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REVIEW ARTICLE

The Neuroprotective Role of Neuroserpin in Ischemic and Hemorrhagic Stroke

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Abstract: Tissue plasminogen activator (tPA) is commonly used to treat acute ischemic stroke within an appropriate therapeutic window. Its inhibitor, neuroserpin (NSP), is reported to exhibit neuroprotective effects on stroke. This review aims to summarize, from literature, the available evidence, potential mechanisms, and knowledge limitations regarding the neuroprotective role of NSP in stroke. All the available evidence indicates that the regulation of the inflammatory response may play a key role in the mechanisms of NSP, which involve all the constituents of the neuroimmune axis. The neuroinflammatory response triggered by stroke can be reversed by NSP, with complicated mechanisms such as maintenance and reconstruction of the structure and function of the blood-brain barrier (BBB), protection of the cells in the central nervous system, and suppression of cell death in both ischemic and hemorrhagic stroke. Moreover, available evidence strongly suggests a tPA-independent mechanism is involved in NSP. However, there are many important issues that are still unclear and need further investigation, such as the effects of NSP on hemorrhagic stroke, the role of the tPA-independent neuroprotective mechanisms, and the clinical application prospects of NSP. We believe our work will be helpful to further understand the neuroprotective role of NSP.

Keywords: Stroke, tPA, neuroserpin, neuroprotective effects, neuroinflammation, blood-brain barrier.

1. INTRODUCTION

Stroke is a common disease that results in high rates of disability and mortality. It has been reported that every year there are over 13 million new onset strokes worldwide, and it is the second leading cause of death [1]. Many survivors suffer from various neurological deficits that require lifelong rehabilitation [2]. Stroke is a global public health concern with a large economic burden for both the patient's family and the government.

Although there is no specific treatment for either ischemic or hemorrhagic stroke, many promising treatments are emerging, such as neuroserpin (NSP). Thrombolysis using tissue plasminogen activator (tPA) has been widely adopted to treat ischemic stroke within an appropriate therapeutic window. However, tPA is commonly associated with many adverse events, hence NSP, an inhibitor of tPA, is gradually introduced. However, the role of NSP is complicated and multidimensional and cannot be explained simply by saying it suppresses tPA.

NSP is a member of the serpin family of serine protease inhibitors. It is essentially a glycoprotein composed of 394 amino acids with molecular weight of 54-60 kDa. NSP is mainly expressed in both the central and peripheral nervous system neurons, starting from the late stage of neural development and continuing into adulthood. It is also known as protease inhibitor 12 (PI12) or SERPINI in the official terminology of serpins [3]. It is well known as a tPA inhibitor, and its neuroprotective role in stroke has been increasingly investigated since 2000. Yepes *et al*. first reported the neuroprotective effects of NSP [4]. Later, a number of studies elucidated the neuroprotective effects of NSP. Cinelli *et al*. found that NSP's neuroprotective effects work by suppressing the activation of microglia [5]. Zhang *et al*. reported that NSP extends the thrombolysis therapeutic window in rat cerebral ischemia (CI) models [6]. Lebeurrier *et al*. demonstrated that NSP can prevent N-methyl-D-aspartate (NMDA)-induced neurotoxicity [7]. Later, they also found that NSP may influence the migration, plasticity, and death

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of neurons [8]. Wu *et al*. found that NSP administration improves ischemic tolerance. In addition, NSP reduces the infarct volume in animal CI models [9].

Rodriguez-Gonzalez and his colleagues also studied this topic. They first reported that NSP exhibits neuroprotective effects in patients with CI [10]. They also reported that higher serum NSP levels were associated with better clinical outcomes in patients with CI [11]. In a bench study, they found that NSP was neuroprotective by suppressing tPA-mediated inflammation along with the damage of the blood-brain barrier (BBB) [12]. Ma *et al*. found that NSP presented a dose-dependent neuroprotective effect *via* suppression of tPA-mediated acute neurotoxicity [13]. Gelderblom *et al*. reported that unbalanced expression of NSP and tPA was associated with a poorer clinical outcome in experimental CI models, due to activation of microglia [14].

Beginning in 2004, our laboratory also carried out a series of experiments concerning the neuroprotective effects of NSP. Early in 2004, we found that suppressing the effects of NSP may deteriorate ischemia-mediated brain injury in diabetic rats [15]. Later, we verified the neuroprotective effects of NSP in ischemic astrocytes *via* the nuclear factor (NF)-κB signaling pathways [16]. We also verified the neuroprotective role of NSP on neurons *via* suppression of the mitogen-activated protein kinase (MAPK) signaling pathways [17]. Later, we found NSP can reduce brain edema and BBB permeability in mouse cerebral hemorrhage (CH) models. It may play a role in protecting and/or repairing the injured vascular endothelial cells [18]. In the clinical study, we found that serum NSP level negatively correlated with the levels of inflammatory markers and positively correlated with the clinical outcomes in patients with CI [19].

Although these studies provided the evidence, in cells, animal models, and patients, of the neuroprotective effects of NSP in both ischemic and hemorrhagic stroke, we believe the neuroprotective effects of NSP are quite complicated and not fully understood. Summarizing what is already known is therefore extremely important for future investigations. However, review papers concerning the neuroprotective effects of NSP are limited.

