Reperfusion brain injury

Focus on cellular bioenergetics

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ABSTRACT

Energy production for the maintenance of brain function fails rapidly with the onset of ischemia and is reinstituted with timely reperfusion. The key bioenergetic organelle, the mitochondrion, is strongly affected by a cascade of events occurring with ischemia and reperfusion. Enhanced production of reactive oxygen species, disruption of calcium homeostasis, and an inflammatory response are induced by reperfusion and have a profound effect on cellular bioenergetics in reversible stroke. The impact of perturbed bioenergetics on cellular homeostasis/function during and after ischemia are discussed. Because mitochondrial function can be compromised by derangements at more than one of the susceptible sites on this organelle, we propose that a combination therapy is needed for the restoration and maintenance of cellular bioenergetics after reperfusion. *Neurology*® **2012;79 (Suppl 1):S44–S51**

GLOSSARY

ATP = adenosine triphosphate; **DAMP** = danger-associated molecular pattern molecule; **MPTP** = mitochondrial permeability transition pore; **ROS** = reactive oxygen species; **SAINT** = Stroke-Acute Ischemic NXY Treatment; **SEF** = secondary energy failure; **STAIR** = Stroke Therapy Academic Industry Roundtable; **TNFα** = tumor necrosis factor-α.

Stroke is a complex and dynamic disease of the brain that rates fourth in mortality and first in disability in the United States. The brain has certain unique physiologic properties that make it extremely sensitive to the loss of blood flow. In general, the energy demands of the brain are high, requiring a continual supply of oxygen and its principal substrate, glucose, mainly from the blood. Occluding blood flow to the brain disrupts the delicate balance between the energy generated by glucose oxidation and energy needed for cell processes, which leads to a rapid loss of function and cell homeostasis.

The imbalance of the brain bioenergetics induced by the loss of blood flow has been shown to lead to cellular infarction of all brain cells, including neurons, astrocytes, endothelial cells, oligodendrocytes, and subpopulations of these cells. Conversely, a pronounced deranged cellular milieu resulting from ischemia elicits a plethora of reactions upon re-establishment of reflow. It is increasingly evident that many of the events center on the neurovascular unit, a functional composite of microvessels, pericytes, astrocytes, neurons, axons, and other supporting cells such as microglia and oligodendrocytes. Although many of the reflow-induced events may be pathologic and their prevention potentially beneficial, it is our contention that the status of cellular bioenergetics is the major determinant of many of the pathophysiologic sequelae manifested in the neurovascular unit and therefore is fundamental to the outcome for the tissue, following reversible focal ischemia.

In the past 50 years, basic science investigations first established that loss of blood flow to the brain resulted in rapid failure of cell bioenergetics, followed by an ever-increasing list of cellular perturbations. A schematic of ischemia-induced events is shown in figure 1. Rapid energy depletion reflects very low energy reserves within the brain, a high metabolic rate, and almost a total reliance on glucose oxidation for energy production. The ischemic cascade is initiated during ischemia. Ischemia depletes adenosine triphosphate (ATP) within minutes, leading to a failure of a

