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Research Article

Post-Stroke Recovery of Motor Function with a New Combination of Medicines–A Pilot Study

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Abstract

Objectives: Recovery of motor function after moderate to severe stroke is challenging given the paucity of therapeutic choices; we propose an effective treatment with a new combination of drugs which protect neuronal mitochondria from oxidative stress, inflammation, and subsequent apoptosis; also decrease excitotoxicity mostly by modulating the brain derived neurotrophic factor (BDNF), insulin growth factor-1 (IGF-1), and transforming growth factor-β (TGF-β).

Methods: The new combination consists of medications approved for human use in multiple pathologies: glutathione, oxytocin, dimethylsulfoxide (DMSO), deproteinated veal serum (Actovegin), vitamins C, B1, B6, B12, which were administered intravenously in an open-label, pilot study. Motor function was evaluated with the National Institutes of Health Stroke Scale (NIHSS) in 15 consecutive hemiplegic patients initially and at 1 month after administering first intravenous treatment, and subsequently.

Results: When treatment was administered during days 10-35 post-stroke, motor improvement at 1 month evaluation post-treatment (mean ΔNIHSS score=-3.6, n=5) was significantly better than when administered at 35-100 days post-stroke (mean ΔNIHSS=-0.83, n=6, p=0.02), or when given after 3 months post-stroke (mean ΔNIHSS=0, n=4). Motor improvements at 2 and 3 months post-treatment were seen only in the group treated at 10-35 days post-stroke, with one complete recovery of hemiplegia at 6 months.

Conclusion: Excellent results were obtained in subacute stroke patients with hemorrhagic transformation of ischemic stroke, recommending it as a much needed addition to the current treatment options for stroke and more ample clinical trials.

Keywords: Hemiplegia treatment, hemorrhagic transformation, motor recovery in stroke, post-stroke recovery, stroke treatment

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Stroke (cerebrovascular accident, CVA) is a pathology with high incidence, with an estimated 1 occurrence every 40 seconds in US alone,[1] and an important cause of mortality with an estimated 6.5 million stroke deaths worldwide in 2013.[2] The accumulated costs for patient care due to the long-term disability are surpassing all other pathologies [3–6]; these facts make the diagnosis and treatment of stroke both very important and challenging.

On the diagnosis front, being able to promptly distinguish between ischemic (which accounts for about 80% of strokes) and hemorrhagic stroke is essential, as they require opposing treatment strategies. Differentiating between the two is done by computer tomography or magnetic resonance, modalities which may not be available for some patients within the critical first 2-3 hours from the onset of a severe stroke. However, an important advance was made

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recently with a mobile device which uses volumetric impedance phase shift spectroscopy (VIPS)[7] to compare the volumes of fluid (blood) between the cerebral hemispheres and which can detect the presence/increase of interstitial liquid and edema, characteristic of hemorrhagic stroke, or decreased vascular volume specific for ischemic stroke, an approach proposed by us in 2016.[8] Equipping ambulances with this device can make a significant difference in stroke outcome because specific but mutually exclusive medications, such as tPA, vitamin K, tranexamic acid, etc., can be administered promptly to limit neuronal damage and improve the outcome.

On the treatment side, the focus in the acute phase of ischemic stroke is currently mostly on the vascular component of the stroke–i.e., repermeabilizing the obstructed blood vessel, via enzymatic (tissue plasminogen activator, tPA) or mechanical (thrombendarterectomy) means. These treatments have important limitations: they can be administered only in the first few hours from CVA occurrence (3-6 h) by specialized doctors and equipment, so only a fraction of patients can benefit; about 5% of stroke patients can benefit from tPA and even less for thrombendarterectomy. Furthermore the possibility of hemorrhagic transformation of ischemic stroke after tPA limits its overall effectiveness; to counteract this possibly fatal complication it was proposed the co-administration of drugs which enhance the blood-brain barrier (minocycline, candesartan, atorvastatin, fasudil, cilostazol, etc), augment cerebral vascularization (granulocyte-colony stimulating factor G-CSF, etc), ascorbic acid, oxygen transporters, stem cell treatments, etc.[9, 10]

The difficulties in treating stroke stem not only from the fact that ischemic brain damage is multifactorial (involving vascular endothelial regulation, coagulation and platelet aggregation; inflammation, modifications of metabolic pathways of glucose, lipoproteins, nucleotide and acid-base, lipid peroxidation, neurotransmitters synthesis and release, calcium regulation, second messenger and cell signaling, protein chaperone and repair, estrogen action, cell volume regulation, apoptosis), but also evolves in different stages where different molecules have beneficial, deleterious and even dual actions on stroke neuronal structure and function. On top of this there are important genetic individual variations which can significantly alter the course of patient recovery.[11] In order to show the two latter aspects (stages and genetic variation), serum levels of neurofilament H and S100B proteins were measured during cardiopulmonary bypass surgery at 1 hour and 24 hours post cerebral ischemia and their levels were correlated with the presence of 92 SNPs (single nucleotide polymorphism) affecting expression and function of various genes) of proteins involved in the above-mentioned pathways. Molecules which correlated positively with recovery were Superoxide dismutase 2-SOD2, Natriuretic peptide B-NPPB, Selectin E-SELE, fibrinogen alpha chain-FGA), and those with deleterious roles were Calpain 10-CAPN10, Serpin family E member 1-SERPINE1, Small ubiquitin-like modifier 4-SUMO4, Adrenoreceptor alpha 2A-ADRA2A, BCL2 associated X–BAX, apoptosis regulator, Solute carrier family 4 member 7-SLC4A7, Heat shock protein family A member 1B–HSPA1B.

Considering all this, the author proposed a first-hour neuroprotective combination consisting of an antioxidant (tirilazad), enalapril (vasorelaxation), cyclosporine (mitochondrial and apoptosis modulator), nasitiride (glucose control), tranexamic acid (antifibrinolytic) and an anti-apoptotic agent which needs to be developed, followed at 24 hours by another combination consisting of dexmedetomidine (adrenergic receptor modulator), cyclosporine, glucocorticoids (anti-inflammatory, anti-leucocyte adhesion), tromethamine (increasing cell pH), and a drug supporting heat shock protein or gene therapy.

To address both the aspects of stroke pathogenesis and recovery, we have put together a combination of drugs which focuses on the acute and subacute events triggered by neuronal ischemia, and secondary on neurogenesis, and below we present the results obtained with this new combination of medicines during a pilot study. Informed consent was obtained from all patients or family before treatment. This clinical study was registered on www.clinicaltrials.gov with the title "Post-Stroke Improvement of Motor Function" and ID number NCT03543917 and Protocol ID "PSIOM".