Ma *et al*. summarized the mechanisms of neuroprotective effects of NSP involved in the ischemic brain. They believed that NSP is associated with a number of mechanisms, such as regulation of tumorigenesis, inhibition of urokinase-type plasminogen activator and fibrinolysin, and modification of immune cells [20]. Lee and his colleagues also discussed the physiological and pathological roles of NSP. Their 2015 paper focused on the mechanisms of tPA [21], and the 2017 paper discussed the roles of NSP in the emotional behavior, synaptic plasticity, and neuroprotection in stroke and excitotoxicity models [22]. Lee *et al*. comprehensively discussed the physiological and pathological roles of NSP from the angles of NSP inclusion bodies, Alzheimer's disease (AD), and brain metastasis, but this paper did not focus on stroke [22]. There is no review study particularly summarizing the newest knowledge regarding the neuroprotective effects of NSP on stroke. Based on our experience with this topic, we attempted to summarize the existing knowledge and insights regarding the neuroprotective role of NSP in stroke. We believe these "take home message" might be helpful in understanding the known mechanisms of NSP.

2. EXISTING EVIDENCE REGARDING THE EFFECTS OF NSP ON STROKE

Although clinically, thrombolysis using tPA is a common treatment for acute CI within 3 hours of stroke onset, literature investigating the effects of NSP on stroke is limited (Table **1**). We summarized evidence from available literature, including *in vivo* and *in vitro* studies in cells, animals, and humans.

2.1. Experimental Research Evidence

Most studies verified the effects of NSP on ischemia models; only 1 study involved CH models.

2.1.1. In vitro Verification

Lebeurrier *et al*. first performed *in vitro* verification in neurons. They employed an NMDA-induced excitotoxic neuronal death model and found that exposure of 12.5 μM NMDA significantly enhanced neuronal death, while administration of NSP (1 μM) significantly reduced neuronal death. However, they also found that NSP failed to protect neurons from apoptosis induced by serum deprivation [7].

The process of oxygen-glucose deprivation (OGD) followed by reoxygenation (OGD/R) was the most commonly used technique to establish a cellular ischemic model. Later, Ma *et al*. found that both OGD/R treatment and NMDA exposure reduced the cell viability of cortical neurons, which could be significantly reversed by NSP administration. They believed that this neuroprotection was associated with suppression of the tPA-mediated neurotoxicity [13]. A study by Rodriguez-Gonzalez *et al*. investigated the effects of NSP on OGD/R-treated astrocytes and neurons. They found that the lactate dehydrogenase (LDH) release, active matrix metallopeptidase (MMP)-9, membrane cofactor protein (MCP)-1, and macrophage inflammatory protein (MIP)-2 levels significantly increased with tPA treatment after OGD, while NSP administration significantly decreased the LDH release and active MMP-9 after OGD. Administration of tPA along with NSP decreased LDH release, active MMP-9, and MIP-2. They thus concluded that the neuroprotective effects of NSP were associated with tPA-induced inflammation [12].

Our previous studies also employed the OGD/R treatment cellular models to verify the effects of NSP. In astrocytes, we found that OGD/R treatment enhanced LDH release and increased the apoptosis of astrocytes, whereas NSP treatment significantly reversed these OGD-induced injuries. Moreover, we found that the potential mechanisms may be associated with the NF-κB pathway, but not the MAPK and phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt) pathways [16]. In neurons and microglia, we found that OGD/R treatment induced morphological changes of neurons and microglia. The cell viability of neurons was significantly reduced by OGD/R. All of these changes were significantly reversed by NSP, which is associated with inhibition of the MAPK pathway [17].

Cheng *et al*. found that NSP exhibited neuroprotective effects on stress-induced dysfunction and death in hippocampal

Table 1. The main findings in the studies of the neuroprotective effects of neuroserpin (NSP) on stroke.

Authors	Subjects	Findings
Cellular studies		
Lebeurrier *et al*, 2005	Primary mouse cortical neurons	Exogenous NSP protects the cortex and the striatum against NMDA induced injury. This effect is associated to a decrease in NMDA receptor-mediated intracellular calcium influx.
Lebeurrier *et al*, 2008	Primary mouse neurons and astrocyte	AMH is considered as a new cytokine or hormone in the brain, with the NSP as one of its target genes, which could confer neuroprotection against excitotoxicity.
Wu *et al*, 2010	Primary mouse hippocampal and cortical neurons	The neuroprotective effect of NSP is due to inhibition of plasmin-mediated excitotoxin-induced cell death and is independent of neuroserpin's ability to inhibit tPA activity.
Rodriguez-Gonzalez *et al*, 2011	Oxygen-glucose deprivation model of primary rat mixed cortical cell (astrocytes and neurons)	NSP treatment after oxygen-glucose deprivation decreases the expression of tPA-enhanced mediators of inflammation and BBB disruption, thus promoting cell survival.
Ma *et al*, 2012	Oxygen-glucose deprivation and reoxygenation model of rat cortical neurons, NMDA-induced neuronal excitatory injury model	NSP protects neurons against oxygen-glucose deprivation and reoxygenation mainly by inhibiting tPA-mediated acute neuronal excitotoxicity
Wang *et al*, 2015	Oxygen-glucose deprivation and reoxygenation model of primary neonatal rat astrocytes	NSP exerts neuroprotective effects in OGD/R-treated astrocytes, and these potential neuroprotective mechanisms may lie in the inhibition of release of TNF-α and NO, along with inhibition of the NF-κB pathway.
Yang *et al*, 2016	Oxygen-glucose deprivation and reoxygenation model of primary neonatal rat neurons and microglia	NSP can reverse abnormal variations in neurons and microglia mediated inflammatory response induced by OGD/R. It may be related to the MAPK signaling pathway.
Cheng *et al*, 2017	H_2O_2-induced neurotoxicity model of primary cultured hippocampal neurons	NSP protects against oxidative stress-induced dysfunction and death of primary cultured hippocampal neurons through the Akt-BCL-2 signaling pathway and leads to inhibition of caspase-3 activation.
Animal studies		
Yepes *et al*, 2000	Focal cerebral ischemia/reperfusion rat model	Intracerebral administration of NSP after stroke decreases stroke volume, reduces basement membrane proteolysis, and diminishes the number of cells with apoptotic features in the area of ischemic penumbra.
Cinelli *et al*, 2001	Middle cerebral artery occlusion mouse model	NSP reduces microglial activation, and therefore, the tPA and uPA activity and has a neuroprotective role after focal ischemic stroke
Zhang *et al*, 2002	Middle cerebral artery occlusion rat model	Administration of NSP in combination with tPA significantly reduced BBB leakage and ischemic lesion volume, suggesting that adjuvant administration of NSP blocks the extravascular effect of tPA, leading to subsequent decrease in stroke volume and widening of the therapeutic window for the thrombolytic effect of tPA
Yepes *et al*, 2003	Middle cerebral artery occlusion rat and mouse model	In initial stages of cerebral ischemia, the opening of the BBB is mediated directly by tPA; this activity is independent of either Plg or MMP-9, but requires interaction with LRP. The effect is suppressed by NSP.
Lebeurrier *et al*, 2005	NMDA-induced excitotoxicity mouse mode	Exogenous NSP protects the cortex and the striatum against NMDA induced injury. This effect is associated to a decrease in NMDA receptor-mediated intracellular calcium influx.
Munuswamy-Ramanujam *et al*, 2010	Mouse aortic allograft transplant model	NSP possesses anti-inflammatory activity in systemic arteries, modifying Th cell responses and significantly reducing plaque growth in mouse aortic allografts.
Wu *et al*, 2010	Middle cerebral artery occlusion mouse model (lethal injury or sublethal injury)	The neuroprotective effect of NSP is due to inhibition of plasmin-mediated excitotoxin-induced cell death and is independent of neuroserpin's ability to inhibit tPA activity.

