Toll-Like Receptors in Stroke

Chuanfu Li, Tuanzhu Ha and Race L Kao*
Department of Surgery, East Tennessee State University, USA

Abstract

Each year about 795,000 Americans will suffer a new or recurrent stroke which remains as one of the leading causes of mortality, morbidity and disability in USA. The treatment goal of acute ischemic stroke is relieving the arterial occlusion (recanalization) and restoring cerebral blood flow (reperfusion) as soon as possible. Indeed, shorten the time from symptom onset to thrombolytic therapy and applying endovascular clot retrieval can achieve excellent reperfusion for most patients. The successes of these emerging therapies open the opportunity to treat ischemia/reperfusion (I/R) injury for improving outcomes after acute ischemic stroke. Toll-like receptor (TLR) mediated innate immune and inflammatory responses play a central role in cerebral I/R injury. We have found that activation of TLR3 or TLR9 by their agonists protect the brain from injury. Drugs that can be given locally during reperfusion to reduce I/R injury, to provide neuroprotection, and to salvage the tissue at risk may provide invaluable information for treatment and management of stroke patients.

Keywords: Stroke; Ischemia/reperfusion; Toll-Like receptors; Nanoparticles

Introduction

Epidemiology of stroke

An estimated 6.6 million Americans ≥ 20 years of age have had a stroke [1]. Despite significant progress has been made for stroke; it still remains as one of the leading causes of mortality, morbidity, and disability in USA [1]. Each year, about 795,000 Americans continue to experience a new or recurrent stroke (ischemic or hemorrhagic), with 87% of strokes are ischemic in nature due to thromboembolic occlusion that blocked cerebral arterial blood supply. On average, every 40 seconds an American has a stroke and every four minutes one will die from it [2]. Stroke is the major cause of acquired adult disability in the United States alone with substantial healthcare expenditure (> $33 billion/year) [1]. At present, there is no effective treatment for cerebral ischemia/reperfusion (I/R) injury. This can be attributed to the lack of understanding for the pathophysiological mechanisms of I/R injury after stroke. This paper briefly covers the current and emerging therapies for stroke and our recent findings with possible improvements in the treatment for stroke.

Treatment for patients with Ischemic stroke

Intra-venous (IV) recombinant tissue plasminogen activator (rtPA; alteplase) is the only FDA approved treatment for patients within 3 hours of ischemic stroke [3-5]. This is based upon the results of National Institute of Neurological Disorders and Stroke rtPA trial [6]. Although IV rtPA given between 3 to 4.5 hours after acute ischemic stroke has been proved beneficial [7-9] and recommended by American Heart Association Stroke Council [10], FDA has not approved the use of IVrtPA beyond 3 hours of symptom onset or any new drugs [11]. After cerebral ischemia, progression to brain infarction occurs quickly and reperfusion therapies are not effective after several hours [12-16]. Earlier thrombolytic treatment of acute ischemic stroke is associated with greater benefit to the patients [3,7,12,17-19]. Despite a significant increase of IV rtPA usage through the years, only 3.4% to 5.2% of acute ischemic stroke patients received thrombolytic treatment in the USA [20]. The narrow therapeutic time window, delays in activating emergency medical services, suboptimal hospital infrastructure and processes, as well as medical contraindications (i.e., recent major surgery or bleeding problems) all contribute to the low treatment rate [12,21-24]. Effective therapy without adverse complication that can be applied to most stroke patients is desperately needed.

