine 200 mM, gentamicin 50 µg/ml, amphotericin B 2.5 µg/ml, and 10% fetal bovine serum (FBS). All of the reagents were obtained from HiMedia (Mumbai, India). Cells were cultured at 37°C in 5% CO₂ and passed in a 1:2 ratio when they reached 80-90% confluence. Cells were cultured on uncoated tissue culture-grade plastic dishes, either 24-well plates or T25 flasks, as needed.

Estimation of cytotoxicity and viability

The results of the various stages of the investigation were examined by calculating the cytotoxicity of the cells and their viability. After the treatment period, 100 µl of DMEM with MTT (0.5 mg/ml) was added to each well and incubated in 5% CO₂ at 37°C for three hours. The media were then withdrawn, and the cells were washed twice with phosphate-buffered saline (PBS). Living cells can convert tetrazolium into dark blue, insoluble formazan crystals. The crystals were solubilized with dimethyl sulfoxide (DMSO) and measured at 570 nm with a microplate reader. The live, healthy cells were expressed as a proportion of the control Neuro-2A cells using the following formula: the absorbance of treated cells × 100 = viability of cells in %.

The absorbance of control cells

Following phase contrast microscope observations, cells were stained with AO and EtBr to assess cell viability, as described by Mironova et al. [11]. The results were evaluated with a Nikon inverted microscope (Minato City, Tokyo, Japan) equipped with epifluorescence attachments.

Method of oxidative stress induction

Cells were grown in media supplemented with various concentrations of FBS (0%, 2%, 10%, and 20%). Both 0% and 20% serum were found to be harmful to the cells. 10% serum was beneficial to the cells and promoted growth. Because 2% serum was discovered to not affect the cells while also limiting cell proliferation, we opted to utilize it for tests because we preferred a steady population of cells without self-proliferation.

Tert-butyl hydroperoxide (tBuOOH) has been shown to cause oxidative stress in Neuro-2A cells, resulting in necrotic cell death that simulates neuronal death following an injury [12]. As a result, tBuOOH at various concentrations was employed to calculate the LD₅₀ in cells after 24 hours. Exposure to tBuOOH at a concentration of 250 µM for 45 minutes caused 50% cell death after 24 hours after reperfusion.

Determination of the optimal dose for cerebrolysin and citicoline

Cells were seeded in media containing 2% serum and allowed to attach to the dish for 24 hours prior to drug administration. The participants were treated with various amounts of cerebrolysin (1.6, 0.8, 0.4, and 0.2 µg/ml) and citicoline (10, 1, 0.1, and 0.01 mM). Cerebrolysin at 0.2 µg/ml and citicoline at 0.1 mM dosages showed the least cytotoxicity and were employed in additional investigations.

Effects of cerebrolysin and citicoline on neurons subjected to oxidative injury

Cells were seeded in a medium with 2% serum and were allowed to attach to the dish for 24 hours. The oxidative damage was caused by exposure to 250 µM of tBuOOH for 45 minutes, as previously described (vide supra). To simulate reperfusion after injury, the medium containing tBuOOH was replaced with either cerebrolysin (0.2 µg/ml) or citicoline (0.1 µM). After 24 hours, cultures were terminated and analyzed using the MTT assay, AO-EtBr staining, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The experimental design can be described as follows.

Analyses of mRNA levels using qRT-PCR

At the end of the experiment (48 hours), the total RNA from the cells was extracted using TRIzol reagent (Sigma-Aldrich, St. Louis, Missouri, United States) according to the manufacturer's procedure. After confirming RNA quality with NanoDrop (Thermo Fisher Scientific, Karlsruhe, Germany), known quantities were reverse-transcribed into cDNA with the Omniscript Kit from Qiagen (Germantown, Maryland, United States). The cDNA was utilized to detect the mRNA levels of many genes related to apoptosis and necrosis. BDNF and neuregulin were also tested using the primer sequences provided in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as an internal control to standardize the expressions.