(Table 1) contd….

Authors	Subjects	Findings
Animal studies		
Gelderblom *et al*, 2013	Middle cerebral artery occlusion mouse model	NSP has a protective role of in cerebral ischemia, and the unbalanced expression of NSP and tPA in Ns2/2 mice leads to worse outcome in experimental stroke, which is associated with increased microglia activation.
Li *et al*, 2017	Intracerebral hemorrhage mouse model	NSP has a neuroprotective effect in non-tPA-induced ICH brains. NSP can reduces brain edema through attenuating the permeability of the BBB possibly by protecting and/or repairing the injured blood vessel endothelium in the ICH state.
Zhao *et al*, 2020	A rat model of traumatic brain injury established by weight-drop method	The inhibition of NSP for endogenous tissue plasminogen activator aggravates neuronal apoptosis and axonal injury after traumatic brain injury, and activates microglia and astrocytes.
Human studies		
Cole *et al*, 2007	Female patients 15 to 49 years of age with a first cerebral infarction	A specific NSP SNP (rs6797312), and haplotypes including this SNP, are associated with ischemic stroke risk in Caucasian women.
Rodriguez-Gonzalez *et al*, 2011	Patients with acute ischemic stroke (with or without tPA treatment)	High serum NSP levels before intravenous tPA and NSP levels decrease at 24 h after ischemic stroke, independently of tPA treatment, are associated with good functional outcome of cerebral ischemia.
Wu *et al*, 2017	Patients with acute cerebral infarction	Decrease of Serum NSP level and NSP level at admission may be considered as potential predictive factors for outcome of acute ischemic stroke.

neurons *via* the Akt-Bcl-2 signaling pathway [23]. All of these *in vitro* verifications suggested that NSP exhibited neuroprotective effects on various cells in the central nervous system (CNS), and suppression of the inflammatory signaling pathway may be a potential mechanism.

2.1.2. In vivo Verification

Most of the *in vivo* verifications employed a middle cerebral artery occlusion (MCAO) rodent animal as the experimental CI model. Yepes *et al*. found that direct injection of NSP to the brain after MCAO achieved a reduction of 64% ischemic volume in CI rats [4]. These results were reproduced by another study using MCAO mice [5]. Zhang *et al*. (2002) observed changes in BBB leakage and infarct volume induced by NSP in rats undergoing administration of tPA 4 hours after MCAO. They found that NSP significantly reduced BBB leakage, brain edema, and ischemic lesion volume (*vs*. tPA alone). They therefore concluded that NSP widens the therapeutic window during tPA thrombolysis, and this effect is associated with inhibition of the extravascular effects of tPA [6]. Gelderblom *et al*. found that NSP administration significantly improved behavioral performance 3 days after MCAO in CI rats, and activation of microglia was increased in the rats without NSP [14]. Thus, the evidence above suggests that NSP may reduce infarct size and improve neurological deficits. Although there is no direct evidence that NSP suppresses activation of microglia, Gelderblom's data in NSP knockout mice indicated that the activation of microglia might be suppressed by NSP; however, this requires further verification.

Verification from hemorrhagic models is limited. Only 1 of our previous studies involved the neuroprotective effects of NSP on CH [18]. By employing a non-tPA-induced intracerebral hemorrhage (ICH) mouse model, we found that expression of NSP was upregulated in the ICH group. NSP administration significantly ameliorated neurological deficits, brain edema, and BBB leakage induced by the ICH surgery. NSP also contributed to the reconstruction of injured vascular endothelial cells [18].

2.2. Clinical Investigation Evidence

Clinical studies involving NSP are limited; those that were carried out were in patients with CI. Cole *et al*. reported the first clinical study of NSP, evaluating the relationship between the NSP single nucleotide polymorphisms (SNPs) and risk of CI onset. They found that a specific SNP of NSP (rs6797312), along with the haplotypes including rs6797312, were associated with the risk of CI onset in Caucasian women [24].

Later, Rodriguez-Gonzalez and his colleagues reported 2 clinical studies.

