



Physiological and pathological roles of tissue plasminogen activator and its inhibitor neuroserpin in the nervous system

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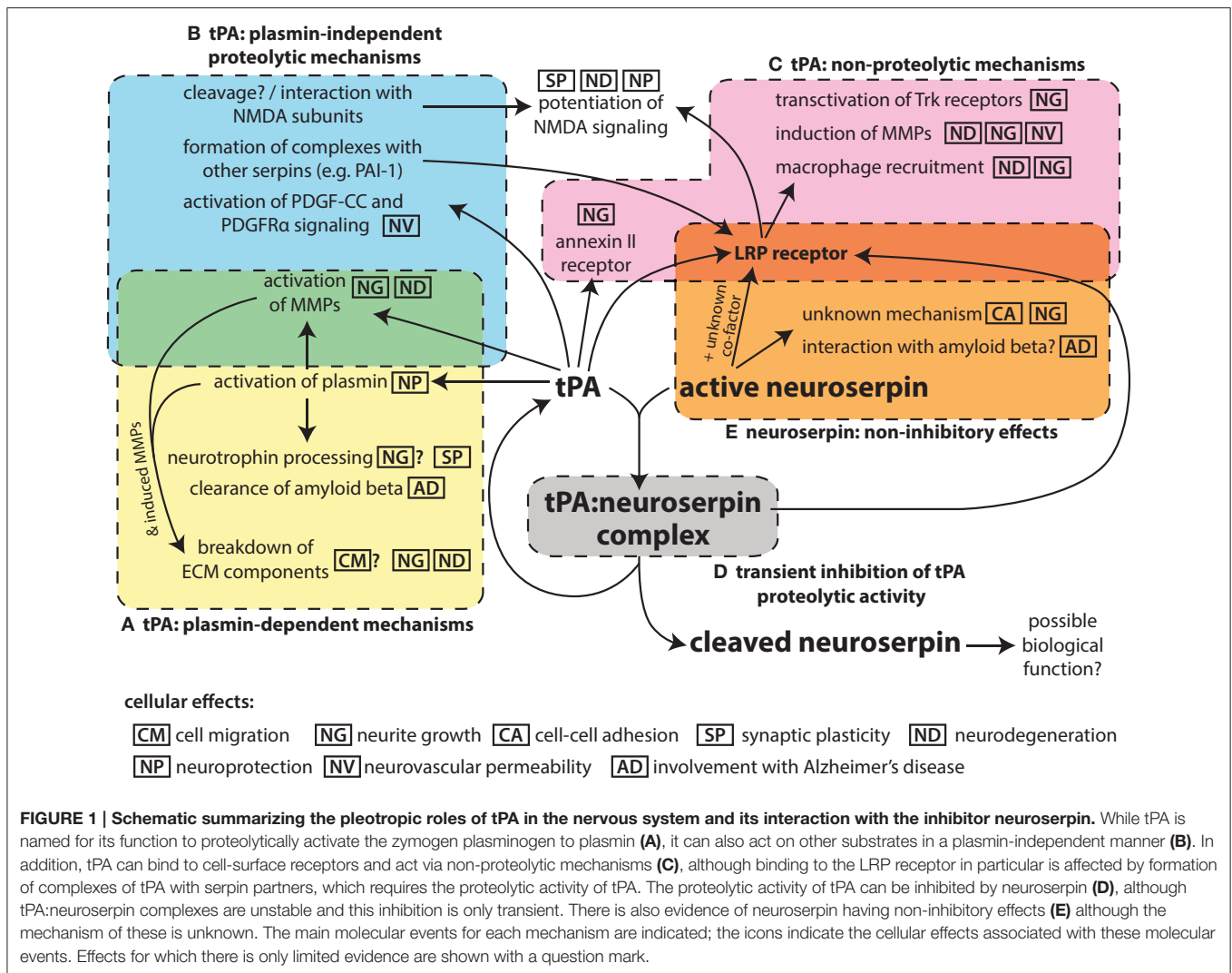
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Although its roles in the vascular space are most well-known, tissue plasminogen activator (tPA) is widely expressed in the developing and adult nervous system, where its activity is believed to be regulated by neuroserpin, a predominantly brain-specific member of the serpin family of protease inhibitors. In the normal physiological state, tPA has been shown to play roles in the development and plasticity of the nervous system. Ischemic damage, however, may lead to excess tPA activity in the brain and this is believed to contribute to neurodegeneration. In this article, we briefly review the physiological and pathological roles of tPA in the nervous system, which includes neuronal migration, axonal growth, synaptic plasticity, neuroprotection and neurodegeneration, as well as a contribution to neurological disease. We summarize tPA's multiple mechanisms of action and also highlight the contributions of the inhibitor neuroserpin to these processes.

