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Prospective, double blinded, comparative assessment of the pharmacological activity of Cerebrolysin and distinct peptide preparations for the treatment of embolic stroke

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ABSTRACT

Background: Our previous work in acute ischemic stroke and TBI models focused on efficacy and pharmacological parameters of Cerebrolysin®. In this prospective, randomized, blinded, placebo-controlled study we compared efficacy of neuropeptide preparations with putative neurotrophic potential to the reference product Cerebrolysin® by assessing functional outcome and lesion volumes after embolic stroke in a rodent model.

Methods: Male Wistar rats were subjected to embolic right middle cerebral artery occlusion and were treated with: 1) Cognistar® (Cerebroprotein Hydrolysate) 2.5 ml/kg, 2) Cerebrolysat® 2.5 ml/kg, 3) Cortexin® 1.7 mg/kg, 4) Cerebrolysin® 2.5 ml/kg, or 5) 1 ml of saline according to a pre-generated randomization plan. Dosages were defined according to the packet leaflet of the corresponding preparation and were adapted to the animal model as previously described. All enrolled rats received intraperitoneal injections once daily for 10 consecutive days, starting 4 h after occlusion. Functional outcome was assessed once weekly over four weeks by using a battery of behavioral tests. Infarct volume was measured four weeks after occlusion. Generalized Estimation Equations (GEE) was performed to study the treatment effect on overall functional recovery at day 28 (primary outcome), compared to saline controls.

Results: Similar functional outcome was observed for saline control, Cognistar®, Cerebrolysat® and Cortexin®; in contrast, a significantly improved neurological outcome was observed with Cerebrolysin® treatment in comparison to saline as well as to the comparator drug treatment ($p < .002$). However, there was no significant difference in lesion volumes between rats treated with either Cortexin® (33.5 ± 1.9%), Cerebrolysat® (28.5 ± 2.4%), Cognistar® (34.7 ± 2.0%), or Cerebrolysin® (26.5 ± 2.3%) compared to saline-treated rats (30.8 ± 2.1%).

Conclusion: Among all tested neuropeptide preparations, Cerebrolysin® was the only agent that was associated with a significant improvement of neurological outcome after stroke.

1. Introduction

The most common cause of disability is stroke, leaving 65% of stroke survivors with sensory, motor, and coordinative disabilities [1,2]. Pharmacological interventions in the acute management of stroke aim to restore blood flowin order to limit brain damage and post-stroke complications and neuroprotective drugs aim to support these effects. Cerebrolysin® is a neuropeptide preparation that mimics the action of endogenous neurotrophic factors by protecting the brain against the detrimental impact of the ischemic cascade and by augmenting cerebral reorganization processes. Preclinical studies have shown a modulatory effect of Cerebrolysin® on neuroplasticity such as synaptic remodeling [3–5] and facilitated synaptic transmission [6–9], neurite outgrowth [10,11], oligodendrogenesis [12,13], and neurogenesis [14–19], and a beneficial effect on endogenous brain recovery processes [13–15,17,19]. In experimental stroke, we and others have demonstrated that treatment acute stroke with Cerebrolysin® dose dependently reduces ischemic lesion volume and neurological deficits. Further investigation reveals that delayed Cerebrolysin® treatment accelerates stroke recovery by promoting Sonic hedgehog and PI3K/Akt

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signaling pathways which enhance brain remodeling processes. More importantly, with an excellent safety and tolerability profile in stroke patients, randomized clinical trials have demonstrated beneficial effects of Cerebrolysin® treatment on stroke recovery [20–25].

Overall, there is comprehensive scientific evidence available that shows neuroprotective and neurorestorative effects of Cerebrolysin® in various model systems of stroke and traumatic brain injury [13,15,26–29]. Similar claims are made by other marketed drugs; however, the level of evidence is rather limited or unclear. To the best of our knowledge, we are not aware any published studies that have made direct comparisons among individual neuroprotective peptides for stroke and other types of brain injury. We therefore established the first head-to-head experimental comparison study using a prospective, blinded, randomized and placebo-controlled design to assess the efficacy of four peptidergic drugs on functional post-stroke outcome and infarct volume in the same stroke model.

2. Materials and methods

All experimental procedures were carried out according to the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital [30].