They found that the serum NSP levels within 24 hours of CI onset were negatively correlated with the serum levels of inflammatory biomarkers such as glutamate, interleukin (IL)-6, intercellular adhesion molecule (ICAM)-1, active MMP-9, and cellular fibronectin (cFn) [10]. They also found that the higher serum NSP levels after CI onset were associated with lower National Institute of Health Stroke Scale (NIHSS) scores (better outcome), whereas the lower serum NSP levels were associated with higher NIHSS scores (poorer outcomes) [11]. Our previous study found that a reduction in NSP level (10 days after admission − on admission) exhibited a negative correlation with the serum levels of IL-6, IL-1β, and ICAM-1. More NSP reduction was associated with lower levels of inflammatory markers. Higher serum NSP levels on admission were associated with smaller infarct volume and lower NIHSS scores [19].

These results have the same tendency, namely higher NSP levels related to lower inflammatory markers and better clinical outcomes and lower NSP levels linked to higher inflammatory markers and poorer clinical outcomes. These clinical data might provide indirect evidence of the neuroprotective role of NSP in patients with CI.

3. POTENTIAL MECHANISMS OF NEUROPROTECTIVE EFFECTS OF NSP ON STROKE

All the available evidence indicates that regulation of the inflammatory response plays a key role in the neuroprotective effects of NSP on stroke subjects. Because of the crucial role of inflammation in the pathophysiological changes after stroke, inflammation is an important target in stroke research [25-27]. However, the neuroinflammation triggered by stroke onset is very complicated and involves all the constituents of the neuroinflammatory system. The inflammatory response is a comprehensive effect of the involvements and interactions of BBB, microglia and macrophages, astrocytes and mast cells, dendritic cells, and other cells. Moreover, many inflammatory-related mechanisms, such as oxidative stress, excitotoxicity, ionic imbalance, and neuronal apoptosis are also involved in the pathophysiology of stroke [26]. Here, we discuss the NSP neuroprotective effects on the neuroimmune axis.

3.1. Mechanisms Associated with BBB

BBB is a natural immunological barrier in the neurovascular unit formed with tight junctions. Normally, BBB serves as a strong mechanical barrier for the CNS and contributes to the maintenance of the specific immune environment in the CNS. Under stroke conditions, BBB breakdown occurs due to attacks from the proinflammatory cytokines such as IL-6, IL-1β, and tumor necrosis factor (TNF)-α. Factors like nitrogen species and reactive oxygen accompanying the inflammatory reaction also contribute to the BBB damage [28]. In ischemic stroke, tPA activates MMPs, which promotes the protein degradation of the vascular basement membrane, leading to BBB breakdown and vasogenic edema. Thus, BBB loses its barrier function, resulting in introduction of the selectins and integrins as well as release of many proinflammatory cytokines [26]. Moreover, circulating cytokines can enter the CNS and trigger the activation of glial cells (astrocytes, microglia, and oligodendrocytes), which subsequently promotes secretion of more proinflammatory cytokines [29]. This vicious circle promotes the inflammatory response in the CNS and finally causes irreversible neurological damage. Hence, BBB damage plays a core role in the inflammatory response after stroke. Several studies demonstrated that NSP exhibits protective effects on BBB.

3.1.1. NSP Restored the Distribution Pattern of Occludin

Anatomically, BBB is composed of many brain microvascular endothelial cells, which are tightly connected with tight junctions (TJs). Occludin is a TJ-specific integral membrane protein, which is expressed in the cell-cell junction between epithelial and endothelial cells [30], and it plays a crucial role in maintaining barrier integrity. Occludin expression levels are correlated with the quality of the endothelial barrier [31]. Downregulation of occluding expression may result in enhanced endothelial permeability [32]. Any changes in the localization and structure of occludin may cause BBB breakdown [33]. Our previous study found that the experimental ICH processes damaged the normal distribution pattern of occludin-expressing cells in brain tissues. NSP administration may reconstruct this pattern, namely the occludin-expressing cells regularly distributed surrounding the capillaries. Meanwhile, by checking the Evans blue leakage, we found that the damaged BBB's permeability was reduced [18]. These data suggested that NSP administration partially repairs the damaged TJs, which may contribute to ameliorate the BBB function in the stroke state. However, the TJ-specific integral membrane protein family includes many proteins besides occludin, such as claudins. The effects of NSP on the other TJ-related proteins require further investigation.

3.1.2. NSP Reduces the Breakdown of Laminin in the Basement Membrane

The basement membrane is a special part of the extracellular matrix (ECM) that connects the endothelial cell compartment to the surrounding cellular layer, which is an essential part of BBB [34]. Laminin is an important component of the basement membrane, and it plays a key role in cell-ECM interactions, such as promoting the growth of protuberances; cell attachment, proliferation, and differentiation; and the development and regeneration of CNS [35-37]. Yepes's study indicated that proteolysis of laminin in the basement membrane was induced by tPA-related reperfusion. Intracerebral NSP administration significantly reduced the proteolysis [4]. It is easy to understand that in the CI state, tPA activates MMPs, which promote protein degradation of vascular basement membrane, leading to BBB "rupture" and vasogenic edema. NSP can suppress these processes by reducing the proteolysis of laminin, thus maintaining the integrity of BBB.

The above direct evidence regarding the effects of NSP on occludin and laminin indicates that NSP is protective for TJs and ECM, which might be the physical basis of the protection of the BBB. These effects finally lead to a comprehensive result, namely reducing the permeability of BBB enhanced by stroke, which has been verified by a tPA-related ischemic model [4, 9] and a non-tPA-related ICH model [18]. However, we believe the above evidence is just the tip of the evolutionary iceberg. The effects of NSP on BBB are far more complicated because the mechanisms between tPA and BBB are multifold. For example, there is a unique interaction between tPA and low-density lipoprotein receptor-related protein (LRP), which leads to strict control of cerebrovascular permeability [38]. tPA interacts with LRP and is an endogenous regulator of cerebrovascular permeability under pathological and physiological conditions [39]. NSP may inhibit the effects of tPA, but as yet there is no direct evidence to elucidate the role of NSP in the interaction between tPA and LRP. More experiments are needed to clarify the complicated effects of NSP on BBB.