Keywords: serine protease, serpin, neuronal migration, neurite growth, synaptic plasticity, neurodegeneration and neuroprotection, Alzheimer's disease, neurovascular unit

Introduction

Research of tissue plasminogen activator (tPA) in the nervous system has linked this protease to a number of functions, including cell migration, axonal growth, and synaptic plasticity, as well as a contribution to neurodegeneration in pathological states. The main inhibitor of plasminogen activator proteolytic activity in the vascular space is the serpin plasminogen activator inhibitor 1 (PAI-1; SERPINE1). This serpin, however, is only weakly expressed in the brain (Sawdey and Loskutoff, 1991; Masos and Miskin, 1997). Another serpin, protease nexin-1 (PN-1; SERPINE2) is expressed throughout the brain (Sappino et al., 1993; Reinhard et al., 1994). Although PN-1 may play some role in regulating tPA activity (Kvajo et al., 2004; Samson et al., 2008), its inhibitory kinetics suggest that it mainly functions as inhibitor of thrombin (Scott et al., 1985). Instead, the predominant inhibitor of neuronal tPA activity is believed to be the neuroserpin (SERPINI1), a serpin that is largely specific to the nervous system (Osterwalder et al., 1996; Hastings et al., 1997; Krueger et al., 1997). This review will focus on the interplay of these two players in the nervous system (**Figure 1**).



Neuroserpin as an Inhibitor of tPA

Analysis of neuroserpin sequence indicated that it was likely to be an inhibitor of trypsin-like serine proteases (Osterwalder et al., 1996). Biochemical evidence subsequently showed strong inhibition of tPA by neuroserpin and considerably less efficient inhibition of urokinase plasminogen activator (uPA), trypsin, NGF- γ , plasmin, and thrombin (Hastings et al., 1997; Osterwalder et al., 1998). The function of neuroserpin as an inhibitor of tPA is supported by their similar expression patterns in the nervous system (Hastings et al., 1997; Krueger et al., 1997; Teesalu et al., 2004) and data showing that tPA activity levels are decreased by over-expression of neuroserpin in the brain (Cinelli et al., 2001).

Other results, however, indicate that neuroserpin does not behave as a classical inhibitory serpin toward tPA. Unlike most covalent serpin:protease complexes, tPA:neuroserpin is unstable and dissociates within minutes to release cleaved neuroserpin

and active tPA (Barker-Carlson et al., 2002; Ricagno et al., 2009; Lee et al., 2015). As complex dissociation is expected to occur prior to clearance (Barker-Carlson et al., 2002), these data suggest that neuroserpin is likely to function as a transient inhibitor of tPA *in vivo*. Interestingly, evolutionarily conserved residues in neuroserpin regulate the half-life of tPA:neuroserpin complexes, suggesting that the precise half-life of the complexes may be physiologically important (Lee et al., 2015). tPA:neuroserpin interactions may involve other players, such as an unknown co-factor that stabilizes the complex (Barker-Carlson et al., 2002). Neuroserpin's weak inhibition of other proteases, such as plasmin, may also be physiologically important (Wu et al., 2010) and it is also possible that neuroserpin may have other protease targets that have not yet been determined. There is evidence of non-inhibitory functions of neuroserpin (Lee et al., 2008), raising the alternate possibility that tPA modulates neuroserpin activity by cleaving neuroserpin to produce a form with distinct (non-inhibitory) biological activity.