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Figure 1   A schematic of key ischemia and reperfusion-induced processes leading to mitochondrial dysfunction and cell death
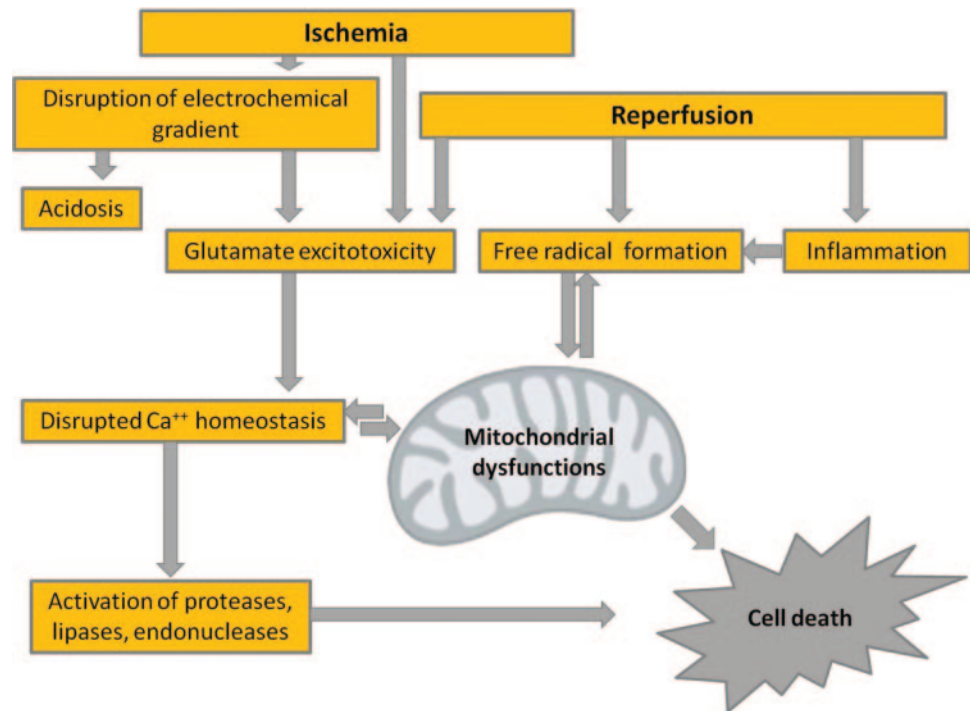
multitude of energy-dependent cellular processes critical to cellular homeostasis and brain function.[1–3] Parenthetically, most physiologic processes within the cell are either directly or indirectly impacted by the loss of energy. The immediate consequence of diminished energy reserves is perturbation of membrane electrochemical gradients, leading to depolarization. The influx of sodium, calcium, and chloride into the intracellular space and potassium efflux result in increased intracellular water and cytotoxic brain edema.[4] Depolarization increases intracellular calcium, activating transcription factors, phospholipases, endonucleases, and proteases and leading to deranged intracellular signaling, compromised cellular function, and loss of structural integrity.[5] Depolarization also triggers excessive release of glutamate, which results in an increased activation of glutamate receptors and an additional influx of calcium, accentuating cellular injury.[6] The cascade of ischemic events disrupts cellular homeostasis, and if not reversed, it causes tissue necrosis. If reflow is initiated in a timely fashion, however, tissue recovers. Early reperfusion with thrombolytics is the only US Food and Drug Administration–approved treatment for ischemic stroke.[7,8] The duration of ischemia is a major determinant of the magnitude of the pathophysiologic response, and time of the ischemic episode cannot be overemphasized when discussing the outcome following stroke.

Restoring blood flow to the brain elicited multiple cellular and physiologic events, and it was initially expected that reperfusion would simply be a reversal of the ischemia-induced disruption of the cellular milieu to re-establish function; however, this clearly is not the case. Experimental evidence shows that reperfusion triggers a set of unique, potentially pathologic events including, for example, increased prostaglandin synthesis, elevated production of second messengers, inflammation, and mitochondrial dysfunction, as indicated by elevated reactive oxygen species (ROS) and the opening of the mitochondrial permeability transition pore (MPTP).[9–13] It is our contention that if the bioenergetics of the brain are not normalized, the tissue will die, and rectifying the other potentially pathogenic events would be of little value. In spite of the collapse of cellular homeostasis during ischemia, the brain has the capacity to re-establish energy metabolism and then to

counteract the ischemia-induced events, leading to the restoration of function if reperfusion occurs within certain time constraints. In this context, it has been a challenge to determine which of the reflow events unrelated to bioenergetics are pathologic, vs those that are merely epiphenomenal.

The central event to the evolution of irreversible mitochondrial injury appears to be the formation of MPTP.[14] The MPTP, when open, becomes permeable to ions and large molecules (<1,500 Kd), disrupting the mitochondrial electrochemical gradient necessary to drive oxidative phosphorylation and ATP synthesis.[10] Although the MPTP is present in the normal brain and subtle reversible openings of the pore are detectable even at the early stages of reperfusion, evidence suggests that the massive pore opening is the final irreversible step in the evolution of brain damage.[15] From the literature, there is evidence to suggest that mitochondrial dysfunction is associated with free radical formation, calcium overload, and inflammation during reflow, which may contribute to the massive opening of the MPTP.[11] These will be discussed below with the idea that concerted interventions in these factors may produce neuroprotection. One factor that generally has been overlooked is that partial recovery of bioenergetics of the tissue is transiently evident upon reperfusion, and if the compromised energy metabolism persists, secondary energy failure (SEF) is manifested and cell death ensues.