Methods

The new combination has multiple actions on the pathways known to be activated in stroke: inflammation, oxidative stress, apoptosis, and vascular, neuroendocrine and immune responses induced by ischemic stress. It is is based on previous results with stroke patients;[12] it consists of: glutathion, oxytocin, Actovegin, dimethylsulfoxide (DMSO), vitamins C B1, B6, B12; their individual actions are summarized as follows:

Glutathione plays an essential role in cell function and survival, being the main defense mechanism against oxidative stress intracellularly (has a higher concentration by about 100-1000 fold than that of thioredoxin); it is also pivotal for cell-cycle regulation, proliferation, and apoptosis. Mitochondria and glutathion have a central role in triggering and unfolding cell apoptosis through the sequence: I. membrane permeabilisation, ii. cytochrome c release, iii.

caspase 3 activation; more importantly it was shown that these events occur only after mitochondrial glutathion depletion.[13]

During ischemic stress astrocytes act as glutathion donors for neurons until depletion[14] and administering glutathione to the ischemic rat brain was followed by a reduction of approximately 60% of the infarct size;[15] for these reasons it is expected that administration of glutathione post-stroke will have significant benefits for avoiding apoptosis in the acute and subacute stages of stroke and subsequently promote neuronal recovery and function.

Dimethylsulfoxide (DMSO) is an amphipathic molecule able to solubilize polar and nonpolar substances and to cross hydrophobic membranes, is widely used to solubilize therapeutic drugs, and it has pleiotropic actions;[16] it has mainly anti-inflammatory, anti-edematous, and anti-oxidant effects, but also produces vasodilation, muscle relaxation, inhibition of platelet aggregation, has analgesic effects, inhibits cholinesterase, modulates cholesterol metabolism, and the action of other medications, and offers overall cellular protection against ischemic injury. In USA DMSO is FDA-approved for human use in chronic interstitial cystitis, and in EU has orphan drug status from EMA for treating traumatic brain injury[17] where it reduces brain edema, increases neuronal oxygenation, and lowers the activation of sodium channels involved in excitotoxicity.[18, 19]

Dimethylsulfoxide is widely utilized for protecting and maintaining vitality of transplanted organs and stem cells during transport and cryopreservation, and its pharmacokinetics is well-known from its use in stem cell transplantation.[20] In our combination we used lower concentrations than commonly used in stem cell transplants, practically eliminating most adverse reactions-hemolysis when concentrations of 40% or higher are administered intravenously, or transient encephalopathy, cardiac and gastrointestinal toxicities when much higher volumes are administered to immunosupressed, oncological patients in advanced stages of disease.[18]

The cellular actions of DMSO are determined by its concentration through 3 main different mechanisms, which were observed empirically and characterized more recently at molecular level:[21] low concentrations produce cell membrane thinning and increased membrane fluidity; higher concentrations of DMSO induce into the membrane transient water pores, while at even higher concentrations (above 20% v/v), the bi-layer structure of the membrane disintegrates after dissociation of individual lipid molecules from the membrane.

DMSO is a very potent molecule in biological systems; it was reported that even at very low concentrations–between 0.00025%-0.1% v/v-it stimulates neuronal mitochondrial respiration and brain metabolism.[22] In motor nerve endings DMSO has fusogenic activity [23] and it facilitates the release of neurotransmitters from the vesicles in the synaptic space; recently it was reported that it induces fusion of cytoplasmic vesicles with the cell membrane.[24] It was also shown that DMSO has antiepileptic effect by reducing the glutamatergic excitotoxic phenomena[25] and that it reduces lipid peroxidation as well as protein carbonyl produced by ferrous chloride/hydrogen peroxide oxidative system in the rat brain.[26]

DMSO inhibits platelet aggregation by reducing thrombin formation, inhibits proliferation of vascular endothelial cells and increases their apoptosis, and these actions may restrict expansion of ischemic vascular beds and help with re-permeabilization in the early stages of thrombosis.[27]

DMSO was recently shown to increase the expression of transforming growth factor beta (TGF-β) on the surface of cellular membranes by 3-4 fold; a molecule with potent anti-inflammatory cytokine and anti-apoptotic activity, and which was also shown to increase survival of different cell types,[24] a very useful action in the ischemic neurons.

The specific pro-inflammatory molecules and pathways modulated by DMSO-tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukin 2 (IL-2), as well as the decrease in activation and proliferation of cluster of differentiation CD4+ and CD8+ T lymphocytes,[28] prostaglandin E2 (PGE2) at concentrations ranging from 0.5–2%,[29] make this substance an essential component of the stroke treatment combination; these actions together with other substances and molecules are presented for a better overall picture in Figure 1.

Actovegin (deproteinated veal serum) is well-known and used in some European countries for treating stroke patients, diabetic neuropathy and burn wounds,[30] and it was shown that early administration (6h) after ischemic injury improves neuron survival in hippocampus.[31]

It consists of more than 200 substances with molecular weight of up to 5000 Daltons. It has pleiotropic effects: improves utilization of glucose by various cells through insulin-like growth factor 1 (IGF-1), and subsequently improves mitochondrial function, which in turn is involved in neuronal survival, axonal growth and new neuron formation.[32, 33] Its efficacy in improving recovery and neurological status of stroke patients was shown in multiple clinical trials;[34, 35] however when given as monotherapy its efficacy is limited. This aspect was shown in a recent double-blind, placebo-controlled study[36] in which Actovegin 2000 mg/day was given intravenously for 20 days and afterward 1200 mg per day orally for 6 months to 248 patients, and 503 pa-

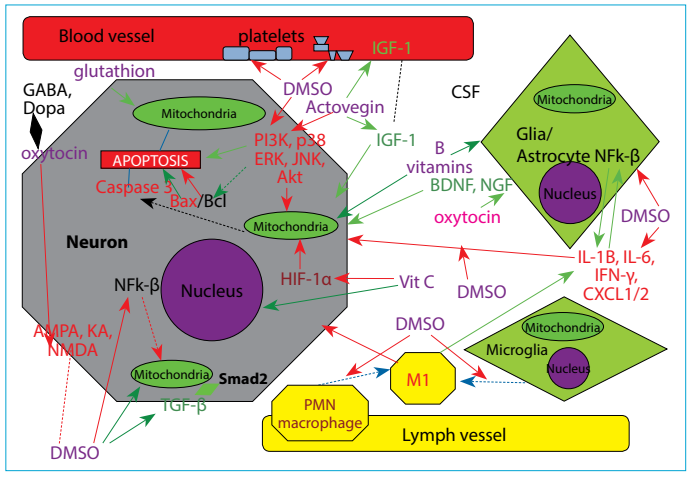

Figure 1. Actions of the individual components of the neurotrofic intravenous combination containing glutathion, oxytocin, DMSO, deproteinated veal serum (Actovegin), vitamins C and B.