Expression of Neuroserpin and tPA in the Nervous System

Both tPA and neuroserpin are expressed in neurons throughout the developing and the adult nervous systems (Sappino et al., 1993; Friedman and Seeds, 1994; Ware et al., 1995; Osterwalder et al., 1996; Krueger et al., 1997; Teesalu et al., 2004), with spatial and temporal expression patterns suggesting roles in neuronal migration, axonal growth, synaptic development, neuronal plasticity and regulation of neurovascular responses. High neuroserpin expression has been shown in post-mitotic cells undergoing neurogenesis in the adult (Yamada et al., 2010), also suggesting a function in neuronal maturation.

Neuroserpin and tPA are targeted to the regulated secretory pathway, being sorted to dense core secretory granules and released in response to stimulation (Parmer et al., 1997; Lochner et al., 1998; Hill et al., 2000; Parmar et al., 2002; Silverman et al., 2005; Ishigami et al., 2007; Miranda et al., 2008). At a subcellular level, tPA has been localized to neuronal growth cones (Lochner et al., 1998; Silverman et al., 2005) and dendritic spines (Lochner et al., 2006), while neuroserpin has been localized to the neurite tips of differentiated PC12 cells (Parmar et al., 2002; Miranda et al., 2008), as well as axons, dendrites and presynaptic terminals of cultured neurons (Ishigami et al., 2007; Borges et al., 2010). Two unique features of neuroserpin that are important for regulated secretion are a targeting sequence at the C-terminus (Ishigami et al., 2007) and a resistance to polymerization at low pH (Belorgey et al., 2010).

In addition to activity-dependent secretion, it has been shown that the expression of tPA is regulated by several forms of neuronal activity including long-term potentiation (LTP) (Qian et al., 1993) and long term depression (LTD) (Napolitano et al., 1999; Calabresi et al., 2000). The expression of neuroserpin has also been shown to be regulated by neuronal depolarization (Berger et al., 1999), neuronal activity during visual cortex development (Wannier-Morino et al., 2003), and several signaling factors and hormones including nerve growth factor (NGF), anti-Müllerian hormone (AMH), thyroid hormone, and progesterone (Berger et al., 1999; Navarro-Yubero et al., 2004; Lebeurrier et al., 2008; Vanlandingham et al., 2008).

Functions of tPA and Neuroserpin in Neuronal Migration and Axonal Growth

A role for tPA in neuronal migration is supported by results showing that migration of cerebellar granule neurons is perturbed in tPA-deficient mice (Seeds et al., 1999). While it has been hypothesized that tPA regulates neuronal migration by activating plasmin to break down cell adhesions or extracellular matrix (ECM) (Seeds et al., 1999; Basham and Seeds, 2001), there is no direct evidence to support this role.

Evidence for a function of tPA and neuroserpin in regulating axonal growth has come from studies of cultured cells. Inhibition of tPA activity or tPA knockout have been shown to block axonal growth in cultured neurons (Pittman et al., 1989; Baranes et al., 1998; Minor et al., 2009), while exogenous tPA or tPA

over-expression causes increased neurite outgrowth (Pittman and Dibenedetto, 1995; Baranes et al., 1998; Lee et al., 2007a). Similarly, altered expression of neuroserpin has been shown to trigger changes in the extension of neurite-like processes of AtT-20 cells (Hill et al., 2000) and NGF-mediated neurite outgrowth in PC12 cells (Parmar et al., 2002; Navarro-Yubero et al., 2004).

A role of tPA in axonal growth has also been shown *in vivo*. In one study, tPA-knockout mice show abnormal growth of mossy fiber axons in the dentate gyrus following seizure (Wu et al., 2000). Other reports have focused on the role of tPA in axonal regeneration following damage. In studies using the sciatic nerve crush model of peripheral nervous system regeneration, tPA is induced in the neurons and supporting cells of the nerve following crush damage, while axonal regeneration and functional recovery is reduced in tPA or plasminogen knockout animals and improved with exogenous tPA or tPA/plasminogen (Akassoglou et al., 2000; Siconolfi and Seeds, 2001, 2003; Zou et al., 2006).