Evidence for the existence of reflow-induced SEF comes from 2 different models of reversible ischemia. The first study comes from a global ischemia model of delayed neuronal death in the gerbil.[16] A short-term (5-minute) episode of global ischemia caused a generalized loss of ATP and P-creatine, which were readily replenished with reperfusion. Certain vulnerable cells (i.e., hippocampal CA1 pyramidal neurons) survived, with normal energy reserves for several days after the insult, and then between days 2 and 4 of reflow, levels of high-energy phosphates significantly dropped, concomitant with death of the CA1 pyramidal neurons. The data support the relationship between delayed energy depletion and neuronal death during reperfusion.[16] Of interest, there is evidence of selective delayed neuronal death phenomenon following cardiac arrest and resuscitation in humans.[17]

In the second model, delayed secondary loss of bioenergetics (i.e., SEF) and cell death have been demonstrated in a focal model of reversible ischemia. The middle cerebral artery in the rat was transiently occluded for 2 hours, and energy stores of the ischemic core and surrounding region (i.e., penumbra) were variably depleted.[18,19] Upon reperfusion, the concentration of ATP in the cerebral cortex was transiently restored, only to fail several hours later (figure 2). In contrast, levels of glucose remain elevated, as levels of ATP decreased, indicating the ATP effect was unrelated to changes in substrate availability. These findings have been confirmed by several laboratories, which also found that the magnitude of the changes was more pronounced in the ischemic core than in the penumbra.[18,19] Noticeably, there was a complete restoration of blood flow in both models of ischemia, suggesting that deprivation of glucose and oxygen was unlikely to be the precipitating cause of secondary energy depletion and cell death.

From the experimental results on ischemic and reperfusion events, our current knowledge of the pathophysiology of ischemia can be summarized as follows. First, cessation of mitochondrial respiration and lactic acidosis without reflow results in cell necrosis. Second, ischemia primes certain mitochondrial components, making mitochondria more susceptible to a variety of reflow-induced pathogenic events, as evidenced by increased free radical formation and calcium-induced uncoupling of oxidative phosphorylation. Third, unique adverse reactions initiated during reflow are energy-dependent, since they occur only if mitochondria are transiently active during reperfusion. Therefore, energy production by mitochondria appears to be a prerequisite for the evolution of cell damage, allowing certain pathogenic events such as apoptosis to occur. Subsequently, mitochondrial failure, medi-

Figure 2 Time course of adenosine triphosphate (ATP), glucose, and mitochondrial respiratory function following 2 hours of focal ischemia and 0 to 8 hours of reperfusion
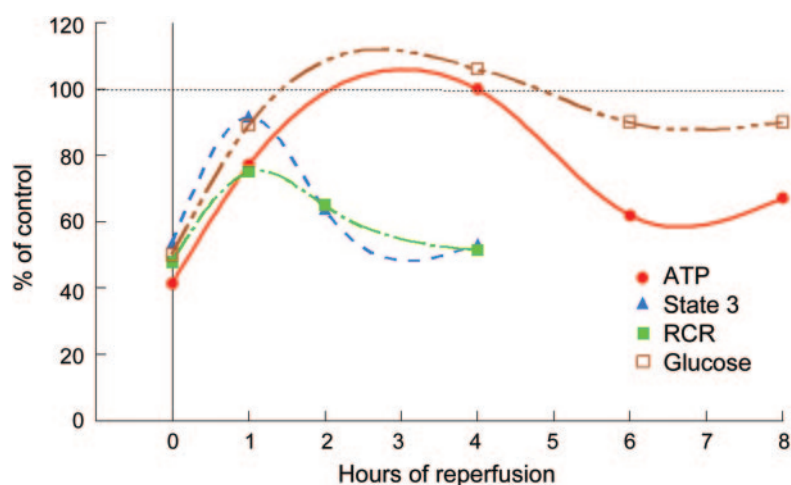
These data are presented as percentages of those of the contralateral cerebral cortex. Mitochondrial respiratory function was indicated by oxidative phosphorylation, assessed by measurement of oxygen consumption by mitochondria in the presence of nicotinamide adenine dinucleotide–linked or flavin adenine dinucleotide–linked substrates. Oxidative rates (state 3: adenosine diphosphate [ADP]-stimulated; state 4: resting state, ADP-limited) were calculated and expressed as nanoatom oxygen/minute/mg protein. The respiratory control ratio (RCR) was determined by the equation RCR = state 3 activity/state 4 activity. Data are from Lust et al. (2002),[19] Kuroda et al. (1996), [22] and Nakai et al. (1997).[23]

ated by the opening of the MPTP, leads to cellular death.