Green line (or font)–stimulating action; red line (font)-impairment; black line (font)–both positive or negative modulation possible; solid line–direct effect; dotted line–indirect, mediated effect; blue line–transport/transformation, glutathion–inhibition of apoptosis induced by mitochondria (Mari 2009); Bax/Bcl modulation of apopotosis (Jonas 2013); oxytocin inhibition of AMPA, KA, NMDA receptor activity-(Caruso, 1993); modulation via GABA, dopamine–(Carter, 2017); BDNF, NGF–brain-derived neurotrofic factor, nerve growth factor increase (Havranek 2015, Bakos 2018); B vitamins–Jadavi, 2017 & 2018; Ullegaddi 2004 & 2006; DMSO–inhibits proliferation of vascular endothelial cells and platelet aggregation (Yi, 2017); stimulates mitochondrial respiration (Nasrallah, 2008); stimulatory modulation of TGF-β, Smad2–(Huang 2016); inhibits macrophage migration and function, transformation in M1 subtype, PI3K, p38 ERK, JNK, Akt pathways (Elisia 2016); DMSO inhibits production and actions of IL-1β, IL-6, IFN-γ, CXCL1/2 (de Abreu Costa, 2017); inhibition of glutamatergic excitotoxicity (Carletti 2013); Actovegin–stimulation of IGF-1, inhibition of inflammatory cytokine production and actions (Machichao, 2012); inhibitory modulation of PI3K and p38 MAPK pathways–(Yurinskaya 2016); Vit C–activates aprox. 800 genes via histone demethylases (Padayatty 2016); inhibitory action on HIF-1α-(Zhang 2018), its intracellular transport–SVCT1/2–is inhibited by inflammatory cytokines (Subramanian 2018, Ang 2018).

tients were followed up for 1 year. There were significant improvements with Actovegin on the Montreal Cognitive Assessment and the Alzheimer Disease Assessment scales, on Beck Depression Inventory and on EuroQol EQ5D after 12 months, also in presence of moderate-severe-extreme anxiety (5.6 vs 11.8%); less patients in the Actovegin group had severe mobility problems (3.2% vs 7.5%) and severe/unable self-care problem (2.9% vs 4.7%); serious adverse events were affecting 8.8% of Actovegin group and 6.7 of placebo treated patients. On the downside there was essentially no difference in the improvement of the NIHSS scores at 1, 3, 6 and 12 months between Actovegin and placebo and there was increased incidence of ischemic stroke occurrence–5.2% in the Actovegin and 1.98% in the placebo group.[37]

B vitamins (B1, B6, B12, etc) are commonly administered in stroke patients, and were shown to decrease inflammation (levels of C-reactive protein); lower homocysteine levels, a molecule associated with poor vascular status and poor outcome in stroke patients, and through various actions to improve post-stroke recovery.[38] Their actions were shown to be independent and additive to that of anti-oxidants or lowering of homocysteine in a placebo-controlled study on 96 stroke patients[39] who were given antioxidants (vit E and C) and/or vit B2, B6, B12. Upon administration of supplements patients had decreased inflammatory marker CRP, an increase in antioxidant status (measured by total plasma antioxidant capacity, malondialdehyde) and decreased homocysteine levels. Antioxidants did not reduce homocysteine levels and B vitamins did not improve antioxidant status, so both of them are required for overall improvement.

A meta-analysis on vitamin B supplementation which included 12 studies and 7474 patients[40] concluded that patients taking the vitamins had significantly lower plasma homocysteine as well as lower combined incidence of vascular events which included recurrent strokes and vascular deaths.

The HOPE 2 trial, where 5522 patients were followed for 5 years[41] showed that in adults with cardiovascular disease, daily administration of folic acid, B6 and B12 decreased risk of stroke (but not stroke severity or disability) simultaneously with lowering of homocysteine levels.

Also worth mentioning are the observations that in patients with severe cerebrovascular disease who were given B vitamins, on MRI there was a significant decrease in the volume of white matter hyperintensities,[42] and that supplementation with B vitamins for lowering homocysteine and secondary prevention of vascular events is not efficacious in association with antiplatelet therapy.[43]

The importance of B vitamins for the normal structure and function of neurons is highlighted by the observation that the deficiency in the enzyme methyltetrahydrofolate reductase (MTHFR) followed by an alteration in one-carbon metabolism significantly impairs stroke recovery by reducing neuronal and astrocyte viability.[44] The pathways associated with MTHFR deficiency lead to neuronal impairment depend upon activation of caspase -3, hypoxia-inducible factor 1-alpha (HIF-1α), and p53, all of which increase apoptosis.[45]

Vitamin C has well-known antioxidant properties, being the main cellular redox system alongside glutathione and thioredoxine; furthermore it is an essential co-factor for collagen synthesis, the scaffold of all tissues including the blood vessels; its extended absence leads to the petechiae and bleeding seen in scurvy. It has essential roles in catecholamine synthesis as cofactor for tyrosine- and dopamine β-hydroxylase; protects against lipid peroxidation, important protection for lipid membranes, and is in-

volved in detoxification of exogenous substances and cytochrome P-450 activity.[44]

It is an essential cofactor for 8 known important intracellular enzymes involved in the hydroxylation/ activation of various molecules including vitamin D3: Cu(+) -dependent monooxygenases as well as Fe(2+)-dependent dioxygenase, where it maintains the respective metallic atom in its active, reduced state by donating an electron. Enzymes of the 2-oxoglutarate-dependent dioxygenase family (2-OGDDs) regulate the hypoxic response via hypoxia-inducible factor 1α (HIF-1α), angiogenesis, stem cell phenotype and migration, and the epigenetic histone and DNA demethylases[46] all of which are essential aspects in neurogenesis and neuroplasticity, which are the most important mechanisms for recovery of neurological function after stroke.