Gene	Forward primer sequences	Reverse primer sequences
BDNF	5' CGAGAGGTCTGACGACGACATC 3'	5' AACCTTCTGGTCTCATCCAGC 3'
Neuregulin	Forward 5' CACCCAAGTCAGGAAGTCAAGCC 3'	Reverse 5' TGGTCCCAGTCGTGGATGTAGATG 3'
GAPDH	Forward 5' CGACTTCAACAGCAACTCCCACTC 3'	Reverse 5' GTAGCCGATTTCATTGTACATCCAGG 3'

TABLE 1: Forward and reverse primer sequences for gene expression studies

BDNF: brain-derived neurotrophic factor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase

Real-time PCR was carried out on a StepOne platform (Applied Biosystems, Waltham, Massachusetts, United States) with Faststart Universal SYBR Green Master Mix (Roche, Indianapolis, Indiana, United States). After the amplification cycles, melt curve analyses were undertaken to rule out any non-specific amplifications. mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ technique [13], with the control group as the reference. mRNA expression levels were expressed as the mean of fold changes from three different experiments plus the standard error of the mean. One-way analysis of variance (ANOVA) was used to examine expression level differences at a significance threshold of $p < 0.05$.

Results

Optimizing the cell culture system

Neuro-2A, as an immortalized cell line, tends to divide following a toxic exposure, making cytoprotective tests challenging. To address this issue, different serum supplementation concentrations in the media were evaluated for their proliferative and toxic effects. As expected, no serum supplementation (0%) resulted in cell death. Interestingly, a high serum concentration of 20% was also proven to be harmful. While both 2% and 10% were found to be good, serum cells thrived at 2% but did not increase in quantity much (Figure 1). The results suggest that 2% serum was found to produce a viable and stable population of Neuro-2A, which could be excellent for assessing the neuroprotective properties of the drugs to be evaluated.

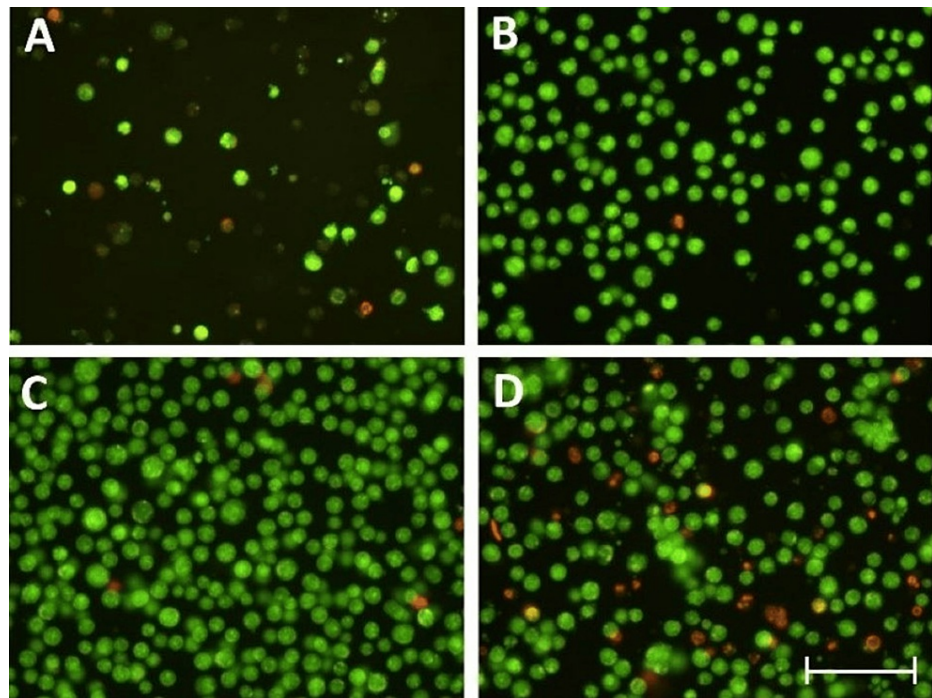


FIGURE 1: Effect of different serum concentrations (A: 0%, B: 2%, C: 10%, and D: 20%) on Neuro-2A cell population after AO-EtBr staining

AO-EtBr: acridine orange/ethidium bromide

Induction of oxidative stress

Neuro-2A cells that had been cultured in 2% serum for 24 hours were treated with various doses of tBuOOH for 45 minutes before being returned to the original medium. This stimulates the production of oxidative stress and the reperfusion conditions for neurons. After reperfusion, 250 μ M of tBuOOH caused approximately 50% cell death (Figure 2).