Emerging therapies for patients suffering acute ischemic stroke

Cerebral vascular occlusion deprives tissue oxygen and energy supply, forms reactive oxygen species, releases glutamate, accumulates intracellular calcium, induces inflammatory processes, and leads to irreversible tissue injury (infarction) [25]. In patients experiencing a typical large vessel acute
ischemic stroke, 1.9 million neurons, 14 billion synapses, and 714 km of myelinated fibers are destroyed every minute [26]. Even a small reduction in stroke thrombolysis delay will result in significant and robust health benefits (i.e. 15 minutes decrease = 1 month additional disability-free life) [27]. The treatment goal of acute ischemic stroke is relieving the arterial occlusion (recanalization) and restoring cerebral blood flow (reperfusion) as soon as possible. Indeed, other than testing different thrombolytic agents [11,27] shorten the time from symptom onset to alteplase administration has been the major recent effort in treating ischemic stroke with the “golden hour” concept [28-31]. Although IV rtPA has proved its safety and efficacy for ischemic stroke patients, unfortunately IV rtPA can only benefit a minute population of the stroke patients with relative low recanalization rate especially for patients with intracranial large artery occlusion [12,20-24]. Endovascular treatment has developed to overcome the limitation of IV thrombolytic therapy. Endovascular treatment covers two different modalities: intra-arterial administration of thrombolytic drugs that offers no major advantages [32] and mechanical thrombectomy. From the five recent randomized controlled trials provide overwhelming evidences that endovascular clot retrieval improves outcomes after acute ischemic stroke [17,33-36]. Following the Thrombolysis in Myocardial Infarction Scale (TIMI) the modified Treatment in Cerebral Ischemia Scale (TICI) has been developed [5] to determine the reperfusion (Table 1). From 3 of the recent clinical trials (ESCAPE [34], EXTEND-IA [35], SWIFT PRIME [36]), better than 90% of treated patients have achieved TICI grade of 2 or 3 [7]. This translates to modified Rankin Scale (mRS) of 0-2 at 90 days almost double as compare to the control groups [3,7,13,23,37-39]. In another word, favorable 90-day outcome is significantly increased for the endovascular clot retrieval group. A new paradigm of stroke treatment may have arrived with mechanical thrombectomy. The advances in IV rtPA treatment by early administration of thrombolytic agent (golden hour concept) and application of mechanical thrombectomy to qualified individuals markedly increase the population of patients can be benefited from the therapies with significantly better outcomes. Although excellent recanalization and reperfusion (>90%) [7] have been achieved, the improvement in functional independence (mRS 0-2 only 53.0–71.4%) does not match the reperfusion rate. Even using a higher reperfusion scale (mTICI 2b-3; 72.4–88.0%) [38,39], 90-day functional independence (mRS 0–2; 53.0–71.4%) still lagged behind. The emerging therapies open the
opportunity to study I/R injury for improving outcomes after acute ischemic stroke by using specific drugs to modulate toll-like receptor (TLR) signaling during reperfusion for ameliorating cerebral I/R injury.

**Innate Immune and Inflammatory Responses Play a Critical Role in Cerebral I/R Injury**

Recent studies have demonstrated that innate immune and inflammatory responses participate in the pathogenesis of cerebral ischemic injury [40-42]. Cerebral I/R leads to a robust *in situ* inflammatory response, resulting in brain tissue damage, including neuronal death and white matter damage. Early cerebral ischemia particularly stimulates the activation of microglia, which are the resident macrophages in brain tissues [43-51]. Activated microglia secretes inflammatory cytokines (TNFα, IL-1β, etc.) and chemokines (MCP-1, etc.) which not only cause neuronal damage but also attract neutrophil and macrophage infiltration into the ischemic areas [43-51], resulting in greater inflammatory responses. We and other investigators have demonstrated that modulation of innate immune and inflammatory responses significantly attenuates cerebral I/R injury [52-57]. However, the mechanisms by which innate immune and inflammatory responses contribute to cerebral I/R injury have not been completely elucidated. TLRs play a critical role in the induction of innate immune and inflammatory responses [58,59].

**Toll-Like receptors in cerebral ischemia/Reperfusion injury**

Human TLR4 was first discovered in 1997 [60]. Since then, more than ten TLRs have been identified in mammals [61]. TLRs play a critical role in the induction of innate immune and inflammatory responses [60-63] by recognition of pathogen-associated molecular patterns (PAMPs) and endogenous danger- or damage-associated molecular patterns (DAMPs) which are released from damaged tissue [64-67].