The importance of vitamin C and its intracellular transporters: sodium-dependent vitamin C transporters 1/2 (SVCT1/2) is highlighted by the fact that mice without the gene which codes for SVCT2 die immediately after birth due to intraparenchymal brain hemorrhage.[47] Studies on population genetics showed that the gene coding for SVCT1-solute carrier family member SLC23A1-, tolerates SNP variations better than SLC23A2, and this indicates it has a higher physiological importance.[48] The recent finding that mitochondrial transport of vitamin C is mediated also by SVCT2[49] points in the same direction and makes the plasma levels of ascorbate less important than its intracellular concentration. The brain and the adrenal glands have the highest tissue concentration of vitamin C–(between 2-12 mM), the similitude is probably due to the fact that both are sites for catecholamine synthesis), compared to muscle 0.4mM, liver 0.8-1mM, plasma 40-60 μM, CSF around 160 μM.[50]

Ascorbate enters from blood into CSF through choroid plexus epithelium, with SVCT2 as transporter, and possibly as dehydroascorbate (DHA) which is reduced inside cells to ascorbate.[51] From CSF it is transported via SVCT2 inside neurons where it achieves 10 mM, and much less into glia, where its concentration is about 1 mM. Ascorbate can be released from both neurons and glia int CSF, and this homeostatic mechanism is coupled with uptake of glutamate.

This needs to be considered from the perspective that total body stores of vitamin C estimated with radiolabeled molecules amount to around 1500 mg, that scurvy symptoms starting with lassitude and neurological deterioration begin when vitamin C is depleted to about 300 mg, which corresponds to a plasma concentration around 10 μM,[51] that humans, few primates and guinea pigs are the sole mammals unable to synthesize vitamin C, and that animals produce vitamin C daily in the gram range[52] with more being synthesized during infectious episodes.

A possible explanation is that the activation of immune responses in infection increases both leukocyte number and their intracellular concentration of vitamin C by as much as 10-fold,-activated neutrophils from about 1mM to 10-12 mM[51] while inflammatory cytokines impair transport of vitamin C in other cells (TNF-a inhibits SVCT1 transcription, mRNA levels via the NF-kB pathway[53] and nitric oxide activation of inflammation pathways via NF-kBinhibits SVCT2 as well.[54]

Sustained presence of inflammatory cells or pro-inflammatory molecules in CSF and brain interstitium produced by activated microglia is followed by a severe impairment in the antioxidant status of neurons and glia, followed by the activation of apoptosis pathways.[55]

Further support for the importance of ascorbate in countering cellular stress is provided by multiple clinical studies which show that when vitamin C was administered intravenously to critically ill patients it was followed by a decrease in plasma levels of the C-reactive protein, malondialdehyde, procalcitonin and the pro-apoptosis marker poly(ADP-ribose) polymerase, and reduced the need for mechanical ventilation and vassopressor medication.[56]

Oxytocin was shown to have stimulatory effects on dopaminergic pathways and is an important positive modulator of neuroendocrine activity via the hypotalamic-pituitary-adrenal axis, which effectuates and controls the response to stress, including ischemic and oxidative stress. Oxytocin activity is modulated by, and in turn influences activity of various molecules–CRH, GABA, dopamine, serotonin[57] as well as the neurotrophic factors which control neural plasticity: brain-derived neurotrophic factor (BDNF) and the nerve growth factor (NGF).[58]

Oxytocin modulates activity in multiple brain areas; in a metaanalysis of 39 fMRI studies[59] it was shown that intranasal oxytocin administration was followed by an increase in the activity of insula, amygdala, the temporal and occipital lobes, and it positively modulates activity of the dopaminergic systems in the midbrain and basal ganglia, as well as activity in amygdala, midbrain, prefrontal, and temporal cortex.

Finally, oxytocin exerts inhibitory effects on NMDA receptors,[60] which is a very important activity in post-stroke recovery by helping to mitigate and/or abolish glutamate excitotoxicty.

Patients and Evaluations

For this study were enrolled all consecutive patients with a first or second stroke and agreed to be treated with the new combination treatment; a total of 15 patients were included between August 2017 and January 2018. Patients

had to be over 18 years old and not pregnant, to have the stroke assessed by computer tomography (CT) or MRI and to have neurological assessment at initial admission and before being discharged or transferred done by the respective neurologist, agree to the administration of the intravenous treatment, and make themselves available for further evaluations including imaging.

Standard neurological evaluation was done initially, before administration of treatment, the day after administering the treatments, at one month after first treatment and subsequently at variable intervals.

One patient was initially enrolled but excluded because she was not available for evaluation after treatment.

Besides the evaluation of the neurological status done by a hospital neurologist during the initial treatment for stroke, which was performed as part of the standard medical records and discharge summary without knowledge of future treatments, patients were evaluated for motor function and overall neurological status by two medical doctors: the neurologist (RM) recorded the evaluation of the patient neurological status in the respective medical record and independently the patient status was quantified with the National Institutes of Health Stroke Scale (NIHSS) by the other doctor (FS). Subsequently the evaluations were compared and no important discrepancies were observed.

The total NIHSS score thus obtained was used for comparing motor and neurological function before and after the intravenous treatments.

Results

The 15 patients received a total of 68 intravenous treatments in a 6-month interval (August 2017-February 2018). The patients were separated and analyzed in 3 groups based on the number of days between stroke occurrence and administration of first intravenous treatment: 0-35 days- (group 1); 36-100 days-(group 2); 101-360 days-(-group 3); a compound group representing patients treated in the chronic stage post-stroke was formed by adding the latter 2 groups in-36-360 days (group 2+3).

There were 3 females, 2 in the 0-35 group and 1 in the 101-360 days group.

Group 1 had patients more advanced in age and received less treatments during first months of treatment compared to patients in the chronic stage of stroke The initial severity of neurological impairments were similar between groups (mean initial NIHSS score around 16 for groups 1 and 2), Table 1.

Comparing group 1–subacute patients (0-35 days) and group 2–chronic patients (36-100 days), patients in the former group had significantly more improvement in the mean NIHSS scores–3.6 vs 0.83. Also, even though the mean initial NIHSS scores were similar [16], and they received fewer treatments (2.8 average per patient vs 4.1), administering the treatment earlier (in the subacute versus chronic phase), led to significantly better improvement in NIHSS scores–(mean Δ NIHSS 3.6 vs 0.83).