3.2. Effects of NSP on Cells in the CNS

CNS is mainly composed of neurons, glia cells, and other cells. Globally, glia cells can be classified as astrocytes, mi-

croglia, and oligodendrocytes [17]. The most important cells influenced by stroke are neurons, microglia, astrocytes, and cerebrovascular endothelial cells [40]. Recently, many *in vitro* studies have elucidated the effects of NSP on these cells. We believe that clarifying the interactions between NSP and these cells will help the NSP mechanisms be understood.

3.2.1. NSP Regulates Cell Death with Various Mechanisms

The key role of apoptosis in ischemic brain injury has been well documented [41, 42]. Thus, apoptosis has been an important index to measure the cytotoxicity of a certain pathogenic factor, whereas suppression of apoptosis has been used to elucidate the neuroprotective effect of a certain therapy. Apoptosis is executed by the proteins in the B cell lymphoma (Bcl) factor family, which are involved in the caspase dependent and/or independent apoptotic pathways [43]. Bcl-2 (anti-apoptotic) and caspase-3 have been widely used as indices to evaluate cell death in stroke [42, 44].

3.2.1.1. Neurotoxicity

Alleviation of the neurotoxicity plays a role in the neuroprotective effects of NSP. Previous studies verified that NSP is effective to alleviate the NMDA-induced neurotoxicity in neurons [7, 13, 14]. NMDA receptors regulate neuronal survival, development of neuronal axons, and dendritic structure, and participate in the formation of synaptic plasticity [45]. Fracture of the NMDA receptor NR1 subunit causes an increase of Ca^{2+} and Ca^{2+}-mediated cell damage. In the brain parenchyma, tPA aggravates NMDA-mediated neurotoxicity *via* cleavage of the NMDA receptor NR1 subunit [45], which can be alleviated by NSP.

3.2.1.2. Oxidative Stress

Oxidative stress plays a deleterious role in ischemic stroke [46]. In the CI state, oxidative stress occurs for enhanced reactive oxygen species and reactive nitrogen species, which results in cellular damage of the proteins, lipids, and DNA [47]. Cheng *et al*. used an H_2O_2-induced neuron model to establish the cellular oxidative stress model. Their results showed that NSP reversed the H_2O_2-induced Bcl-2 reduction and caspase-3 enhancement. Thus, they verified that the NSP-caused reduction in cell death may be associated with attenuation of the influence of oxidative stress [23]. However, there is no study providing direct evidence of NSP using the biomarkers of oxidative stress.

3.2.1.3. Hypoxic Depolarization

Many previous studies have shown that NSP decreases apoptotic cells in the ischemic penumbra. In the hypoxic depolarization of the ischemic brain, tPA is released into extracellular space along with glutamate, then several excitotoxic pathways are involved and finally cause ischemic neuronal death, while depletion of the glutamate may aggravate the cortical infarction and edema [48]. Yepes's study found that neurons secrete NSP in response to the hypoxia depolarization in the ischemic brain, which reduces the infarct and the number of apoptotic cells [4]. Thus, release of NSP might be a compensatory response against hypoxia depolarization, which is considered a mechanism of NSP to attenuate cell death.

3.2.2. NSP Exhibits Neuroprotection of Neurons

Neurons are the key components of the CNS. They can connect *via* dendrites and axons and form a so-called neural network. Neurons and the neural network are the material basis to realize various neurological functions. Likewise, damage or depletion of neurons is the pathophysiological basis of many neurological diseases. Hence, preventing neurons from being damaged by any pathological factor is the most important concept of "neuroprotection."

Rodriguez-Gonzalez's study employed a mixed *in vitro* model including neurons and astrocytes exposed to OGD and tPA. They found that both OGD and tPA exhibited an increase of cellular toxicity. Only LDH release and active MMP-9 were reversed by NSP; the other biomarkers, including caspase-3 activity, were unchanged by NSP. They thus speculated that the neuroprotective effects of NSP were highly related to the cellular toxicity of tPA. However, this experiment could not distinguish if the effects were on neurons or astrocytes [12].

Later, our laboratory solely verified these neuroprotective effects of NSP on neurons. Interestingly, expression of the MAPK pathway-related proteins (extracellular signal-regulated kinases [ERK], p38, and Jun N-terminal kinase [JNK]) was significantly upregulated by OGD and downregulated by NSP, indicating a MAPK-related mechanism involved in the neuroprotective effects of NSP on neurons. However, this study did not involve tPA. We did not know the role of tPA in NSP neuroprotection.

Cheng *et al*. employed an H_2O_2 induced oxidative stress model to observe the neuroprotective effects on primary cultured hippocampal neurons. They found that cell viability and expression of Bcl-2 were significantly reduced by H_2O_2 exposure, whereas cell cytotoxicity, cell death, and expression of caspase-3 were significantly enhanced by H_2O_2 exposure. Administration of NSP can reverse these changes. Thus, the neuroprotective effects of NSP on these neurons in oxidative stress were verified, and they were associated with the Akt-BCL-2 signaling pathway without the involvement of Trk receptors [23].