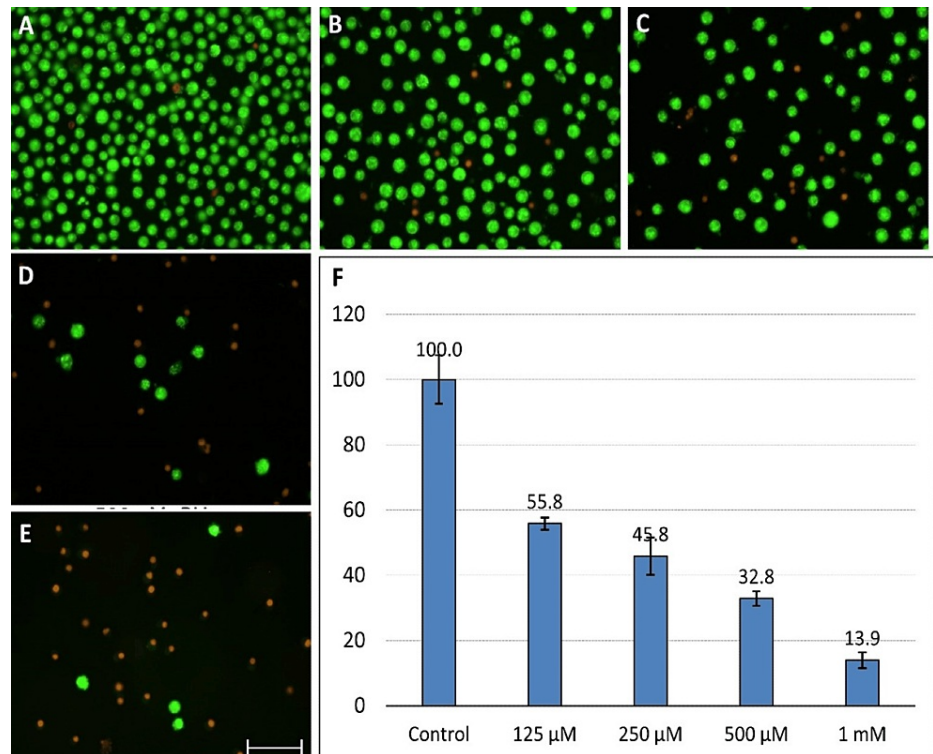


FIGURE 2: A comparative study of control (A) cells treated with different concentrations of tBuOOH (B: 125 μM, C: 250 μM, D: 500 μM, and E: 1 mM) indicates an increased degree of necrosis with concentration. (F) MTT assay indicates 45.8% of viable healthy cells with 250 μM of tBuOOH

tBuOOH: tert-butyl hydroperoxide; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

The optimal dose of citicoline and cerebrolysin

Cells cultured with 2% serum for 24 hours were treated with various concentrations of citicoline and cerebrolysin using the same technique utilized to optimize serum concentration and determine the tBuOOH LD50 dosage. Cerebrolysin at 0.2 μg/ml and citicoline at 0.1 μM were determined to have the least cytotoxic effects (Figure 3A, 3B) and considered for further studies.

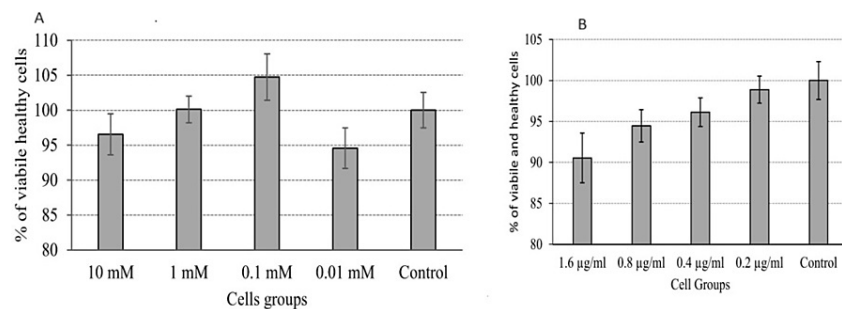


FIGURE 3: (A) Effect of citicoline on Neuro-2A cells. (B) Effect of cerebrolysin on Neuro-2A cells

Neuroprotective effects of citicoline and cerebrolysin

After optimizing serum concentration, tBuOOH dose to cause oxidative stress, and citicoline and cerebrolysin doses, the neuroprotective effects of these drugs were investigated. Following the experimental

design described in the Material and Methods section, it was discovered that both citicoline and cerebrolysin improved the survival of Neuro-2A cells after oxidative injury. The difference in neuroprotective impact between citicoline and cerebrolysin was not significant, indicating that their protective effects are nearly identical (Figure 4 and Figure 5).