There are TLRs (TLR1, TLR2, TLR4, TLR5 and TLR6) which are expressed on the cell membrane surface but there are also TLRs that are localized in intracellular compartments (TLR3, TLR7, TLR8, and TLR9) [68,69]. TLR-mediated signaling pathways predominately activate nuclear factor kappa-B (NFκB) which is a critical transcription factor regulating gene expression involved in innate immune and inflammatory responses [62,63]. With the exception of TLR3, all TLRs signal mediate through the adaptor protein myeloid differentiation factor 88 (MyD88) into the NFκB pathway [60-63]. Figure 1 shows a schematic diagram of TLR-mediated MyD88-dependent and TRAM/TRIF-dependent pathways. In MyD88-dependent signaling, MyD88 associates with the IL-1 receptor associated kinase family (IRAK1-4), which will interact with TRAF6 (TNF receptor-associated factor 6). TRAF6 activates transforming growth factor-β (TGF-β)-activating kinase (TAK1), which in turn activates IκB kinases (IKKaand IKKβ). The IκKs directly phosphorylate IκBα, which sequesters NFκB in the cytosol in an inactive form. The phosphorylated IκBα is then degraded, resulting in the activation and nuclear translocation of NFκB. In the nucleus, NFκB binds to specific DNA motifs to stimulate transcription of mRNA for immune-regulatory and pro-inflammatory mediators [62,63]. Both TLR3 (Figure 1) and TLR4 activate TRIF-dependent signaling to stimulate IRF3 phosphorylation and nuclear translocation, resulting in expression of interferon (IFN)-β and IFN-inducible genes [63,68-71].

**Novel Nanoparticles**

Novel Nanoparticles are novel vectors for transferring drugs

Nanoscience and nanotechnology begin to emerge about 20 years ago focus on materials with domain dimensions below 100 nm [72]. A major challenge in the therapeutic treatment of central nervous system is the delivery of drugs to the target site in the brain [73]. Nanoparticles can readily cross the blood-brain barrier (BBB) without compromising their integrity [73-77]. Most importantly, the biodegradable nanoparticles can deliver their therapeutic agents into the intracellular space via endocytosis. Both TLR3 and TLR9 are intracellular receptors, using the biodegradable nanoparticles carrying their agonists to cross BBB for intracellular delivery of the drug is highly innovative and novel idea.

In the past 20 years, a number of nanoparticle-based therapeutic and diagnostic agents have been developed for the treatment of cancer, diabetes, pain, asthma, allergy and infectious [78-81]. So far nanotechnology-based therapeutic products have been approved for clinical usage [80,81]. Poly(lactic-co-glycolic acid) (PLGA) nanoparticles are safe, biodegradable and FDA approved [82-85] and have a long history of success in enhancing the delivery of therapeutic agents [82-86]. Investigate the therapeutic potential of drugs carried by FDA approved synthetic nanoparticles will be a logical approach. Treatment modalities for ischemic stroke are far from ideal and nanoparticles offer emerging therapeutic strategies that may represent the next frontier over traditional treatments for stroke.