3.2.3. NSP Exhibits Neuroprotection of Astrocytes

Astrocytes account for 20%-40% of the cells in CNS [49]. They play a crucial role in the survival of neurons [50] and in maintaining the function and integrity of BBB [51] during ischemic stroke. Once ischemic stroke occurs, the astrocytes are activated to protect the ischemic brain. Based on this, we believe the neuroprotective effects on astrocytes are important because the astrocytes greatly contribute to brain protection after stroke. Other than the Rodriguez-Gonzalez study involving neurons and astrocytes, our laboratory was the only one to perform verification of the effects of NSP on astrocytes. We obtained evidence of NSP neuroprotection from ameliorations of the cell survival rate, LDH release, cellular apoptosis, the nuclear morphology, and the cellular morphology. The levels of nitric oxide (NO) and TNF-α were significantly enhanced by OGD and reduced by NSP. Importantly, during the experiments exploring the inflammatory-related mechanisms by using sc3060, an antagonist of the NF-κB signaling pathway, we verified that sup-

pressing the NF-κB signaling pathway plays a key role in the NSP mechanisms on astrocytes. The NF-κB signaling pathway can be directly activated by TNF-α, which subsequently results in NO release by stimulation of the NO synthase [52]. Thus, TNF-α might be a trigger of the NF-κB related inflammatory response on astrocytes, induced by OGD. First, OGD causes the release of TNF-α, which subsequently activates the NF-κB signaling pathway, and finally causes the NO. The entire processes induced by OGD can be significantly reversed by NSP. Interestingly, in the parallel experiments verifying the involvement of the MAPK and PI3K/Akt signaling pathways, we found the expressions of p-ERK1, p-ERK2, and p-Akt were significantly upregulated by OGD but unchanged by administration of NSP. These results indicated low involvement of MAPK and PI3K/Akt signaling pathways in the mechanisms of NSP [16].

3.2.4. NSP Suppresses Activation of Microglia

Microglia are known as the resident macrophages of CNS, and they play a decisive role in active immune defense mechanisms in the CNS. Microglia are very sensitive and are distributed over a large range in the CNS. Normally, microglia are in a "resting" state. However, when they are stimulated by pathological factors such as ischemia, microglia are promptly activated. Many inflammatory-related cytokines and proteins are upregulated, subsequently triggering the anti-inflammatory responses to maintain the stability of the CNS [53, 54].

In terms of the effects of NSP on microglia, 1 study observed the changes of NSP *in vitro* [17], and another study investigated *in vivo* [5]. The *in vitro* study found that the OGD treatment induced morphological changes of microglia and enhanced the release of NO and IL-1β. These changes can be reversed by NSP administration. The changes in p-ERK/ERK, p-P38/P38, and p-JNK/JNK exhibited the same tendency, that is, OGD treatment induced the phosphorylation of the MAPK signaling pathway, which can be suppressed by NSP [17]. However, when only observing the microglia *in vitro* it is difficult to understand the meanings of the microglia changes effected by NSP. Another *in vivo* study employing MCAO mice found that activated microglia congregated at the edge of infarct lesions. Mice with overexpression of NSP achieved 30% smaller infarct volume and milder activation of microglia (*vs*. wild-type). NSP is therefore considered to be able to suppress activation of microglia and thereby exhibit a neuroprotective effect in the experimental CI model [5].

However, the detailed mechanisms are not fully understood. We speculated that during the progression of a stroke, NSP and tPA combine to form a complex, resulting in the decrease of both microglia and the microglia-derived tPA. Thus, we consider that NSP might reduce inflammation by reducing the activation of microglia and play a neuroprotective role in the process of stroke. However, as yet there is no direct evidence showing NSP administration can suppress activation of microglia *in vivo* in an experimental CI model, which is expected in a future study.

Interestingly, the neuroprotective effect of NSP on astrocytes was closely associated with the NF-κB pathway, while hardly related to the MAPK and PI3K/Akt pathways. Yet, for microglia, it was associated with the MAPK pathway. These findings suggested that the suppression of the inflammatory signaling pathway may be a potential mechanism for the neuroprotective effect of NSP; however, different cell types might be associated with different signaling pathways.

There is no study of oligodendrocytes, but one should be planned in the future.

3.3. Other Potential Mechanisms might be Associated with NSP

The previous sections discussed the mechanisms of NSP with direct evidence obtained from stroke subjects (Fig. **1**). The mechanisms of NSP are complicated, and there remain many mechanisms summarized from the diseases other than stroke. Here, we summarize the mechanisms that might be closely associated with stroke, which require verification.

3.3.1. Regulation of the Adaptive Immunity

Suppression of inflammation is the key mechanism involved in NSP's neuroprotection. In the ischemic brain, BBB damage may destroy the encapsulation of the immune environment in the CNS. Thereby, the CNS is also affected by the peripheric immune system [28]. Available evidence indicates that NSP exhibits an anti-inflammatory effect in both CNS and the peripheric immune system. Changes in the extracellular proteolytic environment caused by regulation of plasminogen activity play a crucial role in the regulation of aggregation and proliferation of human T cells. Increasing the plasminogen activity results in the increase of interaction between T cells and T cell proliferation. NSP may regulate the T cell-T cell interactions and proliferation by suppressing the activity of tPA [55]. Thus, NPS may serve as a tPA-mediated regulator to regulate the immune cell function, which promotes the migration of activated dendritic cells to the local lymphoid tissue, and it may stimulate the activation of the T cells by regulating the tPA-mediated extracellular proteolysis [56]. It has been documented that NSP significantly suppressed activation and invasion of inflammatory cells and was associated with T-helper cell differentiation modification after aortic transplantation [57]. However, there is no report investigating the state of adaptive immunity and NSP in stroke brain.

3.3.2. Autophagy

It is known that autophagy can cause cell death, commonly known as type II programmed cell death [58], which is associated with a battery of neurological diseases, including AD, Parkinson's disease (PD), traumatic brain injury (TBI), and spinal cord injury (SCI). Li *et al.* found that NSP treatment ameliorated the BBB state and enhanced the neuron numbers in anterior horn motor in SCI rat models. NSP achieved a reduction of the neuron damage by suppressing the lux of autophagy performance through inhibition of the P62 and LC3-II protein levels and the accumulation of autophagosomes [59].