Patients who received the first treatment at more than 3 months after stroke occurrence (group 3, 101-360 days) had virtually no improvement as assessed with the NIHSS (mean Δ NIHSS=0), but 2 patients in this group had improvements in the Barthel index of 5, respectivley 10 points (1 or 2 items on activities of daily living). The improvements in the NIHSS scores are showed in Figure 2.

Two statistical tests were performed: a) the Pearson correlation test between the improvement of motor function at 1 month after first treatment as measured by Δ NIHSS score

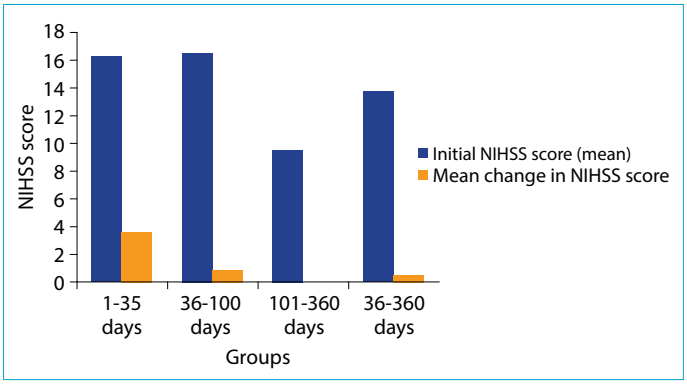

Figure 2. Mean initial NIHSS scores and change post-treatment.

Table 1. Cases of neuraxial anesthesia for ankylosing spondylitis

Days between CVA and first treatment (Tx)	0-35 days (group 1)	36-100 days (group 2)	101-360 days (group 3)	36-360 days (group 2+3)
Number of patients in group	5	6	4	10
Initial NIHSS score: Mean±SD	16.2±6.76	16.5±4.41	9.5±1.91	13.7±5.01
Age: Mean±SD	66.6±13.9	58.3±14.2	61.75±11.6	59.7±13.3
Mean nr of treatments administered per patient/month	2.8	4.1	2	3.3
Motor improvement: Δ NIHSS @ 1 month after 1st Tx: Mean±SD	3.6±2.509	0.83±0.752	0	0.5±0.707

and i. the number of days between stroke occurrence and the date when first treatment was given (promptness of treatment) and ii. the number of treatments administered Table 2.

There was also a much stronger correlation (r=-0.5, p<0.05) between the promptness of intervention and motor improvement than between the number of treatments and improvement in motor function (r=0.01), and this is an argument for the need to administer the treatment as early as possible after stroke; further delays achieve less improvements with more treatments.

Most neurological improvement (movement of fingers, leg, arm, improvement in dysphagia, expressivity, speech) occurred within 48 hrs of treatment administration in most patients, and this improvement was subsequently sustained. The one important exception to this rule was when infection (bacterial and/or viral) was acquired after treatment administration. This situation occurred in 4 patients and was followed by abolished motor progress from previous treatment. In 3 of these patients further treatment was followed by recovery/recuperation of motor improvement, but in 1 patient who had movement of fingers in upper extremity after first treatment, this progress was abolished by a severe respiratory tract infection and subsequent treatment did not bring improvement in upper extremity function.

Best results in motor improvement were observed in Patient A, 65 year old male who had a decrease in the NIHSS score of 8 points at 1 month after first treatment, during which he received 3 intravenous treatments and his NIHSS score improved dramatically, from 18 to 10. He continued to make steady progress, and after 3 and 6 months during which he received 6 intravenous treatments his NIHSS score was 2 (lack of fine motor skills, buttoning and unbuttoning shirt, which he recovered at 9 months).

His initial computer tomography imaging, taken when he was initially admitted to the hospital for disarthria, severe weakness in left upper extremity and difficulty walking, documented an ischemic modification in the territory of the right medial cerebral artery. After a few days on low-molecular weight heparin his condition worsened and he became hemiplegic on his left side. Besides dysartthria he had dysphagia for solids, but he was treated previously for esophageal stenosis for which he had two previous mechanical dilatations, which temporarily improved dysphagia for solids.

This patient also had a history of high blood pressure, cardiac bypass surgery for which he was on antithrombotic (clopidrogel) and before that he had for partial gastric resection for perforated ulcer. with two episodes of hematemesis in the 6 months preceding the stroke despite being on proton pump inhibitor.

Within a few hours after first treatment he was able to pull and bend the left knee; the improvement in motor function continued steadily and after a second treatment he was able to ambulate with a cane and after a third treatment he regained more than 90% of the motor function of both arm and leg on the left side. After recovering from stroke he had a third episode of hematemesis despite being on proton pump inhibitor, and a decision was made to start non-vitamin K anticoagulation instead of antithrombotic and the results were good even after surgery for inguinal herniation, with the patient regaining fine motor skills on left hand (able to button/unbutton shirt) and no further hematemesis or need for esophageal dilation. (Fig. 3)

There were two more patients who received this treatment and had good results despite the complex and severe pathology associated with stroke:

Patient B was a 49-year old man with dilatative cardiomyopathy, severe systolic dysfunction of left ventricle, congestive heart failure NYHA class III/IV, grade II/III regurgitation on both mitral and tricuspid valves, secondary pulmonary hypertension, chronic hepatopathy due to cardiac stasis, permanent atrial fibrillation with bradydysrhythmia and ventricular extrasystolia (pulse of 50-60 bpm on 100-120 RS complexes/minute), high blood pressure stage II and chronic gastritis. His cardiac ejection fraction was low (20%). His medication included Pradaxa, Carvedilol, digoxin, spironolactone, furosemid, hydrochlorothiazide, pantoprazol, ramipril, and he was placed on the waiting list for heart transplant. Soon afterwards he suffered a stroke on the left medial cerebral artery (cortico-subcortical, fronto-temporo-parietal ischemia on CT) with subsequent right hemiplegia, aphasia and mutism, followed on the CT performed 2 days later by ischemia on the cerebellar right side. Also had hyponatremia, hypochloremia, B-na-

Table 2. Statistical comparison of treatments and motor improvement between groups

Correlation between motor improvement and Tx start	(Δ NIHSS) vs (nr of days between CVA and 1st Tx) R=-0.506
Correlation between number of Tx and motor improvement	(Δ NIHSS) vs (number of treatments per patient) R=-0.011
T test and p values for ΔNIHSS scores between groups 1 and 2	T-value=2.58884 p=0.0292
T test and p values for ΔNIHSS scores between groups 1 and (2+3)	T-value=3.74451; p=0.0024