**Selection of specific drugs for acute Ischemic stroke**

Activation of TLR2 and TLR4 have been reported to play a detrimental role in cerebral I/R injury [52-57]. In striking contrast, we have made the novel observation that treatment with the TLR3 ligand, polyinosinic-polycytidylic acid (Poly LC; tlr-pic, Invivo Gen, San Diego, Ca, USA), induces a protection against cerebral I/R injury via attenuation of pro-apoptotic signaling [87]. We have also found that treatment of mice with the TLR9 ligand, CpG-ODN (CpG-ODN; CpG-ODN 1826, Invivo Gen, San Diego, Ca, USA), significantly decreases cerebral I/R injury. CpG-ODN administration activates PI3K/Akt signaling pathway which negatively regulates innate immune and inflammatory responses [88]. Thus, modulation of TLR3 and TLR9 decrease cerebral I/R injury is mediated via different mechanisms. To the best of our knowledge, these are new and novel roles for TLR3 and TLR9 in stroke that has not been tested previously. Furthermore, our observations are supported by others that TLR4 but not TLR3 or TLR9 knock-out mice have neuroprotective effects against cerebral ischemia [59].
TLR3 and TLR9 are intracellular TLRs which are mainly located on intracellular vesicles such as endoplasmic reticulum, endosomes, lysosomes and endolysosomes [89]. To use FDA approved synthetic nanoparticles to cross BBB and for intracellular delivery of specific agents to activate TLR functions has not been tested before. The innovation associated with this research focuses on the role of TLR3 and TLR9 in mediating protection against cerebral I/R injury. This study may significantly advance our knowledge on mechanisms of cerebral I/R injury and how modulation of innate immunity can be harnessed to decrease stroke sequelae.

**Material and Methods**

**Focal cerebral Ischemia/Reperfusion injury**

C57BL/6 mice (23 to 25 g body weight) are obtained from Jackson Laboratory. The mice are maintained in the Division of Laboratory Animal Resources, East Tennessee State University (ETSU). The experiments of this study conform to the Guide for the Care and Use of Laboratory Animals and approved by ETSU Animal Care and Use Committee. To reach statistical significance, the numbers of animal in each experiment are determined by power analysis.

Focal cerebral I/R are induced by middle cerebral artery occlusion (MCAo) on the left side as described in our previous studies [52-54,87,88]. Mice are anesthetized by 5.0% Isoflurane and maintained by inhalation of 1.5% to 2% Isoflurane driven by pressurized air. Mice are intubated and ventilated using a rodent ventilator at a rate of 110 breaths/minute with a tidal volume of 0.5 ml. Body temperature are regulated at 37°C by surface water heating pad. Following the skin incision, the left common carotid artery (CCA), the external carotid artery (ECA) and the internal carotid artery (ICA) are carefully exposed. Microvascular aneurysm clips are applied to the left CCA and the ICA. A coated 6-0 filament (6023PK; Doccol Corp., Sharon, MA) is introduced into an arteriotomy hole, fed distally into the ICA. After the ICA clamp is removed, the filament is advanced 1 mm from the carotid bifurcation, and focal cerebral ischemia started. After ischemia for 60 minutes, the filament is gently removed (reperfusion starts) and a micro catheter inserted to the MCA area for drug administration. After removal of micro catheter and CCA clamp, the collateral suture at the base of the ECA stump is tightened. Regional cerebral blood flow is recorded and expressed as a percentage of pre-ischemic baseline values (Figure 2) by laser Doppler flowmeter (Model PeriFlux system 5000; Perimed, Stockholm, Sweden). The skin is closed, anesthesia discontinued, and the animal allowed to recover in pre-warmed cages.

**Measurement of infarct volume**

The infarct volume is determined as described previously [52-54,87,88]. After completion of reperfusion, mice are terminated and perfused with ice cold PBS via the ascending aorta. Brains are removed and sectioned coronally into 2-mm-thick slices. The slices are stained with 2% triphenyltetrazolium chloride (TTC) solution at 37°C for 15 minutes followed by fixation with 10% formalin neutral buffer solution (pH 7.4). The infarct areas are traced and quantified with an image-analysis system. Unstained areas (pale color) are defined as ischemic lesions. The areas of infarction and the areas of both hemispheres are calculated for each brain slice. An edema index is calculated by dividing the total volume of the left hemisphere by the total volume of the right hemisphere. The actual infarct volume adjusted for edema is calculated by dividing the infarct volume by the edema index [52-54,87,88]. Infarct volumes are expressed as a percentage of the total brain volume ± SEM.