Multiple mechanisms have been suggested to mediate the effects of tPA on axonal growth. Proteolysis of ECM components may create channels for neurites to extend through (Pittman and Dibenedetto, 1995) and/or remove the inhibitory effects of these components (Wu et al., 2000; Bukhari et al., 2011). This is likely to involve activation or induction of additional downstream proteases such as matrix metalloproteinases (MMPs) (Siconolfi and Seeds, 2003; Wang et al., 2003; Hu et al., 2006; Zou et al., 2006). During axonal regeneration, the removal of fibrin deposits by tPA/plasmin also appears to be important (Akassoglou et al., 2000; Zou et al., 2006), as well as macrophage recruitment to remove cellular debris (Zou et al., 2006), which may involve tPA binding to the LDL-related protein (LRP) receptor (Cao et al., 2006). Binding of tPA to the LRP receptor and the annexin II receptor has also been shown to mediate non-proteolytic effects of tPA on neurite outgrowth (Lee et al., 2007a; Shi et al., 2009). Finally, tPA may regulate neurite growth via proteolytic processing of neurotrophins (Pang et al., 2004; Bruno and Cuello, 2006).

The mechanism of neuroserpin's effects on neurite outgrowth are largely undetermined. Neuroserpin may act by modulating tPA activity, for example, neuroserpin has been shown to regulate proteolytic processing of the neurotrophin NGF (Bruno and Cuello, 2006). Interestingly, the neurite outgrowth effects of neuroserpin could be triggered by non-inhibitory mutant forms of neuroserpin (Lee et al., 2008), suggesting neuroserpin may also act independently of tPA, possibly by binding to a cell surface receptor such as LRP (Makarova et al., 2003).

Effects of tPA and Neuroserpin on Neuronal Plasticity

Deficits in hippocampal late phase LTP are seen in tPA-knockout mice (Frey et al., 1996; Huang et al., 1996; Calabresi et al., 2000). Conversely, LTP is increased by exogenous tPA or tPA overexpression (Baranes et al., 1998; Madani et al., 1999). Knockout of the tPA gene also leads to defects in both LTP and LTD in the striatum (Calabresi et al., 2000; Centonze et al.,

2002). Numerous studies have also shown a role of tPA in memory and learning. For example, tPA-knockout mice exhibit deficits in hippocampal-dependent and striatum-dependent tasks (Huang et al., 1996; Calabresi et al., 2000; Pawlak et al., 2002; Benchenane et al., 2007) and cerebellar motor learning (Seeds et al., 2003), while transgenic mice over-expressing tPA were found to have improved spatial learning (Madani et al., 1999). tPA is also required for altered amygdala- and hippocampal-dependent behavioral responses that occur in mice subjected to restraint-stress (Pawlak et al., 2003, 2005b; Norris and Strickland, 2007).

At the cellular level, tPA's involvement in LTP has been associated with the formation of new presynaptic varicosities (Baranes et al., 1998), while activity-dependent formation of perforated synapses in cultured neurons can be blocked by tPA inhibitors (Neuhoff et al., 1999). In animals subjected to restraint stress, induction of the plasticity-related gene GAP43 (Pawlak et al., 2003) and changes in dendritic spine number were absent in tPA knockout mice (Pawlak et al., 2005b). During visual cortex development, experience-dependent plasticity and pruning of dendritic spines is also reduced in tPA-knockout mice and can be partly restored by exogenous tPA (Mataga et al., 2002, 2004).