To iterate, brain function is absolutely dependent on cellular homeostasis that requires a continual supply of energy, but that can be sustained only transiently during ischemia when ATP is supplied primarily by anaerobic glycolysis. Because the evidence from delayed neuronal death and SEF indicate energy availability is essential in the evolution to cell death, the organelle central to brain damage appears to be the mitochondria. This somewhat paradoxical finding indicates that the fate of cells, whether survival or death, is energy dependent. Therefore, it seems likely that other reflow-induced factors, like ROS production and inflammation, become involved in the final tissue outcome following ischemia.

REACTIVE OXYGEN SPECIES The overproduction of reactive oxygen species upon reflow is well-documented and is ubiquitous within the cell. Under physiologic conditions, the mitochondria-generated superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) play important roles in regulating those pathways integral to signaling and

metabolism.[20] These ROS are normally inactivated by endogenous scavenging systems. Excessive ROS production, however, can overwhelm free radical scavenging systems that may have been compromised by ischemia. Perturbations in various components of the respiratory chain during reflow have been attributed to endogenous ROS. The major source of ROS production in the mitochondria is complex I and III of the electron transport chain, and the oxidative damage within the mitochondria is relatively widespread.[21] Experimental results on alterations of state 3 and state 4 respiration and the respiratory control index indicate a normalization of the electron transport system upon reflow and secondary mitochondrial dysfunction following transient cerebral ischemia (figure 2).[22,23] These parameters also decrease at a time prior to SEF. Although the causal relationship between these events has not been established, it is plausible that ROS during reflow reduces the activity of the electron transport chain, resulting in diminished ATP. Other major targets of ROS are lipids. Peroxidative action of these macromolecules promotes inactivation of key metabolic enzymes regulating glucose metabolism, such as α-ketoglutarate dehydrogenase and pyruvate dehydrogenase.[24] Reduced mitochondrial respiratory function in rat brain was further confirmed during the reperfusion period in both focal and global ischemia models,[22,23,25,26] and the extent of the effect varies according to the region examined.[27] Generally, as mitochondria become more dysfunctional, additional free radicals form, increasing the likelihood of cell death.

Many of the aforementioned free radicals are also produced by a number of cytosolic reactions, including cyclo-oxygenase, lipo-oxygenase, and xanthine oxidase.[28] Another highly reactive free radical is peroxynitrite, which is a product of NO and superoxide anion, capable of nitrosylating certain functional proteins. In general, oxidation of proteins, lipids, and nucleic acids by ROS can markedly destabilize cellular homeostasis.

CALCIUM OVERLOAD The intracellular concentration of calcium is normally highly regulated through control of calcium channels and exquisite active sequestering systems, including endoplasmic reticulum and mitochondria.[29] Calcium is one of the most intriguing cations in the brain, and it is not surprising that this multipotent molecule has been implicated in the opening of the MPTP and in cell death. The influx of ionized calcium induced by ischemia has been shown to cause a broad range of events, from lipolysis, proteolysis, and DNA fragmentation to disturbances in axonal transport, edema, and vascular dysfunction.[30] Ionic calcium is also bound to certain

intracellular molecules, and one of the more notable moieties is phosphate, which coincidentally is a product of ATP hydrolysis. Understanding the role of calcium has been hampered by the total tissue concentration being about 1 mM, whereas free resting cytosolic calcium is one-ten-thousandth of that concentration. One report on calcium in ischemic brain damage showed a 0.6-mM increase in intracellular calcium with evolving stroke damage,[31] but such an increase in free calcium seems unlikely. In addition, it would appear that any stimulus to release calcium from a large pool of the bound or sequestered calcium could impact markedly on the many calcium-dependent events in the cytosol. Nevertheless, calcium overload has been implicated as another purported signal for the opening of the MPTP.[29] In both the delayed neuronal death and secondary energy failure models, tissue ATP levels were maintained just prior to energy failure and manifestation of cellular damage. Because the duration from the onset of reflow to energy depletion ranged from hours to days in these 2 animal models, the link between calcium overload and energy failure appears obscure. It is possible, however, that a state may be reached where the levels of calcium exceed the capacity to remove this cation from the cytosol. Under these circumstances, it is possible that energy consumed to support calcium sequestration would exceed the rate of energy production, a condition favoring energy depletion.