**Synthetic nanoparticles containing treatment agents**

FDA approved [84,85] PLGA-nanoparticles (~100 nm in diameter, LG100, Pliosphere, are obtained and loaded with FITC labeled CpG-ODN according to instructions provided by the manufacturer and the published method described by Cheng et al. [78,79,82,86]. PLGA-nanoparticles containing CpG-ODN are prepared using a modified water-in-oil-in-water double emulsion technique. Briefly, CpG-ODN is complexed with spermidine (1:8 ratios) in buffer (10 mMTris-HCl, pH 7.4; 1 mM EDTA). After 20 min, the self-assembled CpG-ODN-spermidine complexes are added drop-wise under vortex to PLGA dissolved in methylene chloride. After sonication this primary water-in-oil emulsion is added into 5% polyvinyl alcohol and sonicated before immediately poured into a solution of 0.3% polyvinyl alcohol to allow nanoparticles harden under continuous stirring for 3–4 hours. Nanoparticles are collected by centrifugation at 16,000 G for 15 min at 4°C and washed with deionized water for three times. The nanoparticles are freeze-dried and stored desiccated at -20°C.

**Data analysis and statistics**

The data are summarized for each study group by the mean, standard deviation (SD), and standard error of the mean (SEM). Differences between study group means are statistically assessed by analysis of variance (ANOVA) and multiple comparisons testing (least significant differences or Tukey’s procedure depending on the ANOVA F-test). A P<0.05 will be considered statistically significant.

**Results**

**TLR3 and TLR9 Ligands Decreased Cerebral I/R Injury**

To examine whether Poly (I:C) could induce protection against cerebral I/R injury without preconditioning, we administrated Poly (I:C) to mice one hour before the mice were subjected to cerebral ischemia (60 min) followed by reperfusion (24 hours). Figure 3 showed that Poly (I:C) administration significantly reduced infarct volume by 57.2% compared with untreated I/R mice. The data indicated that Poly (I:C)-induced neuroprotection occurred rapidly and did not require preconditioning. We also examined the therapeutic effect of Poly (I:C) on cerebral I/R injury. As shown in Figure 3, therapeutic administration of Poly (I:C) (30 min after the beginning of ischemia) also significantly reduced infarct volume by 34.9% (15.2±2.4 versus 24.4±2.7) compared with the untreated I/R group. The data indicated that therapeutic administration of Poly (I:C) during ischemia decreased I/R-induced brain injury.

To determine the role of TLR3 in Poly (I:C)-induced protection, TLR3 knockout (TLR3 KO) mice were treated with or without Poly (I:C) (5 µg/25 g body weight) one hour prior to cerebral ischemia (60 min) followed by reperfusion (24 hours). Infarct size in TLR3 KO mice was comparable to wild type I/R mice. Poly (I:C)-induced protection against cerebral I/R was lost in TLR3 KO mice. The data indicate that TLR3 is required for Poly (I:C)-induced protection against cerebral I/R injury. The data also suggest that the TLR3 ligand would be useful for treatment and management of stroke patients.

To measure the effect of CpG-ODN in focal cerebral I/R injury, we administrated CpG-ODN, control-ODN, inhibitory CpG-ODN (iCpG-ODN) or CpG-ODN + LY294002 (LY) to mice one hour before the mice were subjected to cerebral ischemia (one hour) and reperfusion (24 hours). Figure 3 showed that CpG-ODN administration significantly reduced infarct volume by 69.7%
compared with the untreated I/R group (6.4±1.8% vs. 21.0±2.8%). Administration of either control-ODN or iCpG-ODN (TLR9 antagonist, InvivoGen) to mice did not alter I/R-induced cerebral infarct volume.

Activation of the PI3K/Akt signaling pathway has been reported to protect against cerebral I/R injury. We examined the effect of CpG-ODN (CpG) on activation of PI3K/Akt signaling. When mice were treated with LY (a specific PI3K inhibitor) 15 minutes prior to CpG-ODN administration prevented CpG-ODN induced increase in Akt phosphorylation (P-Akt). Preventing P-Akt increase by inhibition of PI3K with LY also abrogated CpG-ODN induced protection against cerebral I/R injury (Figure 3).