A number of different mechanism underlying tPA's effects on synaptic plasticity have been proposed. Firstly, tPA may contribute to LTP by regulating plasmin-mediated processing of BDNF from its precursor proBDNF to mature BDNF (mBDNF) (Pang et al., 2004; Barnes and Thomas, 2008). It has been shown that tPA is secreted from neurons in response to high-frequency, but not low-frequency, stimulation of neurons, leading to changes in the proBDNF/mBDNF ratio (Nagappan et al., 2009). Since proBDNF has been linked to LTD (Woo et al., 2005) while mBDNF has been linked to LTP, these results suggest that tPA may mediate the differing cellular responses to different patterns of neuronal activity. Secondly, there is general agreement in the literature that tPA can potentiate NMDA-receptor signaling. The manner in which it does so, however, remains unclear. Results from the Vivien group suggest that tPA may act by interacting with the GluN1 subunit of NMDA receptors, particularly in GluN2D-containing receptors (Benchenane et al., 2007; Macrez et al., 2010; Obiang et al., 2012). Other results suggest that tPA modulates NMDA signaling through GluN2B subunits (Pawlak et al., 2005a; Norris and Strickland, 2007; Noel et al., 2011; Ng et al., 2012) or by a mechanism involving LRP (Martin et al., 2008; Samson et al., 2008). The importance of LRP in tPA-mediated neuronal plasticity was also reported in an earlier study on LTP (Zhuo et al., 2000).

Experiments in culture systems have provided some evidence that neuroserpin is involved in cellular plasticity. Firstly, altered neuroserpin expression in PC12 cells has been linked to changes in cell-cell adhesion mediated by the synaptic adhesion molecule N-cadherin (Lee et al., 2008). In addition, overexpression of neuroserpin in cultured neurons has been found to lead to changes in the number and shape of dendritic spines (Borges et al., 2010). Altered neuroserpin expression *in vivo* has also been shown to lead to behavioral changes, with both neuroserpin overexpression and neuroserpin-knockout leading to increased phobic and anxiety-like responses (Madani et al., 2003). Localized

overexpression of neuroserpin in the adult rat hippocampus did not cause any changes in learning and memory, but it altered the expression of postsynaptic scaffolding protein PSD-95 (Tsang et al., 2014). Overall, little is known about neuroserpin's mechanism of action for these effects, however, the results from the PC12 studies show that inhibition of tPA was not required (Lee et al., 2008), and the changes in behavior in neuroserpin-knockout animals were not correlated with altered tPA activity (Madani et al., 2003).

Neuroserpin and tPA in Neurodegeneration and Neuroprotection

Initial evidence for a contribution of tPA to neuronal death came some years ago, when it was shown that tPA knockout mice were resistant to excitotoxin-induced neuronal degeneration (Tsirka et al., 1995) and had reduced ischemic damage in a stroke model (Wang et al., 1998). These results have been independently confirmed by a number of other groups (Strickland, 2001; Kaur et al., 2004).

Three main mechanisms for tPA's effects on neuronal death have been proposed. Firstly, tPA may cause ECM breakdown by proteolytically activating plasmin and/or MMPs (Chen and Strickland, 1997; Tsirka et al., 1997; Sumii and Lo, 2002; Wang et al., 2003). Secondly, the ability of tPA to potentiate NMDA receptor-mediated calcium influx may also contribute by promoting excitotoxic neuronal death (Nicole et al., 2001). In support of this, immunotherapy to block interaction of tPA with NMDA receptors has been shown to reduce neuronal damage in stroke models (Benchenane et al., 2007; Gaberel et al., 2013). Thirdly, tPA may signal through the LRP receptor to trigger a number of inter-related effects including induction of MMP expression (Wang et al., 2003, 2004; Lee et al., 2007b; Sashindranath et al., 2012), opening of the blood-brain barrier (Yepes et al., 2003; Sashindranath et al., 2012) and recruitment and activation of microglia (Rogove and Tsirka, 1998; Rogove et al., 1999; Siao and Tsirka, 2002; Zhang et al., 2009). Paradoxically, the tPA inhibitor PAI-1 has been shown to exacerbate, rather than reduce, some of these effects of tPA, as tPA:PAI-1 complexes bind more strongly to LRP than tPA itself (Sashindranath et al., 2012). The instability of tPA:neuroserpin complexes could therefore be a mechanism to temporarily reduce tPA activity without excessive LRP activation.

There is also evidence of neuroprotective effects of tPA, first shown some time ago (Kim et al., 1999; Yi et al., 2004; Liot et al., 2006) but highlighted by a series of recent results from the *in vitro* oxygen and glucose deprivation (OGD) model of ischemic death, as well as in *in vivo* models of excitotoxic neuronal death (Haile et al., 2012; Wu et al., 2012, 2013). These studies have suggested that lower concentrations of tPA mediate survival instead of neuronal death, through both plasmin-dependent and LRP-dependent/plasmin-independent mechanisms involving NMDA signaling.