INFLAMMATION Cerebral ischemia initiates an inflammatory response that furthers mitochondrial injury. This response occurs sooner and is more robust with reperfusion.[32] The inflammatory response to vessel occlusion is initiated within the vessel immediately and results in activation of complement, platelets, and endothelium.[33] Sequential expression of adhesion molecules, including selectins, intercellular adhesion molecules, and vascular cell adhesion molecules, results in first neutrophil and later monocyte adhesion to the endothelial wall.[34,35] Within the vasculature, activated leukocytes contribute to vessel occlusion both directly and by releasing proinflammatory cytokines, proteases, and ROS, which injure the endothelial surface, leading to thrombus formation, vasospasm, and worsening ischemia.[33] Inflammatory mediators contribute to breakdown of the blood–brain barrier, further promoting the infiltration of leukocytes into the brain.[9]

Within the brain parenchyma, activation of the resident tissue macrophages, microglia, is activated within minutes of ischemic onset. Hypoxia causes depolarization, and extracellular ATP and uridine triphosphate levels rise. These molecules serve as early danger signals, which stimulate the activation of microglia. Microglia are further activated as cells begin to die within the brain. Loss of cell–cell interactions, including the surface proteins CD200 and CX3CL1 on neurons and their receptors on microglia, further promote microglial activation.[36,37] A variety of molecular signals released from dead cells, so-called danger-associated molecular pattern molecules (DAMPs), activate pattern recognition receptors including Toll-like receptors expressed on microglia. DAMPs further contribute to the inflammatory response to stroke by inducing proinflammatory gene expression in infiltrating leukocytes and by priming dendritic cells for antigen presentation.[33] The importance of the immune response to stroke is underlined by the numerous anti-inflammatory interventions that limit injury in animal models.[34]

Interactions between the immune system and mitochondria have been examined in other neurodegenerative diseases, including Alzheimer disease, Parkinson disease, and multiple sclerosis.[38,39] ROS generated by microglia can cause mutations in mtDNA and damage enzyme of the respiratory chain, which can cause dysfunction in oxidative phosphorylation and increased ROS production.[39] Cytokines also directly damage mitochondria. For example, tumor necrosis factor–α (TNFα) and interferon-γ increase inducible nitric oxide synthase expression and cause elevated nitric oxide production in primary cultures of rat oligodendrocytes.[33] TNFα can also trigger excitotoxicity.[33] Timing of the early inflammatory response coincides with the secondary failure of the bioenergetic function. However, further studies are needed for more detailed correlation of inflammatory response with mitochondrial failure during cerebral ischemia/reperfusion.

CLINICAL IMPLICATIONS We summarized 3 key elements of reperfusion-related injury cascade that either directly or indirectly result in mitochondrial dysfunction and failure of cellular bioenergetics. Occurring in parallel, these processes are certain to cause the mitochondrial failure. In order to surmount the challenges in search of effective neuroprotective therapies, it appears that a combination of agents inhibiting the numerous deleterious processes should be a logical next step.

Elucidation of the complex pattern of cellular changes during experimental ischemia led to universal optimism that novel effective therapeutic interventions were imminent. The successful translation of neuroprotection in animal studies into clinical practice, however, has remained elusive. Over 80 putative neuroprotective agents have been tested in clinical trials, but none were effective. The Stroke Therapy Academic Industry Roundtable (STAIR) criteria were offered as guidelines aimed at making preclinical work in animals more reflective of human