**PLGA-nanoparticles loaded with FITC labeled CpG-ODN were prepared according to instructions provided by the manufacturer and the published method [78,79,82,86]. First, we added PLGA-nanoparticles (40 µg/ml) containing FITC-CpG-ODN 1826 (in vivo Gen) to cultured microglial cells (BV2 from ATCC). Four hours after addition of FITC-CpG-ODN 1826 loaded in PLGA-nanoparticles, cultured cells showed significant fluorescence (Figure 4). Next, we determined whether FITC-CpG-ODN 1826 loaded in PLGA-nanoparticles could be delivered into the brain tissue in vivo. We delivered synthetic nanoparticles containing FITC-CpG-ODN 1826 into mouse brain through the left carotid artery near MCA (100 µl, 2 mg/ml). Six hours after administration, brain was harvested, prepared for frozen tissue sections, and examined for FITC fluorescence by fluorescent microscopy. Figure 5 showed that FITC-CpG-ODN fluorescence appeared in the brain tissue. The data indicated that PLGA-nanoparticles could efficiently deliver CpG-ODN 1826 into the brain tissue in vivo.

**Discussion**

This study demonstrated that modulation of TLR3 with its specific ligand, Poly (I:C) significantly attenuates cerebral I/R induced brain injury. Poly (I:C) is a synthetic analog of double-stranded RNA with a molecular pattern mimics viral infection. Poly (I:C) stimulates TLR3-mediated signaling predominantly through IRF3, resulting in expression of type I IFNs (especially IFN-β) and IFN-inducible genes [68,90,91]. From our recent publication [87], when Poly (I:C) was given to TLR3 knockout mice the beneficial effect of this synthetic TLR3 ligand could not be observed. It clearly supports that Poly (I:C) induced protection during focal cerebral I/R is mediated through TLR3. Cytidine has generated significant interest as a potential glutamatergic neuronal-glial glutamate cycling, affecting cerebral phospholipid metabolism, catecholamine synthesis and mitochondrial function. Cytidine has generated significant interest as a potential glutamatergic antidepressant drug [92]. Cerebral vascular occlusion deprives tissue oxygen and energy supply, forms reactive oxygen species, releases glutamate, accumulates intracellular calcium, induces inflammatory processes, and leads to irreversible tissue injury (infarction) [25]. The possible contribution of Poly (I:C) to glutamate cycling can be an interesting area of future study.

Similarly, administration of specific TLR9 ligand, CpG-ODN also induced significant protection against cerebral I/R injury. CpG-ODNs are short synthetic single-stranded DNA molecules containing unmethylated CpG dinucleotides in specific sequence contexts (CpG motifs). CpG-ODNs possess a partially or completely phosphorothioated backbone, as opposed to the natural phosphodiester backbone found in genomic bacterial DNA. CpG-ODN 1826 is a 20 meroligodeoxynucleotides with a sequence of 5’-tcctagctgctgacgt-3’. CpG-ODN 1826 is a class B ODN which contains a full phosphorothioate backbone with CpG dinucleotides that specific for murine TLR9. It strongly activates TLR9-dependent NF-κB signaling and leading to significant immuno-stimulatory effects. Most importantly, we observed that loaded specific TLR9 ligand, CpG-ODN on FDA approved PLAG-nanoparticles could efficiently pass through BBB into the brain tissues. Our data demonstrated that nanoparticles could have significant potential for carrying TLR3 or TLR9 ligands into the brain for the treatment and management of stroke patients.