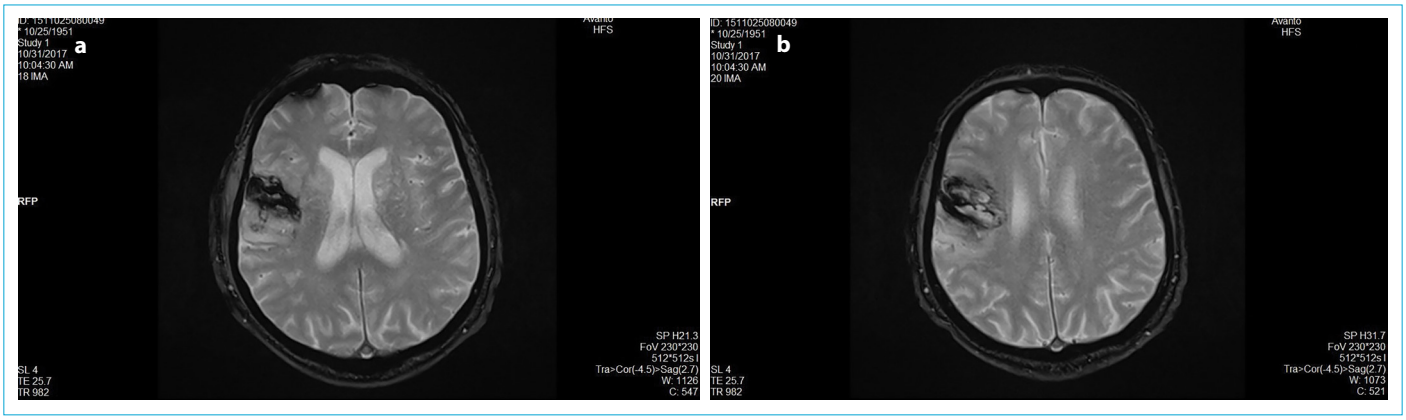

Figure 3. (a, b) MRI for Patient A done at 3 months after ischemic stroke with hemorrhagic transformation, 10 weeks after treatment of ischemic stroke on the right medial cerebral artery; at this time-point patient had recovered over 90% of motor function on left side, less fine motor skills (buttoning/unbuttoning shirt).

triuretic peptide (BNP) of 2344 pg/ml increased bilirubin and transaminases; he was administered first intravenous treatment at day 34 after stroke. He slowly improved and electrolytes, transaminases, bilirubin returned to normal, and BNP decreased to around 1500 pg/mL after 6 weeks. He began moving his right side and at 1 month after first intravenous treatment, Babinsky was negative on the right and there was a 1-point improvement on NIHSS. He was able to ambulate with orthosis and help at 3 months.

Patient C, 71 year old male with a history of gallbladder lithiasis, hypertension, coronary ischemic disease with grade I atrioventricular block, occlusion of right retinal artery with right eye cecity, while on antithrombotic medication had a ponto-mesencephalic stroke with right side hemiplegia, moderate-severe dysphagia, left side ataxia, severe disarthria, bilateral pulmonary thrombembolism, hepatic and right renal thrombembolism, severe pulmonary hypertension, neurological bladder with urethral catheter and repeated urinary tract infections,. He was administered first treatment 80 days after the stroke, with an initial NIHSS score of 20. Even though after the first treatment he was agitated and needed to be administered diazepam and he had emesis, he steadily improved and 1 month afterwards he gained 1 point on NIHSS scale with movements of right hand fingers and leg. After 3 such treatments the urinary catheter was removed, swallowing and speech improved, and after two months he was able to sit unaided at the edge of the bed and after 6 months he was filmed taking steps with help.

These examples in which severely ill patients constantly improved their overall health status upon administration of treatment and did not suffer any notable adverse reaction,, are strong indicators that this treatment is safe to be administered in patients with complex pathologies, including cardiovascular, hepato-renal, gastric, who simultaneously receive antithrombotics or anticoagulation with coumarine, heparin, low molecular weight heparin or non-vitamin K antagonist oral anticoagulants (NOACs).

Adverse Reactions

Besides the 68 intravenous treatments administered in this pilot study, more than 150 such intravenous treatments were administered to more than 35 patients with various pathologies between October 2014 and August 2018, with minimal adverse reactions, both in severity and frequency (less than 10%). There were temporary and mild elevations (10-25 mm Hg) in the systolic blood pressure during administration in about 10% of patients, which did not persist after administration of treatments. In 4 patients we performed simultaneous Holter EKG monitoring and there was no abnormality in rhythm or repolarization; in the patient with dilatative cardiomyopathy there was a decrease in ventricular extrasystoles and the rate of fibrillation in the supraventricular rhythm from around 110-120/minute to less than 100/minute.

In 3 patients there was hyperstimulation and agitation in the first few hours, which in one patient with previous such manifestations and treatment prompted the administration of 5 mg diazepam; poor sleep/insomnia occured when treatment was administered late afternoon. Emesis during or immediatlely after administration occured in 3 patients who had gallblader dyskinesia/lithiasis, and which was supressed by spasmolitic administration–drotaverine 40-80 mg iv). Both the hyperstimulation and emesis in patients with billiary dyskinesia is most probably due to the stimulation of the autonomic (vegetative) nervous system; blocking the overactivity with spasmolytics addresses this issue and emesis was avoided in subsequent treatments in these patients when the spasmolytic was administered simultaneously with the treatment.

Discussion

In the areas affected by hypoxic stress neuronal impairment ranges from complete cell destruction (cell death, in the center of ischemic area) to a temporary suspension of function ("penumbra") due mainly to mitochondrial damage, and similar to the cardiomyocyte "hibernation" seen after myocardial infarction. Neuronal destruction occurs mainly through necrosis and apoptosis, the former being a rapid death occurring in the context of ample ionic and osmotic imbalances between neurons and their environment, while the latter is a protracted deterioration of cell function and structure triggered by various cellular pathways and genes, most important being Bax/Bcl and caspases.[61] Before the initiation of the final, irreversible steps of apoptosis (activation of caspases), the activation and inhibition of cellular pathways via receptors, secondary cell messengers, intracellular enzymatic systems, gene activators and repressors occur in the context of a complex interplay of factors (ex. excitatory aminoacids, cellular redox system, inflammatory pathways, etc) which can be modulated so that neuronal destruction is diminished or avoided.

