A PROTOCOL FOR THE ISCHEMIC STROKE IN PATIENTS TO BE TREATED WITH MAJOR OZONATED AUTOHEMOTHERAPY (MOAHT)

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Vascular disorders such as a stroke are most interesting because involve a high number of patients. Stroke is the third most common cause of death and the consequences of serious disability are very costly from the social-economic aspect.

Embolism of a cerebral artery is often the consequence of atrial fibrillation as well as of atherosclerotic lesions with hypertension and with or without diabetes. Immediately after the embolus has blocked the arterial circulation, the ischemic stroke follows with progressive hypoxia of the nervous system. The typical patient loses 1.9 million neurons for each minute in which stroke is untreated. Thus the earlier, the better and ideally therapy should begin within 90 minutes of stroke onset, a time period very difficult to achieve.

We must note that the brain, although it is only 2% of the normal body weight, consumes about 20% of the total body oxygen consumption or about 3.3 mL/100 g /min. Moreover the utilization of glucose is as much as 5.5 mg/100 g brain/min so that the Central Nervous System (CNS) needs of about 110 g of glucose daily. Thus the maintenance of the oxygen and glucose levels in plasma for the brain is essential. Within 5-8 min after heart arrest, the nervous tissue, not receiving oxygen and glucose undergo irreversible damage characterized by anoxia, consequent oedema and neuronal death.

At the same time a vast area adjacent to the stroke area, called the ***penumbra*** undergo in a few hours a progressive damage characterized by progressive hypoxia, glucose deficiency, increase of lactic acid concentration due to anaerobic glycolysis, hence acidosis, decrease of intracellular ATP, a state of depolarization, oedema, inhibition of the protein synthesis, hence of neurotransmitters. Unless we can intervene, also this large area may become irreversibly damaged with an impendent peripheral damage at various levels. However, while the damage at the “core” is extremely rapid, the nervous tissue in the ***penumbra*** may recover if we can intervene, within 5-12 hours, with an effective therapy. Almost needles to say we are aiming only to treat the stroke due to embolism and not the stroke after a primary cerebral haemorrhage or a subaracnoidal haemorrhage.

Since the discovery of Tissue Plasminogen Activator (TPA, Alteplase) it appeared obvious to test this drug as soon as possible in the embolic stroke because it appeared feasible to induce a rapid dissolution of the embolus that would have allowed a re-opening of the circulation with the hope of limiting the neuronal damage. Thrombolysis was therefore pursued but it was soon realized that only patients arriving at the “stroke unit” within 3-4.5 hours after the stroke onset could have an anatomical and clinical advantage (Wahlgren et al. 2008). It has now been well demonstrated that after Alteplase, IV Injected within 3-4.5 hours the mortality is reduced and both the risk of a successive haemorrhage and functional recovery ranges between 56.3-58.0 %.

The problem is not only to have an efficient and always ready “stroke units” but it is absolutely indispensable to receive the patient within 4.5 hours from the initial symptoms. Unfortunately more than 80% of patients arrive at the hospital much later and therefore trombolysis cannot be performed. In such a case only the best orthodox therapy can be used with aspirin or clopidogrel for preventing further platelet aggregation, and anti-hypertensive therapy with diuretics, cardiotonic therapy etc. As a consequence the nervous tissue within the ***penumbra*** cannot be saved and both mortality and successive spontaneous recovery are limited and disability is extended with the serious consequences of a poor quality of life (Qol) of the patient.

What can we do for the late patients? Our aim should be to re-oxygenate the penumbra as soon as possible because the longer is the delay-time, the worst is the damage due to oxygenation-reperfusion. Several different approaches have been tested among which hypothermia, hyper-oxygenation via oxygen inhalation, albumin infusion as an antioxidant and as an anti oedema factor with minimal results.

