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INVITED REVIEW

Neuroimmunomodulatory effects of transcranial laser therapy combined with intravenous tPA administration for acute cerebral ischemic injury

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Abstract

At present, the only FDA approved treatment for ischemic strokes is intravenous administration of tissue plasminogen activator within 4.5 hours of stroke onset. Owing to this brief window only a small percentage of patients receive tissue plasminogen activator. Transcranial laser therapy has been shown to be effective in animal models of acute ischemic stroke, resulting in significant improvement in neurological score and function. NEST-1 and NEST-2 clinical trials in human patients have demonstrated the safety and positive trends in efficacy of transcranial laser therapy for the treatment of ischemic stroke when initiated close to the time of stroke onset. Combining intravenous tissue plasminogen activator treatment with transcranial laser therapy may provide better functional outcomes. Statins given within 4 weeks of stroke onset improve stroke outcomes at 90 days compared to patients not given statins, and giving statins following transcranial laser therapy may provide an effective treatment for patients not able to be given tissue plasminogen activator due to time constraints.

***Key Words:** ischemic stroke; inflammation injury; cellular changes; laser therapy; neuromodulation*

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Introduction

Stroke is a leading cause of death in the world and a common reason for hospitalization. The two main types of stroke are ischemic stroke and hemorrhagic stroke, with the former representing the most common (80%) type. Ischemic stroke is subclassified as thrombotic or embolic in nature. A thrombotic stroke or infarction occurs when a clot forms in an artery supplying the brain and accounts for approximately 50% of all strokes, whereas an embolic stroke is the result of a clot formed elsewhere in the body and then transported through the bloodstream to the brain. Hemorrhagic stroke entails bleeding within the brain due to a blood vessel or an aneurysm rupturing inside the brain, with consequent damage to surrounding tissues.

Treatment for stroke depends upon whether it is ischemic or hemorrhagic. The only FDA approved treatment for ischemic strokes is intravenous administration of tissue plasminogen activator (tPA *e.g.*, alteplase) that acts by dissolving the blood clot and improving blood flow to the part of the brain being deprived of blood. When administered within 4.5 hours from stroke onset, tPA may improve the chances of recovery. The short time interval for treatment and increased risk of intracranial hemorrhage result in only a small percentage (5–15%) of patients receiving tPA (Lloyd-Jones et al., 2010). Another treatment procedure involves removing the blood clot by introducing a catheter to the site of the blocked blood vessel in the brain. Sometimes this procedure involves tPA being administered directly into the blood clot to help dissolve the blockage. There is clearly a need for alternative treatments for stroke that are effective when applied within a longer time period than 4.5 hours from stroke onset. Transcranial laser therapy (TLT) may provide a suitable treatment option and involves applying low level laser irradiation to the scalp to be transmitted through the skull to penetrate the brain.

Alterations to Cellular Structure and Function in Cerebral Ischemia

Many changes take place at the cellular level when a stroke occurs. The obstruction of a major cerebral vessel (usually the middle cerebral artery, MCA), if not resolved within a short period of time, will lead to a core of severely ischemic brain tissue that may not be salvageable. The ultimate size of the brain infarct depends on the penumbra, a zone of tissue around the core of the infarct where neuronal electrophysiology is not compromised and blood flow is still maintained above a neuronal disabling level (*i.e.*, the critical 20–25% of normal blood flow). If blood flow in the penumbral zone decreases below this critical level and/or energy requirements are exceeded, the infarct zone will expand. Decreased blood flow leads to a reduction in phosphocreatinine and adenosine triphosphate (ATP) and if ischemia is prolonged, the energy depletion will be sufficient to lead to severe impairment of cellular function by disruption of ATP-dependent

processes. The disruption of ionic gradients across excitable (neuronal) and nonexcitable (glial) membranes owing to loss of ATP is characterized by efflux of K^+ from the cells, cellular depolarization, and influx of Na^+, Cl^-, and Ca^{2+} into the cells (Siesjo, 1992a, b). In the phase of increased extracellular K^+ together with a decrease in pH, ATP stores are rapidly depleted and ultimately result in marked changes in ion conductance. The increase in K^+ can reach levels sufficient to release neurotransmitters such as glutamate, which in turn will stimulate Na^+/Ca^{2+} channels coupled to the N-methyl-D-aspartate (NMDA) receptor; these will further lead to Na^+, Cl^-, and H_2O accumulation, cell swelling, and cytotoxic edema. Transient extracellular K^+- induced depolarizations can also contribute to the expansion of the neuronal infarct. Peri-infarct depolarization produces disruption of ionic gradients and transmitter release, with the associated accumulation of Ca^{2+} in the cells leading to rapid and extensive breakdown of phospholipids, proteins, and nucleic acids by activation of calcium-dependent phospholipases, proteases, and endonucleases. Depolarization causes an increase in intracellular Ca^{2+} and an increase in extracellular glutamate. Glutamate, an excitatory neurotransmitter implicated in ischemic neuronal damage, results in excitotoxicity in which excessive extracelluar glutamate kills neurons through an increase in intracellular Ca^{2+} (Barone and Feuerstein, 1999).