2.1. Experimental design

The present study used a prospective, randomized, placebo-controlled, blinded design. Randomization was based on a variable block size and generated by using nQuery3.0 software. Drug preparation and administration as well as data collection and analysis were performed by independent personnel not otherwise involved in the study.

2.2. Animal model

Adult male Wistar rats (n = 115) weighing 350–400 g were subjected to right embolic middle cerebral artery occlusion (MCAO) [31,32]. Briefly, blood clots from a normal donor rat were obtained 24 h prior to MCAO. Under a surgical microscope, a modified PE-50 catheter containing a single clot (40 mm in length) was introduced from the right external carotid artery into the internal carotid artery until its tip reached the origin of the MCA. The clot and 2-5 μl of saline were gently injected and the catheter was immediately withdrawn. The incision was sutured and the rat was allowed to recover from anesthesia.

2.3. Treatment groups and inclusion criteria

According to a pre-generated randomization scheme, rats (n = 23/group) were randomized into one of five treatment groups: 1) Cognistar® 2.5 ml/kg, 2) Cerebrolysat® 2.5 ml/kg, 3) Cortexin® 1.7 mg/kg, 4) Cerebrolysin® 2.5 ml/kg, and 5) saline 1 ml/rat. Dosages were defined according to the packet leaflet of the corresponding preparation and were adapted to the animal model as previously described [29]. The intraperitoneal injection route was chosen based on the demonstrated therapeutic efficacy of cerebrolysin and other marketed peptidergic drugs after IP injection in various animal studies [29,33–35]. Prior to treatment, baseline neurological deficits were assessed 3 h after MCAO by using a five point Zea-Longa score [36]. Animals with a Zea-Longa score of 2 or more (moderate to severe neurological deficits) were eligible for the treatment. Animals that died within 24 h after MCAO (prior to the first neurological functional test) were excluded from further analyses.

The study drug was provided in opaque syringes of identical appearance in a total volume of one milliliter and appropriately labeled to maintain blindness. Treatment was initiated 4 h after MCAO and was administered once daily for a total of 10 consecutive days by intraperitoneal injection.

2.4. Behavioral tests

Functional outcome after stroke was assessed by the modified Neurological Severity Score (mNSS), the Adhesive Removal Test, and the Foot-Fault Test on a weekly basis over four weeks starting one day after onset of MCAO. All behavioral tests were performed by an investigator blinded to the experimental groups [14].

The mNSS rates motor, sensory, balance, and reflex on a scale of 0–18 (0, normal score; 18, maximal deficit) [37].

The Adhesive Removal Test rates somatosensory neglect via bilateral tactile stimuli [38]. Each rat received three trials per testing day and the mean time taken for each rat to remove stimuli from the contralateral (left) forelimb was recorded with a cut-off time of 120 s.

The Foot-Fault Test rates forelimb placement dysfunction [39]. The total number of steps (movement of each forelimb) that the rat made on the grid surface and the number of foot-faults (fall or slip off the grids) for the left forelimb were recorded and presented as a percentage of left foot-faults relative to the total number of steps.

2.5. Histological measurement of infarct volume

Infarct volume was measured on seven hematoxylin & eosin (H&E) stained coronal sections of all enrolled rats either upon death or at end of study (28 days after MCAO). Data are presented as the percentage of the ipsilateral indirect lesion volume relative to the volume of contralateral hemisphere [40,41].

2.6. Statistical analysis

Baseline body weight was used to check the balance among groups prior to the randomization. The weight variable was included in the outcome analysis as a covariate if the body weight was imbalanced among groups. Repeated measurement analysis or Analysis of variance and covariance (ANCOVA) was used to study changes in weight over time by groups.

For the safety evaluation, the log rank test/Kaplan-Meier was used to test mortality differences among groups.