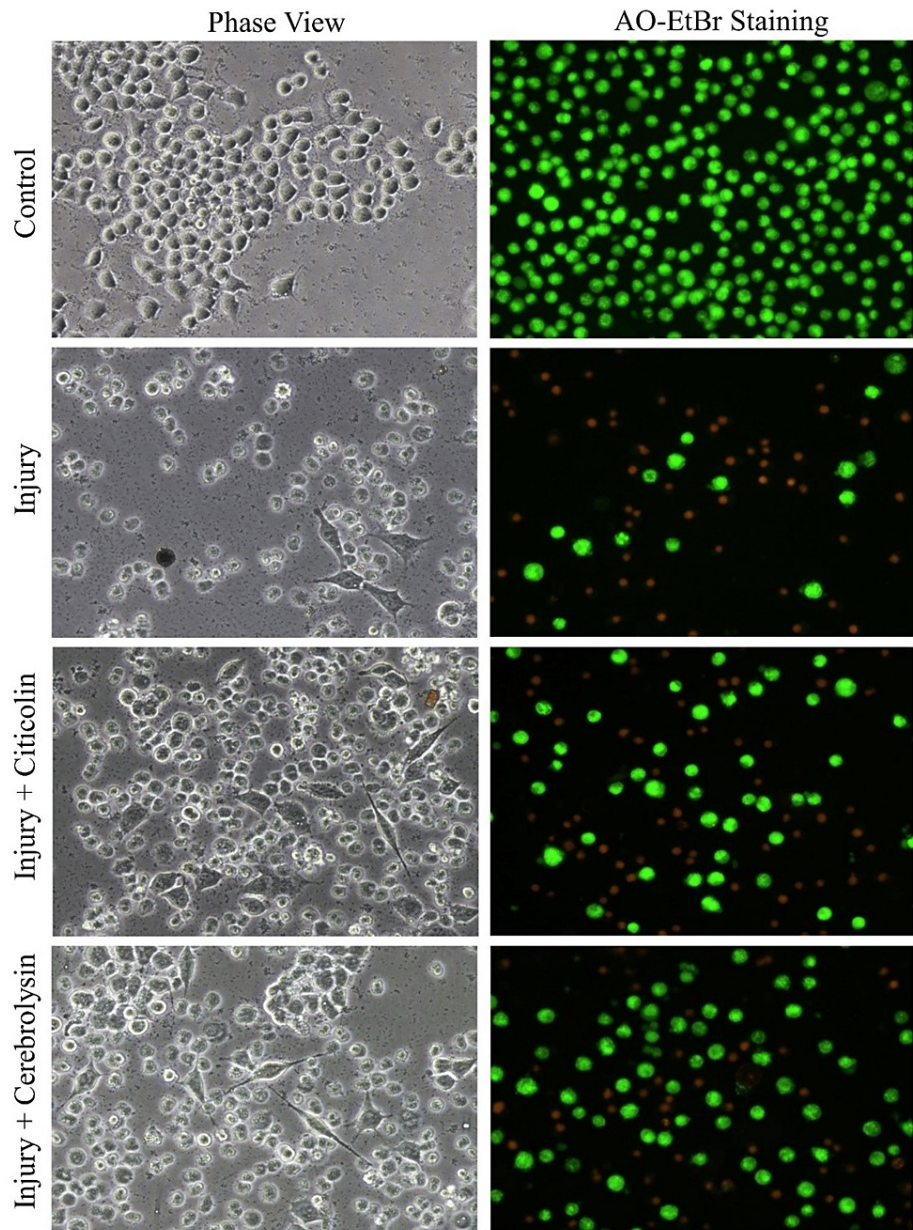


FIGURE 4: Neuroprotective effect of citicoline and cerebrolysin on Neuro-2A cells subjected to oxidative injury as evident from the increase in cell number and reduction of necrotic cells (orange/red) in AO-EtBr staining. Note: images in phase view and AO-EtBr panels do not represent the same microscopic field