Accumulation of products such as free fatty acids that are metabolized to toxic lipid peroxides further contribute to structural and functional alterations of the membrane and cell function. Free radicals which are a group of highly reactive oxygen species (ROS) are formed during ischemia and cause considerable damage to lipids, DNA, and proteins, and contribute to the process of neuronal death. Free radicals also contribute to the breakdown of the blood-brain barrier and brain edema. Levels of free radical scavenging enzymes (*e.g.*, superoxide dismutase, SOD) decrease during ischemia and nitric oxide levels are elevated. Nitric oxide (NO) produced primarily by neuronal and inducible nitric oxide synthases promote neuronal damage after ischemia (Barone and Feuerstein, 1999).

Inflammation Injury in Acute Cerebral Ischemia

Evidence demonstrates that inflammation and immune response play an important role in the outcome of ischemic stroke (Famakin, 2014). Inflammation after stroke involves leukocyte infiltration in brain parenchyma that contributes to cerebral damage. Peripherally derived mononuclear phagocytes, T lymphocytes, natural killer (NK) cells, and polymorphonuclear leukocytes, which produce and secrete cytokines, can all contribute to central nervous system (CNS) inflammation and gliosis (Brea et al., 2009). Blood-derived leukocytes and resident microglia are the more activated inflammatory cells, accumulating in the brain tissue after cerebral ischemia, leading to inflammatory injury (Akopov et al., 1996). Microglia, the major source of cytokines and other immune molecules of the CNS, are the first non-neuronal cells that respond to CNS injury, becoming phagocytic when fully activated by neuronal death. Neutrophils are the first leukocytes recruited to the ischemic brain and occurs between 6 and 12 hours following stroke onset, progressing for up to 24 hours, and then declining (Akopov et al., 1996). Monocytes accumulate in the injury area around 12 to 24 hours after onset of ischemic injury, and are rapidly transformed into tissue macrophages capable of aggressive phagocytosis. Other immune/inflammatory cells such as lymphocytes are present at later time points (Brea et al., 2009).

Mediators released by activated leukocytes, such as ROS, proteases and cytokines, cause damage to neurons and other brain cells (del Zoppo et al., 1991). Studies in rodents have shown that microglia and macrophages are the principal CNS source of cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) (Gregersen et al., 2000). Following ischemia, astrocytes are activated in the brain and are capable of secreting inflammatory factors such as cytokines, chemokines and inducible nitric oxide synthase (iNOS) (Dong and Benveniste, 2001). iNOS from astrocytes has been shown to enhance ischemia-like injury to neurons (Hewett et al., 1996).

Low-level Laser Therapy (LLLT)

Low-level laser therapy or low-level light therapy uses low-power lasers or light-emitting diodes (LEDs) to alter cellular function; this is in contrast to high-power lasers that cause tissue ablation.

The absorption of light by chromophores within cells increases the chemical energy within cells in the form of ATP, increases deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), promotes NO release, increases cytrochrome c oxidase activity, ROS production, intracellular membrane activity particularly in mitochondria, Ca^{2+} flux and stress proteins (Karu et al., 2005; Khuman et al., 2012). Mitochondria are the single most important organelle governing the cellular response to LLLT, with cytochrome c oxidase on the mitochondrial inner membrane being the crucial chromophore. The effects of LLLT on cells and tissues involve a complex cascade of pathways with altered intracellular signaling and changes to redox states. Mitochondrial ROS may act as a modulatable redox signal, reversibly affecting the activity of a range of functions in the mitochondria, cytosol and nucleus. LLLT produces a shift in overall cellular redox potential towards oxidation and which is associated with cellular vitality (Karu, 1999), increased ROS generation and cellular redox activity (Lubart et al., 2005). Several transcription factors are regulated by changes in cellular redox state including nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) that enter the nucleus and cause transcription of a range of new gene products (Meyer et al., 1994).

LLLT Therapy for Stroke

Animal studies

Most studies have used near infrared light with wavelength 808 nm, but a few studies have also employed red visible

light of wavelength 633, 660 or 710 nm.

Several *in vitro* ischemic models have been tested for effect of light irradiation.

