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Anti-inflammatory role of Fetuin-A in Injury and Infection

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Abstract

Infection and injury are two seemingly unrelated processes that often converge on common innate inflammatory responses mediated by pathogen- or damage-associated molecular patterns (PAMPs or DAMPs). If dysregulated, an excessive inflammation manifested by the overproduction and release of proinflammatory mediators (e.g., TNF, IFN-γ, and HMGB1) may adversely lead to many pathogenic consequences. As a counter-regulatory mechanism, the liver strategically re-prioritizes the synthesis and systemic release of acute phase proteins (APP) including the fetuin-A (also termed alpha-2-HS-glycoprotein for the human homologue). Fetuin-A is divergently regulated by different proinflammatory mediators, and functions as a positive or negative APP in injury and infection. It not only facilitates anti-inflammatory actions of cationic polyamines (e.g., spermine), but also directly inhibits PAMP-induced HMGB1 release by innate immune cells. Peripheral administration of fetuin-A promotes a short-term reduction of cerebral ischemic injury, but confers a long-lasting protection against lethal endotoxemia. Furthermore, delayed administration of fetuin-A rescues mice from lethal sepsis even when the first dose is given 24 hours post the onset of disease. Collectively, these findings have reinforced an essential role for fetuin-A in counter-regulating injury- or infection-elicited inflammatory responses.

Keywords

Innate immune cells; PAMP; DAMP; acute phase protein; HMGB1; endotoxemia; sepsis; cerebral ischemic injury

Infection and injury, seemingly unrelated conditions, converge on a common process - inflammation, which is mediated partly by innate immune cells including macrophages and monocytes. These innate immune cells are equipped with pattern recognition receptors (such as TLR2, TLR4, and TLR9) [1–3] that can recognize both pathogen- and damage-associated molecular patterns (PAMPs, such as endotoxin, and DAMPs, such as HMGB1) [4–7]. In response to various PAMPs or DAMPs, innate immune cells release proinflammatory cytokines (such as TNF, IL-1, IFN-γ or HMGB1) to mount inflammatory responses. If dysregulated, an uncontrolled inflammation may adversely lead to detrimental consequences. In this review, we summarize emerging evidence to support fetuin-A as an acute phase protein capable of attenuating infection- and injury-elicited inflammatory responses.

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1. PATHOGENIC ROLES OF DYSREGULATED INFLAMMATION IN INFECTION AND INJURY

1.1 Bacterial Infection

As a syndrome of dysregulated systemic inflammation to a microbial infection, sepsis is partly attributable to the excessive production of various proinflammatory mediators. In animal models of endotoxemia and bacteremia, overexpression of early proinflammatory cytokines, including TNF [8], interleukin (IL)-1 [9], interferon (IFN)-γ [10], individually or in combination, contribute to the pathogenesis of lethal systemic inflammation (Figure 1). However, the therapeutic windows for these early mediators are relatively narrow, prompting the search for other "late" pro-inflammatory mediators that may offer better therapeutic opportunities. A decade ago, we made the seminal finding that high mobility group box-1 (HMGB1) was released from macrophages or monocytes in response to exogenous PAMPs (e.g., endotoxin or CpG-DNA) [7,11] or endogenous cytokines (e.g., TNF or IFN-γ) [7,12]. In addition, HMGB1 can be passively leaked by necrotic cells [13,14], thereby functioning as a damage-associated molecular pattern (DAMP). Upon binding to the receptor for advanced glycation end products (RAGE), TLR2 or TLR4 [15–17], HMGB1 induces the expression of various cytokines, chemokines, and adhesion molecules [15,16,18–24]. Consequently, extracellular HMGB1 functions as an alarmin signal to alert, recruit and activate innate immune cells [4,25–28], thereby sustaining rigorous and potentially injurious lethal systemic inflammation.

In animal models of endotoxemia and sepsis (induced by cecal ligation and puncture, CLP), circulating HMGB1 increased to plateau levels between 24–36 h [7,29]. This late appearance precedes the onset of animal lethality, and distinguishes HMGB1 from TNF and other early cytokines [30]. The pathogenic role of HMGB1 was inferred from the observations that HMGB1-neutralizing antibodies [7,29,31] and inhibitors (e.g., tanshinones, ethyl pyruvate, nicotine, stearoyl lysophosphatidylcholine, epigallocatechin-3-gallate, nicotine, choline, GTS-21, and spermine) [7,32–40] confer protection against lethal endotoxemia and sepsis, even when the first dose of these antidotes was given 24 h after CLP - a time point when mice had developed clear signs of sepsis. Conversely, administration of exogenous HMGB1 to mice recapitulated clinical manifestations of sepsis, including fever [41], derangement of intestinal barrier function [42] and tissue injury [43–46]. Collectively, these data establish HMGB1 as a critical "late" mediator of sepsis with a wider therapeutic window (Figure 1) [30,47–49].

1.2 Cerebral Ischemic Injury

Cerebral ischemic injury (stroke) consists of two stages: i) primary tissue damage in the ischemic core that is mediated by tissue ion (Ca^{2+} and Na^{+}) overload [50] and excitotoxicity [51]; and ii) secondary tissue injury in the surrounding penumbra that is mediated by proinflammatory cytokines (Figure 2) [52]. Within seconds to minutes after cerebral ischemia, decreased ATP production leads to failure of the Na^{+}/K^{+}-ATPase pump, disruption of membrane potentials, influx of sodium and calcium, and subsequent release of excitatory amino acids (such as glutamate). Engagement of glutamate with the ionotropic N-methyl-D-aspartate receptor (NMDA) leads to Ca^{2+} influx and activation of damaging proteases (e.g., phospholipase A_2, nitric oxide synthase, endonucleases, and calpain) that compromise the functional and structural integrity of neuronal cells within 20–60 minutes. Early-stage therapeutics that block ion (Na^{+} and Ca^{2+}) channels [50] and glutamate receptors [53] have failed in clinical trials, partly because of the impracticalities of administering such drugs in a timely fashion. These failures have prompted the search for other downstream targets that also contribute to the pathogenesis of ischemic injury.