Extended hypoxic conditions activate cellular pathways other than HIF-1α in order to ensure cell survival, namely p53, mTOR, the endoplasmic reticulum (ER) stress, and finally the unfolded protein response (UPR), all seemingly activated by miRNA-210;[62] ultimately cell apoptosis becomes inevitable. However in the early stages of hypoxia, vitamin C via direct inhibition of HIF-1α[50] can restore the aerobic metabolic pathways, especially if other antioxidants (glutathione) and anti-inflammatory molecules act in tandem. Additionally, important neurotrofic actions are exerted by Actovegin which stimulates IGF-1 and TGF-b pathways, DMSO increases fusogenic activity at synaptic membranes and TGF-b expression; finally BDNF-1, molecule with essential neurotrofic roles, is positively modulated by DMSO, oxytocin, glutathion and vitamins B and C via various pathways.

Post-stroke recovery was shown to be independently predicted by a few factors: the brain area affected by the stroke, both in size and location (cortical, subortical), age of patient (disadvantage with advancing age), physical condition (ratio between the strength of knee flexion on the unaffected side and body weight), presence of other pathologies.

Another important factor is affecting recovery post-stroke is the presence of infections, which have a significant negative impact on neurons by increasing inflammatory molecules and worsening the neuronal mitochondrial function through activation of anaerobic metabolism.

Finally but not least important, in some patients genetic polymorphism impairs recovery by significantly impairing molecules with important roles in brain function, neurogenesis and neuroplasticity. It was shown that the presence of Apolipoprotein ApoE ε4 polymorphism was associated with poor recovery at one month post-stroke and more patients with disability at 3 months post-stroke.[63]

BDNF gene polymorphism rs6265 results in a change from valine to methionine (val66met) and a reduction in BDNF activity and was associated with worse outcomes at 2 weeks and 1 year after stroke.[64] BDNF polymorphism is present in about 30% of individuals in the United States of European descent; in Italy and Japan at approximately 50% and 65%, respectively.[65] Other neurotransmitters and their receptors can also be affected-dopamine via SNPs in catechol-o-methyltransferase (COMT), dopamine transporter protein, or dopamine receptors D1, D2 and D3.[65]

Other polymorphisms associated with stroke pathology were found in the low-density lipoprotein receptor (LDL-R) genes LDLR rs688, Apolipoprotein A5 (ApoA5) rs662799 A/G and Cholesteryl ester transfer protein CETP rs708272 C/T,[66] MAP2K4 rs3826392 C/A which is linked to higher plasma levels of IL-1,[67] matrix metalloproteases 1, 3 and 12-MMP-1 -1607 1G/2G and MMP-12 -82 A/G gene polymorphisms;[68] MMP-1 -1607 1G/2G and MMP-3 -1612 5A/6A;[69] MTHFR 677C>T and endothelial nitric oxide synthase eNOS intron 4a/b,[70, 71] haptoglobin H2-2.[72]

The list of genetic polymorphisms found to influence the levels of molecules associated with stroke is growing:[73] for vitamin E both APOA5 rs662799 and PAI-1 4G/5G SNPs, for vitamin K-APOE E3/4 and E4/4, for vitamin D in its 1-Hydroxylase coding gene CYP27B1 (R107H) which greatly reduces the conversion to active vitamin D3, the vitamin D receptor VDR rs7968585 (associated with increased risk of myocardial infarction and possible other ischemic conditions); for vitamin B12 the SNP 772G>A in the fucosyl transferase transporter FUT2 (required for cellular uptake of vit B12) gives low concentrations of B12 both in plasma and cells; folic acid besides MTHFR 677C>T there is as importantly SLC19A1 which is involved in transporting folic acid across blood-brain barrier, and the SNP 80A>G rmpairs its function.

Finally, one important genetic polymorphysm which we suggest influences the outcome of stroke although its association was not yet studied, is in the solute carrier family members SLC23A1/2 (coding for sodium-dependent vitamin C transporter 1/2 (SVCT1/2). As mentioned above, brain is concentrating vitamin C, more avidly than any tissue, and the absence of SVCT2 is lethal shortly after birth via intracerebral hemorrhage.[47] Impairement of SVCTs may be more prevalent in patients with low seric vitamin C levels, situation encountered in aproximately 7% of general pop-

ulation[74] and about 47% of hospitalized patients.[75] Genetic polymorphysm in SVCT1 influences levels of plasma vitamin C, and different SVCT1 and SVCT2 genotypes influence the correlation between the intake and serum levels of vitamin C.[76] Furthermore, an impaired vitamin C transporter coupled with simultaneous deficits (MTHFR, BDNF, ApoE, etc) or deleterious circumstances such as infection, can accentuate the vitamin C deficiency and tip the metabolic balancein mitochondria towards anaerobic pathways, increased generation of reactive oxygen species and ultimately apoptosis.

Extensive genetic testing for polymorphisms involved in stroke pathogenesis and recovery for every patients is not yet currently done, although there are already available very affordable options for whole genome or multiple gene sequencing (a few hundred genes can be sequenced for around $100 or less); nevertheless this will impact the care of the stroke patients, as well as the treatment of other chronic, multifactorial diseases.

However, notwithstanding patient confidentiality issues and the insurance costs consequences of genetic testing, the benefit of early treatment (in acute and subacute stroke) clearly surpasses the benefit of precise diagnostic by both limiting the neurological impairment and healthcare costs, and for this reason we strongly advocate the administration of combination therapy promptly after stroke.

Fortunately, a positive fact about the genetic factors which influence post-stroke recovery is that for prognostic and therapeutic purposes some of these genetic polymorphisms (BDNF) can be counteracted by increasing the dose of treatments (kinetotherapy). in others (APOe4) there is no current treatment known to antagonize it, however vitamin/antioxidant supplementation and more intense and prolonged physical exercise may bring similar compensation.

Refocusing from nucleic acids and genes to the cellular level, the treatment administered intravenously in acute and sub-acute stroke aims to achieve three goals: I. Limit neuronal damage; ii. Restore the normal mitochondrial function, and iii. Stimulate neurogenesis.