LED irradiation (710 nm, 4 J/cm^2) of rat primary cortical neurons (isolated from embryonic brain) exposed to oxygen-glucose deprivation, followed by reoxygenation and normal conditions, promoted neurite outgrowth and synaptogenesis through mitogen-activated protein kinase (MAPK) activation and protected against ischemic damage (Choi et al., 2012a). Laser irradiation (810 nm) of mouse primary cortical neurons induced an increase in Ca^{2+}, ATP and mitochondrial membrane potential (MMP) at 3 J/cm^2. ROS was significantly increased at 0.03 and 0.3 J/cm^2, with a peak at 3 J/cm^2, followed by a decrease at 10 J/cm^2 and a second increase at 30 J/cm^2. NO levels followed the same general triphasic pattern as the ROS levels but the increase was less prominent compared to ROS (Sharma et al., 2011). Laser irradiation (633 nm) of rat hippocampal brain slices exposed to perfusion of oxygen-free/low glucose medium increased the time required for loss of electrical excitability and increased recovery from ischemic injury. Injury in this system is known to result at least in part from free radical production (Iwase et al., 1996).

In vivo studies in rats and rabbits have shown that laser irradiation can result in significant clinical improvement when administered at an appropriate time after onset of ischemic stroke.

In one study, rats were subjected to 90-minute middle cerebral artery occlusion (MCAO) followed by reperfusion, LED irradiation (710 nm, 1.8 J/cm^2) applied randomly to each animal 12 hours each day for 3 weeks after MCAO establishment. Helper T cell ($CD4^+$) count was reduced after MCAO but significantly increased by LED irradiation. Infarct sizes were decreased in MCAO + irradiation group compared with MCAO control group. IL-10 mRNA expression and immunoreactivity of regulatory T cells were increased in MCAO + irradiation group compared with MCAO control group. IL-4 mRNA expression tended to be decreased in MCAO + irradiation group compared with MCAO control group but was not significantly changed. Increased microglia activation after MCAO was reduced by irradiation. Irradiation group showed improved neurological severity scores after MCAO (Choi et al., 2012b).

In another rat study, two sets of experiments were performed. In experiment 1, stroke was induced by permanent occlusion of MCA and scored for cumulative neurological deficit at 3 hours post stroke. At 4 and 24 hours post stroke, transcranial laser irradiation (808 nm, 1.8 J/cm^2) of the hemisphere contralateral to the stroke was performed. In experiment 2, stroke was induced by permanent occlusion of MCA by insertion of a filament through the carotid artery and scored for neurological deficit at 24 hours post stroke. Laser irradiation (808 nm, 1.8 J/cm^2) was administered transcranially at 24 hours post stroke. In both models, laser irradiation significantly reduced neurological deficits when applied 24 hours post stroke. Laser treatment at 4 hours post stroke did not affect the neurological outcome of the stroke-induced rats as compared with controls. There was no statistically significant difference in the stroke lesion area between control and laser-irradiated rats. The number of newly formed neuronal cells as well as migrating cells was significantly increased in the subventricular zone of the hemisphere ipsilateral to the induction of stroke when treated by laser (Oron et al., 2006).

In a further study using rats with acute stroke induced by insertion of a filament into MCA and with marked neurological deficit at 24 hours, laser irradiation (808 nm, 1.8 J/cm^2) applied transcranially either ipsilateral, contralateral or to both sides of induced stroke resulted in an improvement in neurological deficit from 14 to 28 days post stroke. At 28 days there was only a 32% reduction in neurological score in the control non-laser group while in the laser-treated groups a 63% reduction occurred. Moreover, at 28 days there were no significant differences in neurological score between the groups of rats to which the laser was applied to different regions of the brain (DeTaboada et al., 2006).

In a rat study involving occlusion of MCA by a surgical clip and release of the artery after 1 hour for reperfusion, laser irradiation (660 nm) was applied through a burr hole to the cerebrum to deliver 2.64 J/cm^2/min, pulse frequency 10 kHz for 1, 5 or 10 minutes. After ischemia and reperfusion, the activity of NOS increased from day 3, becoming significantly greater on days 4 to 6, and then returning to normal levels after day 7. Activity and expression of the three isoforms of NOS at post-injury day 4 were significantly suppressed to different extents after laser irradiation. In addition, the expression of TGF-β at day 4 post-injury was up-regulated after laser irradiation (Leung et al., 2002).