3.3.3. Regulation of the Amyloid β (Aβ)

Many neurodegenerative diseases, such as AD and PD, are strongly associated with NSP. Accumulation and deposi-

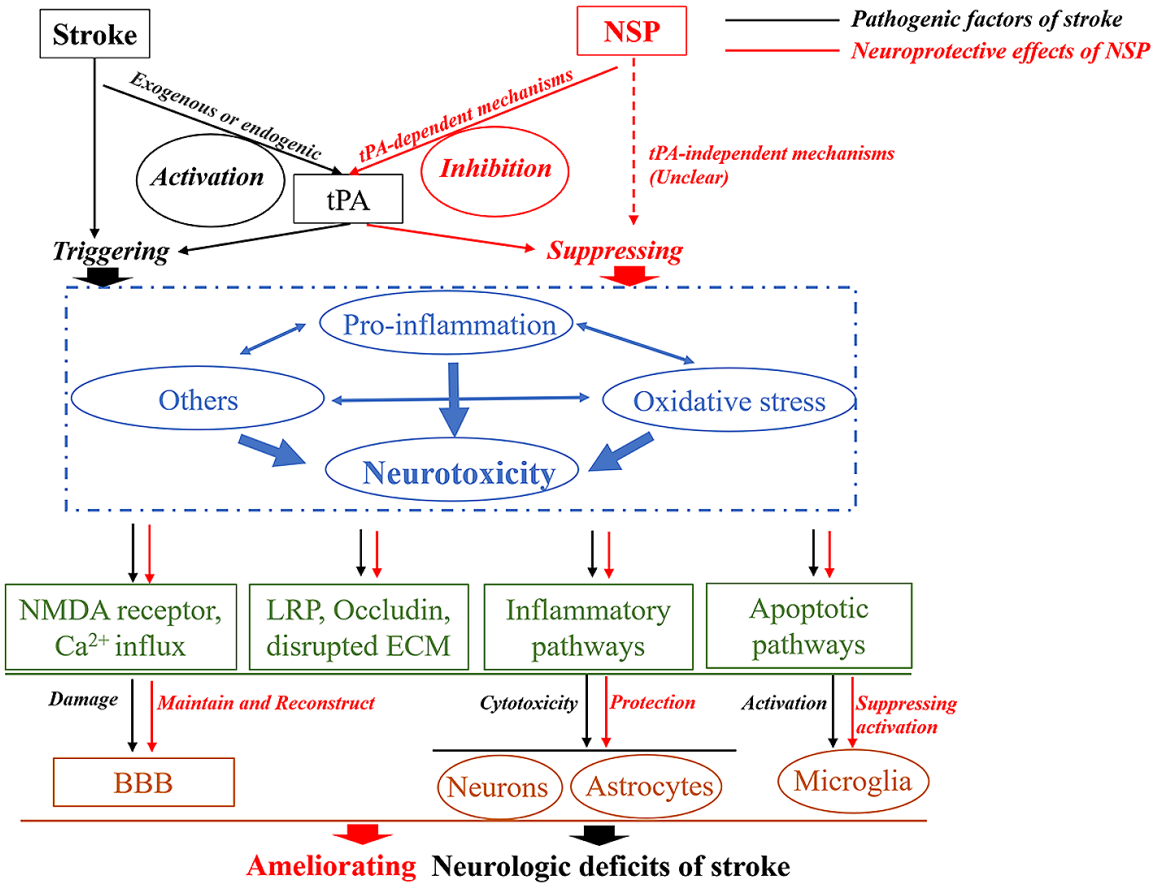

Fig. (1). The potential mechanisms of the neuroprotective effects of neuroserpin (NSP). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

tion of Aβ peptides are pathological hallmarks in AD that trigger a series of processes leading to synaptic dysfunction and neuronal death. It has been reported that NSP contributes to reduce Aβ aggregation and alleviate Aβ toxicity in cell culture; NSP is therefore considered as a potential regulator of the pathogenesis of AD [60]. However, the role of the Aβ-related mechanisms of NSP in stroke remains uncertain.

4. LIMITATIONS AND CONCERNS

This paper focuses on the neuroprotective role of NSP in stroke. However, based on the literature on this topic, many questions cannot be addressed by the available information, although these studies are extremely important for further understanding the principles of NSP. These issues require further discussion and investigation.

4.1. Ischemic *vs*. Hemorrhagic Mechanisms

Most of the studies were based on ischemic brain, with only 1 study of the hemorrhagic brain [18]. CH is another common type of stroke, although there are remarkable differences between CH and CI, including pathophysiology, clinical manifestation, and treatment. CH can be a secondary onset from CI, for example, from an inappropriate intravenous tPA therapy. CI can also be a secondary onset from CH.

CI and CH have a close association: they share the same risk factors and pathogenesis. Involvement of the inflammatory mechanisms, breakdown of BBB, neurological deficits, edema, neuronal death, and so forth, can be found both in CI and CH. In the case of tPA-induced CH, NSP is helpful in reducing the onset of hemorrhage, as NSP is an inhibitor of tPA. However, information on NSP's mechanisms is lacking in the non-tPA-related hemorrhage. Although our previous study demonstrated that NSP reduces brain edema, alleviates BBB leakage, ameliorates neurological deficits, and reconstructs the TJs in the non-tPA hemorrhagic brain [18], the pathophysiologic processes and the detailed mechanisms in the non-tPA hemorrhage remain unclear. For example, in a non-tPA hemorrhagic brain, what is the role of endogenous tPA, and how does it interact with NSP? What is the clinical value in terms of reduction of brain edema and reconstruction of BBB by NSP? More investigations of the non-tPA hemorrhagic brain are therefore expected.