1. Limiting neuronal damage caused by ischemia and/or hemorrhage and subsequent oxidative stress and inflammation. This is achieved by promptly administering antioxidant and antiinflammatory substances, preferably those with known physiological roles (glutathion, vitamin C), preferably intravenously. DMSO has prompt and pleiotrophic antiinflammatory actions, being an excellent solvent allows mixing of all perfusion components, and by increasing membrane permeability increases intracellular access of vital substances (vit C, etc) which may be affected by impaired membrane transport systems (solute carrier family members including sodium-dependent vitamin C transporter SLC23A1/2, receptor kinases, etc) due to age, genetic polymorphism, circumstantial impairment of transcription of certain genes, etc.
2. Restoring the normal function of neuronal mitochondria and aerobic metabolism in the soma of partially damaged neurons and their networking synapses. These are functioning in a low-energy mode induced by the hypoxic conditions, in which the anaerobic, glycolytic pathways are activated by HIF-1α and their mitochondria produces much less energy with more oxidative stress. This fragile functional status is also time-limited, as the functional degradation of cells marked by the accumulation of metabolic debris, free radicals, and defective molecules becomes irreversible at some point, which for most patients and a majority of damaged neurons seem to reside at about 30 days post-stroke.
3. Stimulating neurogenesis as main repair mechanism in post-stroke injury. Stimulating migration of immature neurons from periventricular areas, their migration and subsequent integration in neural network is a complex process involving multiple steps and pathways, and here again DMSO is an important molecule by acting on RhoA/G and Rac1 pathways.

The innovative aspect of this stroke treatment consists in both the use of glutathione and oxytocin, and also their synergistic combinination with DMSO, Actovegin, vitamins C and B. Associating this many substances is made possible by the excellent solvent properties of DMSO, which also facilitates the permeation of cellular membranes and the blood-brain barrier to the substances co-administered.

DMSO and Actovegin were used previously in stroke mostly as monotherapy and sometimes as a 2-substance combination with limited results, due to the complex, multifactorial pathology of stroke.

In animal models of stroke therapy with DMSO at high doses (0.75–3 g/kg) had conflicting results, some studies reporting benefits in rhesus monkeys,[77] cats[78] and in dogs, where it completely prevented cerebral infarction when administered simultaneously with arterial occlusion;[79] while others reported no benefit in baboons.[80] It was also found that beneficial effects of iv DMSO decrease dramatically in time,[81] so that 1.5 g/kg DMSO administered at 1 hour post arterial occlusion reduced infarct volume at 24 hours by 44%, while only a 17% reduction in infarct volume was obtained when infusion was started 2 h post-occlusion.

One important difference was that benefits were observed when DMSO was administered in combination with other substances acting on inflammatory pathways-dexamethasone, prostacycline[77, 78] or metabolic pathways modulating mitochondrial activity-fructose 1.6-disphosphate FDP.[82]

A similar limitation in efficacy of recovery of motor function post-stroke was observed when Actovegin was administered as monotherapy.[36]

We consider that the complexity of stroke pathology and the multitude of cellular pathways involved in recovery from stroke mandates the use of a combination of therapeutic susbstances which act simultaneously, complementary and synergistic on multiple molecules and pathways. By adding strong physiological anti-oxidants (glutathion, vitamin C), the neuromodulator oxytocin to the tried-and-true DMSO, deproteinated veal serum and B vitamins, the benefits are greatly increased with no additional risks for the patients. This point is exemplified by comparing the efficacy of DMSO and Actovegin from previous clinical studies with the results from this study.

In the pilot study which used DMSO and fructose 1,6-disphosphate FDP,[82] improvement of motor function was seen in 7 of 11 patients (63%) treated with DMSO and FDP and 1 of 5 (20%) treated with standard treatment; in this study all 5 patients treated within 35 days of stroke had motor improvemet at 1 month.

Another study[83] showed that in 18 hemiplegic, non-ambulatory patients who had mean NIHSS score of 11.2 initially, after 1 month of treatment the mean decrease in NIHSS was 1.6 points; our results compare favorably with a mean NIHSS score decrease of 3.6. even though the mean initial NIHSS score was higher (16.2).

A similar situation is seen when we compare the results with the ARTEMIDA trial results which used Actovegin only [36]–the mean decrease in the NIHSS score after 1 month of injectable and oral Actovegin was 1.8.

One final aspect of post-stroke treatment is the periodicity of administration, and here we need to consider the essential processes for post-stroke recovery: neurogenesis, angiogenesis and new synapse formation.[84, 11] We consider that the intermittent rather than continuous administration of the treatment is more beneficial, and good results were obtained by administering the perfusions at 5-14 days intervals and no advantage was observed when administered at shorter intervals in two patients. This is probably linked to the periodicity of the processes of neurogenesis and synapse formation, which involves different molecules and paths, and indeed, a 14-day periodicity in supraventricular zone neurogenic activity was shown recently[85] as well as an increase in new cortical neurons in the peri-infarct cortex up to 65 days post-stroke. More studies and information and new biomarkers will be needed in order to individualize treatment periodicity.

Conclusion

This is the first study which documents the benefit of treating stroke patients with the natural antioxidant glutathion, the neuromodulator oxytocin and their use in combination with DMSO, Actovegin, and vitamins C and B, all with beneficial synergistic effects on neuronal structure and function.

The intravenous combination of glutathion, DMSO, Actovegin, oxytocin, vitamins C and B was safe to be administered; more than 200 were administered to date with no major side effects (emesis was observed in patients with gallbladder dysfunction, and hyperstimulation/agitation/insomnia for 12-18 hours in about 20%). The fact that it was administered with positive results in complex and severe pathologies (dilated cardiomyopathy with low ejection fraction and hepatorenal dysfunction, multiple thrombembolism on liver, kidneys and lungs, cardiac bypass and gastric ulcer) also pleads for its safety and benefits.

It was especially efficacious in treating stroke in the acute and subacute stages (up to 35 days from stroke onset), and it had good results in both ischemic stroke and hemorrhagic transformation (patient A and two others); best recovery (complete lower and upper extremity at 6 months, including fine motor skills–buttoning shirt) was seen in the hemorrhagic transformation of ischemic stroke.

A major advantage is that it can be employed both in hemorrhagic and ischemic stroke, and it can be administered in the hemorrhagic transformation of ischemic stroke, which can extend the treatment window of tPA.

Finally, the benefit of early treatment (in acute and subacute stroke) with the antioxidant, anti-inflammatory and anti-apoptotic substances present in this combination supersedes the benefits of screening for molecular and genetic disorders by addressing simultaneously the most important pathways involved in neuronal injury post-stroke, and deleterious dysfunctions and mutations. For this reason we strongly advocate the administration of combination therapy as soon as possible after stroke, for both ischemic and hemorrhagic stroke, even before imaging, in order to limit the damage, promote neuronal regenerative processes and recover neurological functions. At the same time we advocate the need for more ample clinical trials in order to study the benefits and limits of this new combination treatment for stroke.

Disclosures

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