On the basis of our previous experience in chronic limb ischemia, we are proposing to evaluate the use of Major ozonated autohemotherapy (M-AHT) as soon as the patient arrive at the hospital, (within 5-12 hours) actively pursuing this therapy for at least 6-12 days (or even longer) after the initial stroke. Indeed, if we can quickly re-oxygenate the ***penumbra*** area, it may be possible to improve the patient's prognosis

So far we have only a few anecdotal data, which strongly encourage the earliest application of M-AHT. Both Dr Cooper in the Dept of Neurobiology, UCLA, Los Angeles and Dr G. Wasser have applied this therapy achieving impressive results. Several other physicians have privately used this approach surprisingly obtaining useful results even applying this therapy 1-2 weeks later.

Rationale of the study

As ozone has strong oxidizing properties, it seems paradoxical that this gas may display beneficial effects. However, on the basis of this synthesis:
3 O2 + 68,400 Cal 2O3

and the reversibility of the reaction, it has been shown that ozone readily dissolves into the blood water, releases its energy into the hematic components, hence into the body during the reinfusion procedure in the blood donor. Ozone is about ten-fold more soluble in water than oxygen, and all the basic chemical reactions concerning it have been clarified during the last decade.
The therapeutic range has been precisely defined to be within ozone (Bocci et al.2009)concentrations of 20 (0.42 μM) - 80 (1.68 μM) μg/mL of gas (pure O2: 95% and O3: 5%) per mL of human blood. Owing to the potent antioxidant power of blood due to its hydrophilic, lipophilic and cellular enzymes, a small part of the ozone dose dissolved into the water of plasma is instantly quenched by free antioxidants (mainly uric acid, ascorbic acid and albumin), while the bulk of ozone reacts with polyunsaturated fatty acids (PUFA) mostly present in the three hydrophobic tasks of albumin .
-R-CH=CH-R + H2O + O3 □ 2 RCHO + H2O2

Thus the potential energy of ozone is finally transferred into two fundamental messengers such as hydrogen peroxide as a ROS and aldehydic molecules of which 4-hydroxynonenal(4-HNE) is the quantitatively relevant lipid oxidation product:
O3 + plasma □ H2O2 + 4-HNE

Thanks to the high ozone reactivity these biochemical reactions occur in few seconds, in fact within the canonical five minutes during which an average 200 mL of human blood ex vivo in a sterile glass bottle with the 200 mL corresponding volume of the gas mixture (O2+O3), ozone is totally exhausted while oxygen is solubilized in plasma and fully oxygenates hemoglobin:
Hb4O4-6 + O2 □ Hb4O8

Blood oxygenation is useful, but it has little practical relevance because oxygenated-ozonated blood is reinfused via venous route into the donor during the next 20 minutes and it is abundantly diluted with venous blood.

What is the fate of H2O2 and 4-HNE? H2O2, being unionized, rapidly enters all blood cells and the chemical gradient between plasma-cells is about 10% of the extracellular concentration . In other words, when the highest ozone concentration is mixed with blood, depending upon the modest interindividual variability of antioxidant potency (1.28-1.83 mmol/L plasma,) the highest H2O2 concentration measured in plasma is 40-50 μM and therefore it is at most 4-5 μM inside the cells. This sudden inflow of H2O2 inside blood cells is the stimulus necessary to activate a series of biochemical reactions as follows:

1) In Erythrocytes
Activation of glycolysis with increase of ATP and 2,3-diphosphoglycerate. Functionally, the oxyhemoglobin sigmoid curve shifts to the right and increases the release of oxygen at the tissue level. H2O2 is promptly reduced to water by GSH, thioredoxin, catalase and GSH-peroxidase. GSH disulphide is reduced by GSH-reductase or by reducing equivalents (NADPH) generated by dehydrogenation of glucose-6-phosphate at C-1, a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PD).

2) In Leukocytes
Neutrophil phagocytic activity is enhanced. Inside monocytes and lymphocytes, H2O2 activates a tyrosin-kinase with subsequent phosphorylation of IkB, one of the trimeric components at rest of the NF-kB . The phosphorylated IkB detaches from the trimer and it is broken down in the proteasome. The remaining eterodimer p50 -p65 is transferred into the nucleus where it can activate about 100 genes. Of great significance is the final release of some cytokines (IFNgamma, TNFalpha and IL-8) and of some acute-phase proteins.