AO-EtBr: acridine orange/ethidium bromide

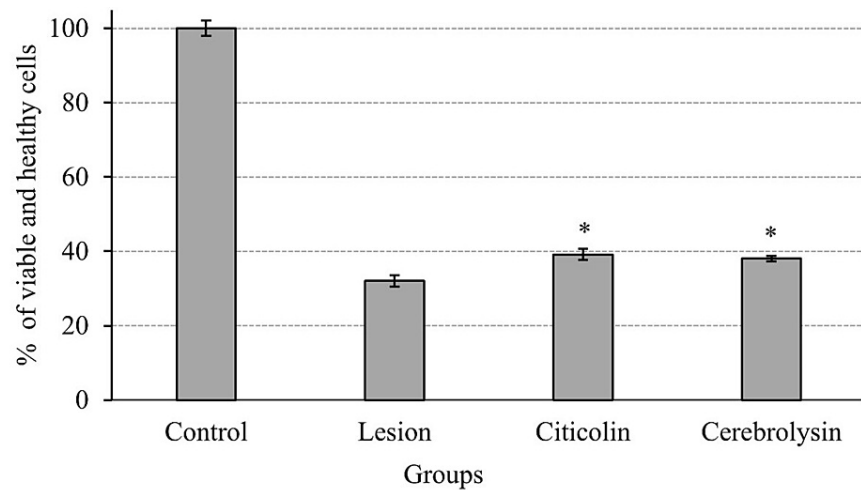


FIGURE 5: MTT assay shows the cytoprotective effect of citicoline and cerebrolysin on oxidative injury. * represents statistical significance compared with the lesion group at $p < 0.05$. There was no significant difference between citicoline and cerebrolysin in the neuroprotective effect

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Gene expression changes induced by citicoline and cerebrolysin

When compared to the control group, the injury/lesion group showed elevated expressions of Neuregulin 1 (NRG-1) and BDNF. As a result, such an increase could constitute a self-defensive elevation of gene expressions triggered by injury. Cerebrolysin was discovered to significantly boost the expression of both of these genes. Citicoline did not boost NRG-1 expression while significantly increasing BDNF gene expression (Figure 6). Both drugs examined did not significantly alter BCL-2 expression or other cell death-related genes (results not shown).

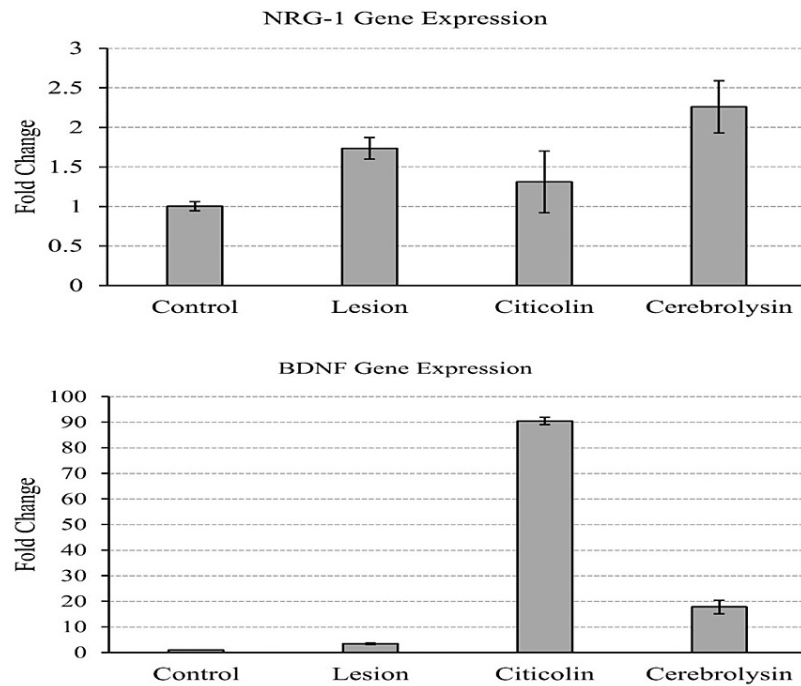


FIGURE 6: Gene expression changes by citicoline and cerebrolysin. Elevated levels of NRG-1 and BDNF expression are observed as a self-defense response to injury

Discussion

Kennedy synthesized citicoline (cytidine 5'-diphosphocholine) and described its properties in 1956 [7]. Animal studies have shown that citicoline has beneficial effects on a variety of neurological diseases [14-16]. Shuaib et al. [17] determined that citicoline has neuroprotective properties using an ischemic stroke model in rats. However, clinical research utilizing citicoline produced conflicting outcomes. Alvarez-Sabín and Román [8] argued that citicoline possesses neurogenesis and neurorepair benefits, while Overgaard [9] acknowledged good results but also highlighted issues surrounding its use. Citicoline was shown to be ineffective in a phase III trial, with only a few post hoc studies indicating benefit [18,19]. Surprisingly, citicoline proved ineffective in two major clinical trials [20,21].