4.2. tPA-dependent *vs*. tPA-independent Neuroprotective Mechanisms

Until now, it has been unclear whether the neuroprotective effects of NSP on stroke are tPA-dependent or tPA-independent. Studies in the early stage verified the mechanisms closely associated with tPA. Certainly tPA, including the endogenous tPA, has adverse effects. It may interact with the NMDA receptor, LRP family, and membrane protein interaction-II and induce neurotoxicity. This results in neuronal death, brain edema, and BBB damage. NSP inhibits

tPA and certainly can alleviate these damages. However, many later reports, particularly some verifications *in vitro*, seem to indicate that there are tPA-independent mechanisms involved in the neuroprotective effects of NSP. In Cheng's study, they found that NSP exhibited neuroprotective effects in the H_2O_2-induced neuron model. However, these effects were not associated with tropomyosin-related kinase (Trk) receptors [23]. tPA activates LRP-1 by transactivation of the Trk receptors and stimulation of Akt activity [61]. These results strongly indicate that NSP presents neuroprotective effects on oxidative stress *via* a tPA-independent mechanism. However, the role of tPA-independent mechanisms in the neuroprotective effects of NSP remains unknown [20]. If the tPA- independent neuroprotective mechanisms can be investigated and evaluated, it will be helpful to understand the potential clinical value of NSP, namely, whether it can be used as an independent neuroprotective agent or only as a tPA inhibitor. Future studies should address this problem using tPA knockout animals.

4.3. How Far is NSP from Actual Clinical Usage?

Although tPA is commonly used to treat acute ischemic stroke within an appropriate therapeutic window, NSP is still far from actual clinical use. Only 3 studies have involved human patients [10, 19, 24]. All of these were "observational studies," investigating the correlation between NSP levels and clinical outcomes. No "intervention study" was carried out to observe the efficacy and safety of direct administration of NSP in patients with stroke. Hence, there is no direct evidence of efficacy and safety of NSP for treating stroke. One animal study in experimental TBI indicated that NSP also exhibited neurotoxicity, such as activation of microglia and astrocytes and deterioration of the neurobehavioral function after TBI [62]. These data indicated that NSP is far from "harmless," and that inappropriate use of NSP may induce adverse events. Chamorro *et al*. reported that although a number of neuroprotectants succeeded with *in vitro* and *in vivo* verifications, they failed to show benefit in randomized controlled trials (RCTs) [46]. It seems there is a gap in the bench-to-beside translation. To promote the clinical usage of NSP for treating stroke, several concerns should be seriously considered: (1) Similar to the failed neuroprotective agents mentioned in Chamorro's study, nowadays almost all *in vivo* studies employ MCAO rodent animals with arteries that are mechanically and reversibly occluded. But this MCAO model has been criticized, for it cannot mimic the natural cause of vascular occlusion in patients with CI [63]. Thus, better animal stroke models, including the non-human primates, need to be involved in the NSP study. (2) Study quality of the experimental studies needs to be improved. A previous study pointed out that many preclinical stroke studies suffered from methodological flaws, such as small sample size and lack of rigorous experimental design [64]. Only when these flaws are addressed in future studies can we obtain convincing evidence regarding the efficacy of NSP in animals. We do not recommend planning the intervention human trial on NSP before convincing evidence is obtained.

CONCLUDING REMARKS

Here, we summarized the existing knowledge and evidence concerning the neuroprotective role of NSP in stroke. Although the available literature and evidence are limited, we believe that inflammation-related mechanisms play a key role in NSP, including maintenance and reconstruction of the structure and function of BBB, protection of the cells in the CNS, and suppression of the cell death in both ischemic and hemorrhagic stroke (Fig. **1**). Available evidence strongly suggests that a tPA-independent mechanism is involved in NSP, which requires further investigation. Nowadays, NSP research on stroke is still in its infancy, and NSP is far from clinical usage. More studies on cerebral hemorrhagic models need to be planned. Studies using more appropriate animal models with rigorous experimental design are expected before human intervention experiments on NSP can be proposed.

LIST OF ABBREVIATIONS

AD	=	Alzheimer's disease
Aβ	=	Amyloid β
BBB	=	Blood-brain barrier
Bcl	=	B cell lymphoma
cFn	=	Cellular fibronectin
CH	=	Cerebral hemorrhage
CI	=	Cerebral ischemia
CNS	=	Central nervous system
ECM	=	Extracellular matrix
ERK	=	Extracellular signal-regulated kinases
ICAM	=	Intercellular adhesion molecule
ICH	=	Intracerebral hemorrhage
IL	=	Interleukin
JNK	=	Jun N-terminal kinase
LDH	=	Lactate dehydrogenase
LRP	=	Receptor-related protein
MAPK	=	Mitogen-activated protein kinase
MCAO	=	Middle cerebral artery occlusion
MCP	=	Membrane cofactor protein
MIP	=	Macrophage inflammatory protein
MMP	=	Matrix metallopeptidase
NF	=	Nuclear factor
NIHSS	=	National Institute of Health Stroke Scale
NMDA	=	N-methyl-D-aspartate
NO	=	Nitric oxide
NSP	=	Neuroserpin
OGD	=	Oxygen-glucose deprivation followed by reoxygenation (OGD/R)
PD	=	Parkinson's disease
PI12	=	Protease inhibitor 12

PI3K	=	Phosphoinositide-3-kinase
RCTs	=	Randomized controlled trials
SCI	=	Spinal cord injury
SNPs	=	Single nucleotide polymorphisms
TBI	=	Traumatic brain injury
TJs	=	Tight junctions
TNF-α	=	Tumor necrosis factor-α
tPA	=	Tissue plasminogen activator
TrK	=	Tropomyosin-related kinase

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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