The Global Test using Generalized Estimating Equations (GEE) was employed to test the group effect on neurological functional deficits (primary outcome), obtained from three behavioral tests (mNSS, Foot-Fault Test, and Adhesive Removal Test) at each time point (days 1, 7, 14, 21, 28) [42–45]. Early death or deterioration that prevents functional testing was assigned the worst scores as an intention-to-treat (ITT) approach. Data were evaluated for normality and ranked data were used for the non-normally distributed neurological functional data analysis. Two controls were considered (Cerebrolysin® and saline), whereas saline treatment is used to validate the effect of Cerebrolysin® and other interventional agents as a secondary analysis. The analysis started testing the overall group effect, followed by pair wise comparisons if the overall group effect was detected at the 0.05 level. Any subgroup analysis was considered exploratory if the overall group effect was not observed.

The analyses for the primary (the global analysis of three behavioral tests) and secondary outcome (lesion volume at day 28) were carried out in SAS version 9 using PROC GENMOD. The CONTRAST statement in SAS was used to estimate the mean difference between two groups based on the ranked data. A significant functional improvement was observed if ranked mean difference was negative with a p-value < .05 when compared to a control group. Similarly GEE/Analysis of variance (ANOA) was used to study the treatment effect on infarct volume at day 28.

Table 1
Study population, baseline characteristics, and mortality rate.

Treatment	N	Body weight (grams)						Mortality
		D0	D1	D7	D14	D21	D28	
Saline	21	376 ± 20	333 ± 23	330 ± 29	352 ± 36	374 ± 43	376 ± 48	5% (1 of 21)
Cerebrolysin®	22	376 ± 17	334 ± 26	339 ± 37	358 ± 50	386 ± 56	412 ± 60	5% (1 of 22)
Cerebrolysat®	20	382 ± 17	332 ± 25	328 ± 50	348 ± 59	372 ± 68	398 ± 70	5% (1 of 20)
Cognistar®	21	376 ± 18	328 ± 19	319 ± 27	332 ± 43	352 ± 56	377 ± 60	10% (2 of 21)
Cortexin®	20	386 ± 15	329 ± 19	315 ± 33	327 ± 44	356 ± 50	383 ± 49	10% (2 of 20)

3. Results

3.1. Study populations, baseline characteristics, safety, and mortality

A total of 115 rats ($n = 23$/group) were randomized into 5 treatment groups. Rats with Zea-Longa scores < 2 ($n = 4$) or that died ($n = 7$) within 24 h after MCAO prior to any neurological functional tests were excluded from the study. A total of 104 rats were included in the outcome analyses (Table 1). Among them, seven rats died between days 2 to 3 after MCAO (Table 1). The mortality rates were comparable among groups. The body weights were balanced among groups at baseline prior to the MCAO. In addition, as a general indication of animal condition and safety, body weight was also recorded weekly starting at day 1 after MCAO. While all animals gained weight gradually, there was no group difference in body weight over time (Table 1).

3.2. Neurological functional outcome

One day after MCAO all rats exhibited a similar degree of neurological functional deficits (Table 2). From day 7 onwards, functional outcomes of rats treated with Cerebrolysin® were significantly superior to saline-treated rats at all assessed time points (Fig. 1). Functional performance of rats treated with Cognistar®, Cerebrolysat®, or Cortexin® were comparable to saline treated animals (no significant difference at any assessed time point) and significantly worse than Cerebrolysin® treated animals (Fig. 1).

3.3. Infarct volume

The indirect infarct volume at day 28 post MCAO was 26.5 ± 2.3% for Cerebrolysin®, 28.5 ± 2.4% for Cerebrolysat®, 30.8 ± 2.1% for saline, 33.5 ± 1.9% for Cortexin®, and 34.7 ± 2.0% for Cognistar®. The F-test with 4 degree freedom (DF = 4) showed the overall treatment effect ($p < .0001$), the subgroup analysis showed that there was no significant difference in lesion volumes between the animals treated with either Cortexin® ($p = .38$), Cerebrolysat® ($p = .45$), Cognistar® ($p = .19$) or Cerebrolysin® ($p = .14$) and animals treated with saline. However, Cerebrolysin® treated animals had significantly smaller lesion volumes than rats treated with Cognistar® (by 30%, $p = .004$) or with Cortexin® (by 26%, $p = .018$) (Fig. 2).

4. Discussion

The present study compared for the first time different neuropeptide preparations on the effectiveness of improving functional outcome in rats after stroke. In the design, this study followed rigorously the STAIR recommendations [46,47].