3) In Platelets
In relation to the ozone concentration, we have measured the release of PDGF-AB, TGFbeta□-1 and IL-8 . It must be said that the H2O2 concentration in the cells (4-5 μM) is the minimal necessary to switch on cellular responses and it probably lasts few seconds since GSH-Px and catalase promptly reduce it to H2O. In plasma, the H2O2 half-life is less than 1 minute and it is absent during blood reinfusion. On the other hand, among a variety of LOPs, 4-HNE remains fairly stable. A small part is broken down at once by enzymes such as GSH-S-transferases and aldehyde dehydrogenase but the bulk is bound to the -SH group of Cys34 present in domain-I of albumin. Furthermore, eleven nucleophilic residues (Lys199 and His146) can also bind up eleven 4-HNE molecules. Thus, owing to the high albumin amount (about 125 g intravascular and 160 g extravascular) the bound alkenals undergo a great dilution inthe body fluids causing a most important loss of toxicity. An interesting aspect is that albumin can transport 4-HNE in ALL body tissues, from liver to endocrine glands and the CNS. Thus 4-HNE-Cys adducts can be released at many sites and inform a variety of cells of a transient, acute oxidative stress. At submicromolar or picomolar levels, 4-HNE can act as a signaling molecule capable of activating the synthesis of □-glutamate cysteine ligase, □-glutamyl transferase, □-glutamyl transpeptidase, HSP-70, and hemeoxygenase-1(HO-1), and antioxidant enzymes such as SOD, GSH-peroxidase, catalase and last but not least, G6PDH, a critical electron-donor enzyme during erythropoiesis in the bone marrow. There is a wide consensus on the relevance of the induction of protective molecules during small but repeated oxidative stress In other words, the concept that a precisely controlled oxidative stress can strengthen the antioxidant defenses is well accepted today.

At the time of ozonated blood infusion, 4-HNE-Cys adduct can also act on the vast expanse of endothelial cells and enhance the production of NO. This crucial mediator on its own or as a nitrosothiol, with a trace of CO released with bilirubin via HO-1 activity allows vasodilation, thus improving tissue oxygenation in ischemic tissues. H2S is another potentially toxic molecule that, when released in trace amounts, it becomes an important physiological vasodilatorlike NO and CO

Moreover, as it happens for the just mentioned physiological traces of gases, the minimal amount of ozone necessary to trigger useful biological effects fits is wholly consistent with the concept of the xenohormesis theory .

Another very interesting aspect observed in 67-78 years old subjects affected by the dry form of age-related macular degeneration (ARMD) is that the majority of them report a feeling of euphoria and a sense of wellness and physical energy throughout the ozonetherapy cycle of 14-16 treatments lasting about two months . Whether these feelings are simply due to faith in the medical treatment (the power of the mind!), i.e. the power of the placebo effect, or is caused by the generated ozone messengers that can modify or improve the hormonal secretion is not yet known. Unfortunately, lack of funds has always prevented researchers from performing a study in normal volunteers where, before and after ozonetherapy, the complete hormonal pattern and cycling in the plasma throughout the 24 hours could have been determined. This study would be very informative and helpful to understand why the patients experiment a feeling of well-being. This may be due to improved oxygenation or/and enhanced secretion of GH, cortisol, and DHEA. If this proves to be true, the treatment would be preferable to the pharmacological ones. Patients with pain caused by arthrosis have also noticed a marked improvement and less pain which may be due to release of ACTH-cortisol or to a limited stimulation of COX-2 with enhanced release of prostacyclin (PGI2). It is also possible for LOPs, by reaching the hypothalamic area, to improve the release of neurotransmitters such as serotonin. dopamine, and endorphins as has been observed after intense physical exercise.

It is important to note that neither acute nor chronic toxicity has ever been observed during or after ozone therapy. Several studies performed to evaluate possible hematochemical, biochemical or enzymatic modifications have clearly demonstrated that ozone concentrations of 20-80 (even up 160) μg/mL of gas per mL of blood do not damage blood cells or other components.
Of the several procedures currently used for ozone administration on the basis of previous data for anti-aging purposes, it is suggested that the classical major ozonated autohemotherapy (M-AHT). is the most useful for the therapy of stroke.