stroke. These guidelines recommended physiologic monitoring during surgeries, clinically relevant time windows, defining minimally effective and toxic drug levels, and outcome measures, which included functional analyses similar to those used in human studies.[40] The Stroke-Acute Ischemic NXY Treatment (SAINT) trials, which tested a free radical scavenger, were reported to have fulfilled them, and when the SAINT I trial reported a positive result, optimism again surged. However, NXY 059 had several shortcomings, including poor blood–brain barrier permeability, nonphysiologic oxidation potential, and low potency.[41] These may explain why an agent that targeted the ROS generated by mitochondria failed to protect the brain in the larger SAINT II trial. Many subsequent studies have analyzed the preclinical studies leading up to the SAINT trials and showed numerous ways in which the STAIR criteria were not fulfilled.[42–44] In addition, the outcome measure used in the trial examined a shift in the Rankin Scale score as opposed to the traditional improvement to a score of 2 or less. This may have allowed a less robust clinical effect to show a significant improvement in the SAINT I trial. Given the methodologic shortcomings of the SAINT trials, their result should not be seen as evidence that targeting ROS is not a potential therapy for cerebral ischemia. The STAIR criteria have been updated to include the removal of bias during preclinical experiments, the use of male and female animals as well as aged or diseased animal models, which may more closely resemble stroke patients, and the inclusion of biomarkers.[45] It is hoped that adherence to these recommendations will lead to the development of effective therapies for neuroprotection; however, it must be remembered that the STAIR criteria are not validated and cannot be until a neuroprotective agent fulfilling the STAIR criteria is proved effective in clinical trials. More simply, STAIR criteria remain a hypothesis that has been tested but not yet proven.

Although the translation problem has not been resolved, interventions aimed at maintaining mitochondrial integrity and optimizing function, while difficult to design, may offer some hope. Agents that attenuate the deleterious insults of excess calcium and ROS on mitochondria should be tested. Stabilization of calcium homeostasis may be in part supported by treatment with lithium,[46] magnesium,[47] and calcium channel blockers such as nimodipine.[48,49] Although these agents may or may not be effective when given alone, they may be combined with free radical scavengers[50,51] such as resveratrol,[52,53] tempol,[54] and edaravone.[55] Another approach is to combine agents that inhibit mitochondrial damage with those that support mitochondrial function. Any and all procedures that improve mitochondrial function should be considered, including hypothermia, use of coenzyme Q, and use of alternative substrates.[56,57] Furthermore, some agents such as the immunosuppressant cyclosporine A have dual mechanisms of action, as an MPTP inhibitor and anti-inflammatory agent.[58] Peroxisome proliferator–activated receptor agonists have been found to have both anti-inflammatory and antioxidative actions. These agents suppress the inflammatory response to cerebral ischemia, including reducing the expression of proinflammatory cytokines and influx of systemic inflammatory cells and increasing the expression of free radical scavengers.[59,60] Agents that act on multiple sites on the mitochondria to reduce organelle dysfunction are more likely to be effective, since if only one pathway of mitochondrial injury is targeted, other avenues of injury will remain susceptible. Combination therapy may prolong the temporal therapeutic window.[61]

In animal studies, combined drug therapy targeted to reduce oxidative stress and mitochondrial dysfunction showed enhanced neuroprotection after stroke, reduced infarct volume, and improved functional-behavioral outcomes.[62–66] It should be emphasized that the strategy we propose is in no way to create a cocktail of all the listed agents, but rather to design a treatment with 2 agents, where each agent shows some indication of efficacy but the effects are not significant. With 2 agents acting at different sites on the mitochondria, it is quite possible that the degree of neuroprotection may become additive or even synergistic. Although such an approach is not without pharmacologic problems, it may be an answer to the ubiquitous pathology engendered by ischemia.

The evidence for a role of mitochondria in the evolution of ischemia-induced cell damage/death is convincing. It seems unlikely that inhibiting one mitochondria-related pathologic process alone would reverse the evolution of cell damage or death. Thus, failure of translation of neuroprotective therapies into clinical practice may be due to the fact that most of the approaches have focused on blocking one aspect of the ischemic cascade, suggesting a multifaceted approach may be more effective at preventing secondary insults imposed on mitochondria. The evidence suggests that effective neuroprotective therapies will eventually evolve with a greater focus on the multiple pathologic pathways that act on the mitochondria to cause dysfunction and cell death.

AUTHOR CONTRIBUTIONS

Dr. Pundik, Dr. Xu, Dr. Sundarajan: literature review, manuscript preparation, figure design.

DISCLOSURE

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