Among the tested agents Cerebrolysin® was the only drug that significantly improved functional outcome in comparison to saline (Fig. 1). This is consistent with findings from clinical studies that assessed the impact of Cerebrolysin® on functional recovery in combination with rehabilitation [20,21]. In these studies, treatment with Cerebrolysin® at a daily dose of 30 ml for 21 consecutive days had a beneficial effect on rehabilitation supported motor recovery. Importantly, these therapeutic effects were still observed even when treatment with Cerebrolysin® was initiated up to 8 days after the stroke event, when the beneficial effects of Cerebrolysin® on stroke recovery were not likely resulting from acute neuroprotection [20]. Indeed, various experimental studies have shown that Cerebrolysin® treatment robustly promotes multiple brain remodeling processes including angiogenesis, neurogenesis, oligodendrogenesis, and axonal outgrowth, all of which are essential for neurological recovery after stroke [48,49]. Thus, it is likely that Cerebrolysin® stimulates multiple brain repair/remodeling processes that in concert contribute to stroke recovery.

We previously demonstrated that Cerebrolysin® at a higher dose of 5.0 ml/kg significantly reduced the infarct volume compared with saline treated rats [28]. However, in the present study, although Cerebrolysin® treated rats had smallest infarct volume among groups; there was no significant difference on infarct volume between Cerebrolysin® and saline groups. These and other previous results suggest that neuroprotective effects of Cerebrolysin® may be more pronounced at higher dosages and/or earlier treatment initiation [27,39]. Nevertheless, Cerebrolysin® significantly reduced the lesion size in comparison to the peptide preparations Cognistar® and Cortexin®. Interestingly, treatment of stroke with Cognistar® and Cortexin® led to a trend of increased lesion volumes as compared to saline (Fig. 2). Thus, our data indicates that none of the peptide preparations including Cerebrolysin® had a therapeutic effect on the lesion volume.

There are some limitations in the present study. First, the fundamental differences in compositions between Cerebrolysin® and the other compounds tested in the present study were not addressed in this study; moreover, the manufacturing process of these compounds appear to differ from Cerebrolysin®. For example, the Cerebroprotein Hydrolysat “Cognistar®” and Cortexin® are lyophilized powders that require dissolution before administration with sterile water or even analgesics such as Novocain. The liquid preparation Cerebrolysat® contains as indicated in the package leaflet, additives such as phenol. Secondly, although other compounds have claimed similarity to

Table 2
Neurological functional outcome at 1 day after MCAO.

Treatment	mNSS (score)	Adhesive removal test (seconds)	Foot-fault test (% of left foot-faults)	p-value (based on global test)
Saline	10.6 ± 1.2	113.1 ± 14.6	28.9 ± 6.8	0.12 (Groups)
Cerebrolysin®	9.6 ± 2.5	98.2 ± 26.9	26.3 ± 8.3	0.31 (vs Saline)
Cerebrolysat®	11.0 ± 1.3	116.0 ± 9.9	30.5 ± 6.2	0.23 (vs Saline)
Cognistar®	11.0 ± 1.3	115.0 ± 12.7	29.4 ± 5.0	0.45 (vs Saline)
Cortexin®	11.3 ± 1.0	119.0 ± 2.2	31.2 ± 5.3	0.08 (vs Saline)