To encapsulate the TLR ligands into PLGA-nanoparticles, spermidine has been used to form the ligand-spermidine complexes. Spermine has been reported to reduce ischemic damage in focal cerebral ischemia animal model [93]. However, 10mg/kg is needed to have a therapeutic effect (lower doses are not effective) and this does is far beyond the concentration that applied in our experiment. Although spermidine has reported to induce BBB disruption and decrease permeability [94,95], when spermidine is formed complexes with TLR ligands and encapsulated inside PLGA-nanoparticles it may not have the effect to modify the BBB.

Despite significant progress has been made for stroke, it still remains as one of the leading causes of mortality, morbidity, and disability in USA [1]. Stroke is the major cause of acquired adult disability in the United States alone with substantial healthcare expenditure and higher lost in healthy life than other major diseases [1]. Shorten the time from symptom onset to thrombolytic therapy and applying endovascular clot retrieval can achieve excellent reperfusion for most patients. The successes of these emerging therapies open the opportunity to treat I/R injury for improving outcomes after acute ischemic stroke.

Recent studies have demonstrated that innate immune and inflammatory responses participate in the pathogenesis of cerebral ischemic injury [40-42]. Cerebral I/R lead to a robust in situ inflammatory response, resulting in brain tissue damage, including neuronal death and white matter damage. TLRs play a critical role in the induction of innate immune and inflammatory responses [58,59]. Our data indicate that TLR3 and TLR9 ligands significantly reduced cerebral I/R injury. TLR3 and TLR9 are intracellular TLRs which are mainly located on intracellular vesicles such as endoplasmic reticulum, endosomes, lysosomes and endolysosomes [89]. To use FDA approved synthetic nanoparticles to cross BBB and for intracellular delivery of specific agents to activate TLR functions is an innovative approach. Using specific drugs to modulate innate immunity and inflammatory responses to decrease stroke sequelae will be highly significant.

The drugs and pharmacologic agents for this project have been used in our previous publications or in our preliminary studies and they do not have toxic or adverse effects to the experimental animals. The FDA approved and commercially available synthetic nanoparticles are safe, biodegradable and have been proven to enhance the delivery of therapeutic agents. Using the synthetic nanoparticles to deliver TLR3 agonist (Poly I:C) or TLR9 agonist (CpG-ODN) for preventing cerebral I/R injury may directly translate for patients suffering ischemic stroke. In addition, we anticipate that the therapeutic efficacy of TLR3 and TLR9 ligands when administered in combination will...
induce an additive or synergistic effect. Poly (I:C) and CpG-ODN are
currently used in clinical trials [96-98]; thus, pharmaceutical grade
TLR3 and TLR9 ligands are available [96-98]. The data acquired may
provide a strong rationale for the use of TLR3 and/or TLR9 ligands in
the treatment of stroke patients especially during reperfusion.