Design of the study

Ideally the study ought to be carried out in a stroke unit which has already an experience of the disease. A neurologist, expert in stroke pathology, a specialized ozonetherapist and well-trained nurses are extremely important for patient's care.

We propose to perform a controlled and randomized study including 60 patients, who arrive at the stroke unit after 5 hours- but not later than 12 hours from the initial symptoms. All of these patients cannot be treated with either systemic or loco-regional Alteplase.

The two groups of male patients will be as homogenous as possible in terms of age (50-82 years old) and time delay. All patients will undergo immediately a CAT(computed axial tomography) examination for excluding a cerebral haemorrhage. Other exams will be carried out when necessary.

All patients will receive the most suitable general medication (aspirin 300 mg daily or clopidogrel, 300 mg the first day and then 75 mg/die and other necessary drugs). Blood will be take for the basic haematological and biochemical evaluation. A complete clinical evaluation will take note of the objective deficiencies such as: headache, paralysis and speech defect . An accurate assessment of swallow status to reduce pneumonia *ab ingestis*, the assessment of hyponutrition and the maintenance of a correct fluid balance are a must.

The M-AHT will be performed only in one group of 30 patients, twice daily (every about 12 hours) as soon as possible for at least 10 days. The urgency of the treatments is obvious because it is crucial to restore a normal metabolism in the ***penumbra***. If it proves to be useful, it can be continued for a further 1-2 weeks, once daily.

If possible 135-180 ml of blood will be drawn in a sterile glass bottle containing already 15-20 ml of Na citrate 2.8 % (1 ml citrate plus 9 ml of blood). Heparin is avoided owing to the risk of provoking a cerebral haemorrhage. Then 135-180 ml of a gas mixture (O2-O3) with an initial concentration of 20 mcg/ml ozone (hence total ozone dose from 2.7 up to 3.6 mg ozone) will be added into the flask, gentle mixed for 5 min and infused into the patient's donor. As this is an autologous transfusion, it can be safely infused in about 20 min. No side effects have been recorded.

The ozone concentration can be slowly but progressively increased up to 40 mcg/ml during the following days.

Scopes of the experimentation.

By considering the small number of patients, for statistical purposes, the two groups must be the more homogeneous as possible.

The objective is to evaluate the effect of O3-AHT on the basis of the modified Rankin scale (6 levels of which the first: 0: No symptoms .Level 5 describes severe disability, bedridden patient, incontinent and in need of constant nursing care and level 6 report the death of the patient. Evaluation will be performed within three months.
Favourable disability (Rankin: 0 or 1)
Unfavourable disability (Rankin > 2)

Moreover other end-points to be noted :

1) Intracranial haemorrhage
2) Cerebral oedema
3) Any other complications or side effects.

Inclusion criteria:

1) Acute ischemic stroke not treated with TPA
2) Age> 50 years.
3) Symptoms noticed no longer than 12 hours before beginning the therapy

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Exclusion criteria;

1) Intracranic haemorrhage
2) Uncertainty about the time of initial symptoms
3) Rapid improvement before beginning the therapy (Probably a transient ischemic attack)
4) Comatous state
5) Arterial pressure > 185 mmHg and 120 mm Hg minimal
6) Glycemia; < 50mg% ml ot > of 400 mg % ml
7) Symptoms suggesting a subarachnoidal hemorrage even if with negative CAT
8) Use of oral anticoagulants

Application of any suitable statistical analysis

Basic evaluation.

Both the neurologist (Specialist in stroke) and an internal medicine specialist will evaluate the patient clinically. The relevance of the neurological conditions may be evaluated by using the National Institute of Health Stroke Scale (NIHSS)

Post-treatment management

The evaluation of the efficacy of the O3-AHT treatment can be evaluated after one week and then after 30 and 90 days with either a CAT scan or MRI

Informed consent.

This will be obtained after consultation with both the patient (if possibile) or the relatives and it must be signed by a responsible adult.

Adverse effects

Although unlikely great attention should be paid to any subjective and objective side effects. These must be reported and considered at the conclusion of the study

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