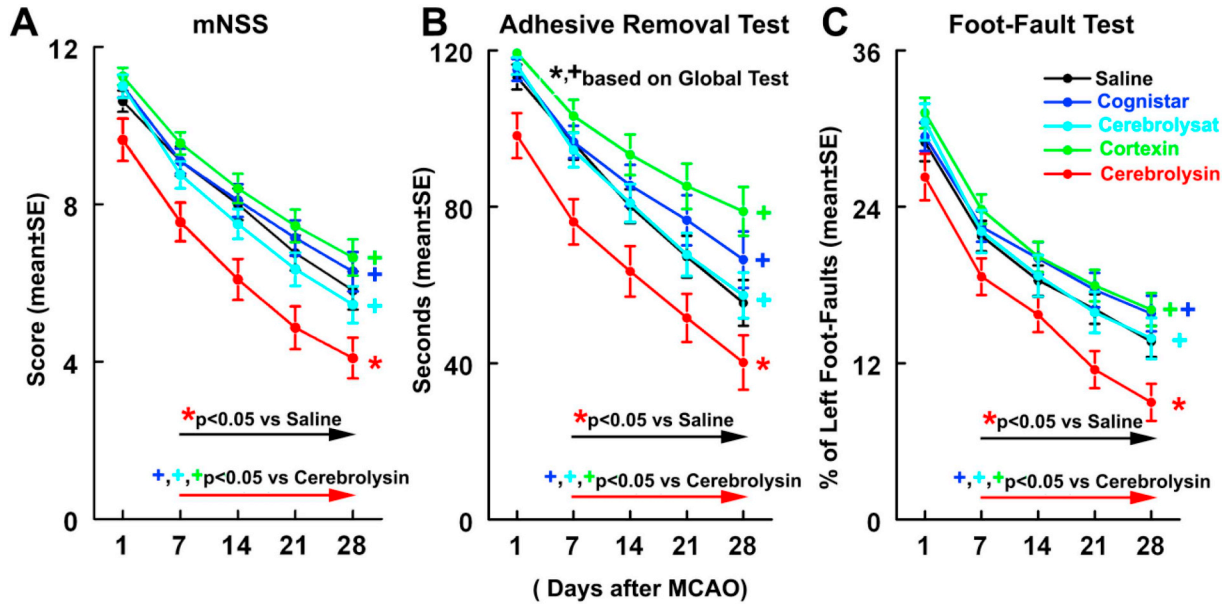

Fig. 1. Neurological functional outcome. Cerebrolysin® treatment significantly improved overall neurological functions measured by mNSS (A), Adhesive Removal Test (B), and Foot-Fault Test (C) at 7, 14, 21, and 28 days after MCAO. Values are mean ± SE. *$P < .05$ versus saline-treated group from day 7 to 28 (black arrows). $^{+}P < .05$ versus Cerebrolysin®-treated group from day 7 to 28 (red arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

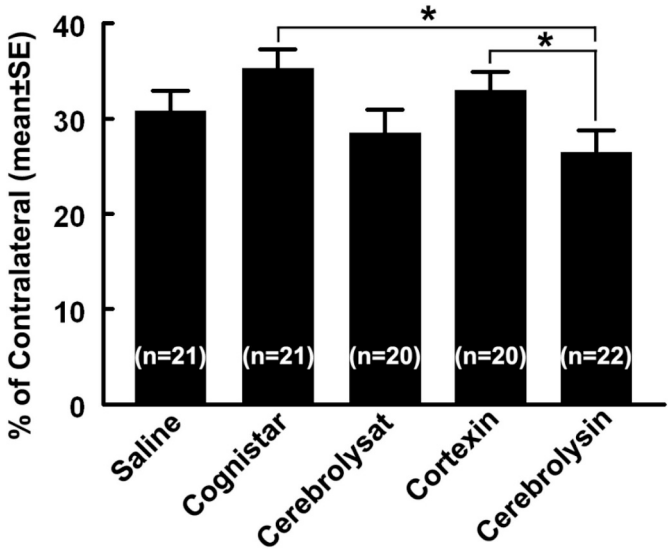

Fig. 2. Infarct volume. Quantitative analysis of infarct volume 28 days after MCAO. Values are mean ± SE.*$P < .05$ versus the Cerebrolysin®-treated group.

Cerebrolysin® such as Cognistar® and Cerebrolysat® for various experimental and clinical implications, their pharmacokinetic/pharmacodynamic characteristics for the treatment of ischemic stroke have yet to be established. Therefore, the therapeutic potentials of Cognistar®, Cerebrolysat®, and Cortexin for stroke recovery warrant further investigation.

The results of the present studies demonstrate that the pharmacological activity differs among multi-peptide preparations, with evident functional benefit compared to saline control only seen with Cerebrolysin®. Therefore, such preparations should not be considered as being exchangeable for the treatment of stroke in terms of efficacy and safety.

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Conflict of interest

M.C. has received speaker honorarium andresearch grants from EVER Pharma GmbH. All other authors (L.Z., C.W., Y.Z., M.L., T.Z., and ZG.Z) declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.All experimental procedures were carried out according to the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital. This article does not contain any studies with human participants.

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