Dr. Gerhart Harrer created cerebrolysin in 1949 through the enzymatic hydrolysis of brain tissue, which he claimed had neurotrophic properties. Many studies have been undertaken since then. Cerebrolysin has been shown to have a variety of favorable effects in both basic science investigations using cell cultures [22,23] and animal studies [24]. Eventually, clinical trial results on the efficacy of cerebrolysin began to arrive [25]. Exhaustive recent research found that cerebrolysin has no beneficial impact and may even pose hazards [26]. More importantly, in 2017, an editorial in the journal *Stroke* [27] found that the use of cerebrolysin for acute stroke therapy is inappropriate and emphasized the need for more randomized trials. The sole consolation was that no major adverse effects were recorded from the usage of cerebrolysin in human patients, despite the likelihood of harm being indicated in an *in vitro* investigation [28].

From these experiences, it is clear that both citicoline and cerebrolysin may have some benefits, but even after five decades of use, there is still no consensus. Both of these medications have only been approved in a few countries, with the majority of others still awaiting approval. Under these circumstances, it may be preferable to take a fresh look at the problem through basic science studies.

Using the culture technique described here, we were able to maintain a stable population of Neuro-2A cells for 48 hours, providing us with sufficient therapeutic windows of 24 hours to investigate the rescue impact of the drugs utilized post injury. The current study found that both citicoline and cerebrolysin rescued neurons subjected to oxidative stress and that this positive effect was small but statistically significant. Real-time PCR-based mRNA estimate studies provided some insights into the probable mechanism responsible for the observed effect. mRNA levels of genes involved in the cell death pathway, such as Casp3, Bcl2, and others, were unaltered. NRG-1 and BDNF levels were altered, as shown.

NRG-1 serves several different functions in the central nervous system. Aside from its recognized role in myelination [29-34], it was discovered to play a significant role in rescue following ischemia damage such as

a stroke [35]. NRG-1 levels were higher in the cerebrolysin group than in the lesion group. BDNF promotes cell survival, differentiation, and synaptic function [36,37]. Alzheimer's disease is associated with BDNF deficiency, which causes neuronal degeneration [38-42]. Its expression has also been shown to be decreased in Parkinson's disease, stress, and anxiety [43,44].

Given the overwhelming support for the role of BDNF in neuronal repair, survival, and degeneration prevention, the observation of significantly upregulated BDNF gene expressions by both citicoline and cerebrolysin could indicate the possible involvement of growth factor-based rescue in oxidatively stressed cells. The lesion group had higher levels of NRG-1 and BDNF than the control group. This could be related to the innate defense mechanisms that these neurons activate in response to the injury. The increased expression of these genes in the treated groups, particularly BDNF levels, may imply that these two medications improve the defense response demonstrated by injured neurons, a hitherto unexplored possibility.

Conclusions

The effects of citicoline are frequently debated, and no particular mechanism was proposed for cerebrolysin. The present investigation reveals that these two medications have a minor therapeutic effect in rescuing neurons following oxidative stress through the elevation of BDNF gene expression rather than direct engagement in cell death or repair mechanisms. However, complex gene expression studies (combining qRT-PCR assessments of other genes with phospho-specific Western blotting) are needed to confirm this hypothesis.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

Concept and design: Sumathi Jones, Anandan P, Santhanam Rengarajan, Sankar Venkatachalam, Prabhu K, Krishna Prasanth

Acquisition, analysis, or interpretation of data: Sumathi Jones, Sasikumar Pattabi, Prabhu K, Vani Krishna

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Critical review of the manuscript for important intellectual content: Sumathi Jones, Prabhu K

Supervision: Sumathi Jones, Prabhu K

Disclosures

Human subjects: All authors have confirmed that this study did not involve human participants or tissue.

Animal subjects: All authors have confirmed that this study did not involve animal subjects or tissue.

Conflicts of interest: In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

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