References
al. American Heart Association Statistics Committee and Stroke Statistics
5. Gomis M, Dávalos A. Recanalization and reperfusion therapies of acute
ischemic stroke: What have we learned, What are the major research
questions, and Where are we headed? Front Neurol. 2014; 5: 1-12.
6. The National Institute of Neurological Disorders and Stroke rt-PA Stroke
Study Group. Tissue plasminogen activator for acute ischemic stroke. N
7. Prabhakaran S, Ruff I, Bernstein RA. Acute stroke intervention: a
al. ECASS Investigators. Thrombolysis with alteplase 3 to 4.5 hours after acute
9. Lansberg MG, Bluhmki E, Thijs VN. Efficacy and safety of tissue
plasminogen activator 3 to 4.5 hours after acute ischemic stroke: a meta-
10. Del Zoppo GI, Saver JL, Jauch EC, Adams HP, American Heart
Association Stroke Council. Expansion of the time window for treatment
of acute stroke with intravenous tissue plasminogen activator: a
science advisory from the American Heart Association/American Stroke
11. Marshall RS. Progress in intravenous thrombolytic therapy for acute
12. Saver JL, Fonarow GC, Smith EE, Reeves MJ, Grau-Sepulveda MV, Pan W,
et al. Time to treatment with intravenous tissue plasminogen activator and
13. Hill MD, Goyal M, Demchuk AM, Fisher M. Ischemic stroke tissue-
window in the new era of endovascular treatment. Stroke. 2015; 46: 2332-
2334.
AM, et al.; IMS III Trialists. Time to angiographic reperfusion and
clinical outcome after acute ischemic stroke: an analysis of data from the
Interventional Management of Stroke (IMS III) phase 3 trial. Lancet Neurol.
al. Time to treatment with intravenous alteplase and outcome in stroke:
an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET
IMS I and II Investigators. Good clinical outcome after ischemic stroke
with successful revascularization is time-dependent. Neurology. 2009; 73:
1066-1072.
17. Jovin TG, Chamorro A, Cobo E, De Miquel MA, Molina A Carlos, Rovira
A, et al; REVASCAT Trial Investigators. Thrombectomy within 8 hours
after symptom onset in ischemic stroke. N Engl J Med. 2015; 372: 2296-
2306.
BM, et al; American Heart Association Stroke Council; Council on
Cardiovascular Nursing; Council on Peripheral Vascular Disease; Council
on Clinical Cardiology. Guidelines for the early management of patients
with acute ischemic stroke: a guideline for healthcare professionals from
the American Heart Association/American Stroke Association. Stroke.
2013; 44: 870-947.
MV, et al. Timeliness of tissue-type plasminogen activator therapy in acute
ischemic stroke: patient characteristics, hospital factors, and outcomes
associated with door-to-needle times within 60 minutes. Circulation. 2011;
123: 750-758.
20. Adeoye O, Hornung R, Khatri P, Kleindorfer D. Recombinant tissue-
type plasminogen activator use for ischemic stroke in the United States:
a doubling of treatment rates over the course of 5 years. Stroke. 2011; 42:
21. Shirakawa M, Yoshimura S, Yamada K, Uchida K, Shindo S. Endovascular
therapy for acute ischemic stroke: Considerations from recent randomized
trials. Interv Neurol. 2015; 5: 115-121.
22. Song D, Cho AH. Previous and recent evidence of endovascular therapy
23. Madsen TE, Khoury JC, Alwell KA, Moomaw CJ, Kissela BM, De Los Rios
La Rosa F, et al. Analysis of tissue plasminogen activator eligibility by sex
in the Greater Cincinnati/Northern Kentucky Stroke Study. Stroke. 2015;
46: 717-721.
24. Cheng NT, Kim AS. Intravenous thrombolysis for acute ischemic stroke
within 3 hours versus between 3 and 4.5 hours of symptom onset.
25. George PM, Steinberg GK. Novel stroke therapeutics: Unraveling stroke
pathophysiology and its impact on clinical treatments. Neuro. 2015; 87:
297-309.
45: 1053-1058.
al. Effects of goldenhour thrombolysis: a Prehospital Acute Neurological
Treatment and Optimization of Medical Care in Stroke (PHANTOM-S)
Door-to-needle times for tissue plasminogen activator administration
and clinical outcomes in acute ischemic stroke before and after a quality
GWTG-Stroke Steering Committee and Investigators. The "golden hour"
and acute brain ischemia: presenting features and lytic therapy in >30,000
patients arriving within 60 minutes of stroke onset. Stroke. 2010; 41:
1431-1439.
32. Nam J, Jing H, O’Reilly D. Intra-arterial thrombolysis vs. standard
treatment or intraoperative thrombolysis in adults with acute ischemic
33. Berkermer OA, Fransen PS, Beumer D, van den Berg LA, Linguma HF,
Race L Kao, et al.,

Journal of Heart and Stroke


96. Makkouk A, Abdulnoor AM. The potential use of Toll-like receptor (TLR) agonists and antagonists as prophylactic and/or therapeutic agents. ImmunopharmacolImmunotoxicol. 2009; 31: 331-338.