In animal models of stroke, administration of exogenous neuroserpin alone, neuroserpin in combination with tPA and neuroserpin overexpression have been shown to reduce ischemic

damage *in vivo* (Yepes et al., 2000; Cinelli et al., 2001; Zhang et al., 2002). In these studies, the effects of neuroserpin were associated with reductions in tPA and uPA activity, ECM degradation, microglia activation and blood brain barrier leakage. Conversely, neuroserpin-knockout mice have worse ischemic damage and neurological outcomes than controls, with the effects attributed to tPA-mediated activation of microglia (Gelderblom et al., 2013). Similarly, studies in the OGD model and a mouse model of motoneuropathy have also shown neuroprotective effects of neuroserpin with results suggesting a mechanism involving tPA inhibition (Simonin et al., 2006; Rodríguez-González et al., 2011). However, neuroserpin has been shown to promote neuronal survival in tPA knockout mice, indicating it can also act through a tPA-independent mechanism, possibly through inhibition of uPA or plasmin (Wu et al., 2010).

Functions of tPA and Neuroserpin in the Neurovascular Unit

There is considerable evidence that tPA in the central nervous system side of the neurovascular unit increases the permeability of the blood-brain barrier (e.g., Yepes et al., 2003; Su et al., 2008; Sashindranath et al., 2012). This effect of tPA may contribute to neurodegeneration following stroke, and recent results also suggest a contribution to seizure propagation (Fredriksson et al., 2015). tPA has also been shown to regulate functional hyperemia (Park et al., 2008). A number of downstream events have been identified for the neurovascular effects of tPA including activation of neuronal nitric oxide synthase (Parathath et al., 2006; Park et al., 2008), proteolytic activation of platelet-derived growth factor-CC (PDGF-CC) and platelet-derived growth factor receptor alpha (PDGFR α) signaling (Su et al., 2008; Fredriksson et al., 2015), LRP signaling and induction of MMPs (Sashindranath et al., 2012). While most of the neurovascular effects of tPA are considered to be plasmin-independent (Yepes et al., 2003), there is also evidence for an involvement of plasmin (Freeman et al., 2014; Niego and Medcalf, 2014). As an inhibitor of tPA, neuroserpin can act as an antagonist of tPA in the neurovascular unit (Yepes et al., 2003; Fredriksson et al., 2015).

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Contributions of tPA and Neuroserpin to Neurological Disease

Mutations in the neuroserpin gene cause a rare autosomal-dominant dementia accompanied by epilepsy called Familial Encephalopathy with Neuroserpin Inclusion Bodies (FENIB), characterized by polymerization of neuroserpin, formation of inclusion bodies and subsequent neuronal degeneration (Davis et al., 1999, 2002; Takao et al., 2000; Gourfinkel-An et al., 2007; Coutelier et al., 2008; Hagen et al., 2011). Other studies have suggested a role for neuroserpin in Alzheimer's disease, with neuroserpin hypothesized to be either beneficial by interacting with amyloid-beta peptides and altering their oligomerization (Kingham et al., 2006) or detrimental by reducing tPA-mediated clearance of amyloid-beta (Fabbro and Seeds, 2009; Fabbro et al., 2011). Changes in the expression of neuroserpin have also been linked to schizophrenia (Hakak et al., 2001; Vawter et al., 2004; Brennand et al., 2011). A recent study also suggests that the expression of neuroserpin by tumor cells may inhibit plasmin-mediated death signals and allow metastasis into the brain (Valiente et al., 2014).

Conclusion

Research over the years has shown that tPA has pleiotropic effects in the nervous system and can act through multiple mechanisms. It is also clear that neuroserpin does not function as a classical serpin inhibitor for tPA and this must be considered when making inferences regarding its function and mode of action. Future research should take a broad view and consider all possible mechanisms of these two players to provide a more complete understanding of their roles in the nervous system.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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