Using the rabbit small clot embolic stroke model (RSCESM), laser treatment (808 nm, 15 J/cm^2) given transcranially 1 to 24 hours post embolization showed laser treatment reduced the neurological deficit if initiated up to 6 hours but not 24 hours post embolization (Lapchak et al., 2004). Furthermore, using this same model and applying continuous wave (CW) or pulse wave (P) laser therapy (808 nm) transcranially at 6 or 12 hours after embolization, there was a decrease in the neurological deficit for P wave but not CW wave administered at 6 hours post embolization. At 12 hours post embolization treatment, there was a trend for an improvement in neurological deficit by CW and P modes (Lapchak et al., 2007).

These animal trials inform studies examining the effects of TLT in human subjects. However, differences in cranial size and skull thickness may be important critical factors for LLLT in rats, rabbits and humans. In humans, the mean cranial thickness at frontal and occipital sites were 7.04 and 7.83 mm for males, and 6.68 and 7.60 mm for females, respectively (Lynnerup, 2001). Rat skull bone ranged in thickness from approximately 0.5–1 mm, while the rabbit skull was thicker than 1.5 mm (O'Reilly et al., 2011).

Human studies

A small number of studies have been performed in patients. The NEST-1 trial showed safety and effectiveness of TLT

for the treatment of ischemic stroke when initiated within 24 hours of stroke onset (median time to treatment of 18 hours, range 2 to 24 hours) (Lampl et al., 2007). The NEST-2 study of patients with moderate to moderately severe ischemic stroke concluded that 36% of patients had a favorable outcome when treated with TLT within 24 hours of stroke onset (median time to treatment of 15 hours, range 3 to 24 hours) (Zivin et al., 2009). The NEST-3 double-blind, randomized, sham-controlled, parallel, multi-centre trial was to demonstrate the safety and efficacy of TLT (808 nm) in the treatment of ischemic stroke when initiated between 4.5 and 24 hours of stroke onset with outcomes assessed at 90 days (Zivin et al., 2014). The study was terminated after a futility analysis found no difference between TLT and sham treatment in the primary endpoint which was disability at 90 days. The patients included in the study were unsuitable for treatment by thrombolytic agent or thrombectomy (Hacke et al., 2014). There are concerns regarding the sites of application of the laser light, the amount of energy that would penetrate the skull and be received by deeper structures in the brain, and the time from stroke onset to treatment initiation of 16 hours, and potentially = or < 24 hours, which extends well beyond the time window at which powerful treatments have been shown to confer benefit. Only a few of the 20 skull sites used for light application were likely to be adjacent to penumbral tissue and a single 2 minute application of laser energy is probably insufficient. The power of the laser was not specified. While penetration of the skull had been confirmed in human cadavers, the energy would reach only 2 cm into the brain cortex. Interestingly there was a lower reporting of serious adverse events in patients with TLT than in patients with sham treatment.

Future Perspectives

The studies in rats and rabbits demonstrated a neuroprotective effect of TLT following ischemic stroke. There is a need to find a way to make TLT effective in improving neurological outcome in human patients with ischemic stroke. The time from stroke onset at which TLT administration can benefit ischemic stroke patients needs to be established, as well as optimal laser parameters such as continuous or pulsed waveform, wavelength, power, duration of irradiation, energy dose, number of sites and days of application. Near infrared laser light has a greater transmission through the skull and greater depth of penetration of the brain than red laser light. In addition, combinatorial treatments may lead to better outcomes. In a small exploratory study involving patients with acute ischemic stroke, TLT was well tolerated in combination with intravenous tPA and no patients experienced intracranial hemorrhage (Hemmen et al., 2014). Statins given within 4 weeks of stroke onset improve stroke outcomes at 90 days compared with patients not given statins (Stead et al., 2009), and may when given following TLT result in more favorable outcomes. The pleiotropic effects of statins are probably more important at least in the first 3 to 7 days after stroke and include dose-dependent elevation of endothelial NOS, enhanced endogenous tPA, exertion of an anti-thrombotic effect, improved collateral blood flow, and decreased inflammatory mediators. Collectively, these dose-dependent effects of statins improve blood flow to the penumbra and promote autolysis of blood clot, decrease the likelihood of reocclusion, decrease infarct size, and improve clinical outcomes (Moonis, 2012). TLT in combination with specific agents to reduce inflammation and/or immune response could be trialed in animal stroke models. Possible agents are anti-IL-1β, anti-IL-6, anti-TNF-α monoclonal antibody or cytokine receptor antagonists, TGF-β and IL-10 analogues. Administration of an inhibitor of TNF-α production up to 6 hours after ischemia reduced brain edema in MCAO rats (Vakili et al., 2011). Intraventricular injection of an adenoviral vector encoding human IL-10 after MCAO in spontaneous hypertensive rats decreased brain infarct volume, with fewer infiltrations of leukocytes and macrophages, and attenuated IL-1β (Ooboshi et al., 2005).

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