Outside of the ischemic core where cells are destined to die are penumbral zones where brain cell death continues slowly for hours and even days after the onset of ischemia. This progressive expansion of cell death in the penumbra (i.e., secondary injury) is mediated by ischemia-elicited inflammatory responses that are orchestrated by both centrally- and peripherally-derived immune cells (Figure 2). For instance, microglia and neurons become activated to produce TNF and other cytokines within a few hours [54,55]. Subsequently, polymorphonuclear cells infiltrate into the ischemic brain tissue within 12–48 hours [56], followed by an influx of monocytes and macrophages over a period of one to several days. Many pro-inflammatory cytokines (e.g., TNF and IL-1) contribute to cerebral ischemic injury [57,58], because inhibition of their production [59,60] or activity [59,61,62] confers protection against cerebral ischemic injury. In addition, HMGB1, can be passively released from the ischemic core, and spilled into the surrounding periphery [63]. In the penumbra, it may amplify a potentially injurious inflammatory response by inducing various cytokines, chemokines, tissue factor and adhesion molecules (Figure 2) [4,22,23]. Indeed, HMGB1-specific neutralizing antibodies and antagonists (e.g., the A box) have been proven protective [64–66], supporting a pathogenic role for HMGB1 in ischemic injury.

2. ENDOGENOUS ANTI-INFLAMMATORY MECHANISMS

Mammals have evolved multiple anti-inflammatory mechanisms to counter-regulate potentially injurious inflammatory responses. For instance, the central nervous system can directly and rapidly attenuate bacterial endotoxin-induced release of TNF through efferent vagus nerve signals to tissue-resident T cells [67] and macrophages [68]. This effect is mediated by acetylcholine, the principle neurotransmitter of the vagus nerve, via nicotinic cholinergic receptors (such as the alpha-7 nAChR) [36,68,69]. Physical (electrical or mechanical) stimulation of the vagus nerve [36,70] reduced serum HMGB1 levels, and consequently improved survival in animal models of sepsis [70]. Similarly, a number of cholinergic chemical agonists (including nicotine, choline, GTS-21, and PHA568487) also conferred protection against lethal endotoxemia, bacteremia, and sepsis partly by attenuating HMGB1 release [36,38,39,71]. Taken together, these findings have suggested an important role of the central nervous system in the counter-regulation of peripheral inflammatory response.

At the sites of infection, various PAMPs also induce the production of many anti-inflammatory cytokines (e.g. IL-10, IL-4 and TGF-β), which participate in the down-regulation of the local inflammatory response [72–74]. As another local counter-regulatory mechanism, a ubiquitous biogenic molecule, spermine, can be passively released by injured cells [75], and thus accumulates at the sites of infection or injury. It effectively attenuates the synthesis and release of various pro-inflammatory cytokines (e.g. TNF, IL-1, MIP-1) from activated macrophages and monocytes [76–79]. Furthemore, the anti-inflammatory effects of spermine are dependent upon the availability of a ubiquitous protein, fetuin-A. That is, spermine fails to inactivate macrophage/monocytes if these cells are deprived of fetuin-A by serum-starvation, or addition of specific fetuin-A-neutralizing antibodies [80]. In contrast, co-addition of highly purified fetuin-A significantly enhances the anti-inflammatory activity of spermine [80], supporting an important role of fetuin-A in the regulation of the innate immune responses [76,79,81].

3. FETUIN-A AS AN ACUTE PHASE PROTEIN (APP)

In response to infection and injury, the liver strategically re-prioritizes the synthesis and systemic release of a group of proteins collectively termed “acute phase proteins” (APPs), whose plasma concentrations are increased (“positive APPs”) or decreased (“negative APPs”) during inflammation. For instance, fetuin-A, also known as the alpha-2-HS-

glycoprotein for the human homologue [82], was first characterized as a major plasma protein in the fetus [83]. During fetal development, it is expressed in many organs such as the liver, kidney, gastrointestinal tract, skin and brain [84–87]. In adults however, fetuin-A is produced primarily by the liver, and its synthesis is divergently regulated in response to injury or infection, classifying it as a negative or positive APP.

3.1 Fetuin-A as a Negative APP in Infection

The hepatic expression of fetuin-A is negatively regulated by several proinflammatory cytokines such as TNF, IL-1, IL-6 and IFN-γ (Figure 1) [88,89]. For instance, at concentrations as low as 10–50 ng/ml, IFN-γ reduced fetuin-A expression levels by > 50–70% in human hepatoma HepG2 cells [89]. In contrast, HMGB1 (1 μg/ml) elevated hepatic fetuin-A expression levels by 2–3 folds, suggesting that different cytokines divergently regulate hepatic fetuin-A expression (Figure 1). In animal models of endotoxemia and sepsis (induced by cecal ligation and puncture, CLP), circulating fetuin-A levels were decreased in a time-dependent fashion, starting between 2–6 h, reaching a nadir (with maximal reduction by 50–60%) around 24–48 h. Afterwards, fetuin-A levels started to increase, returning towards basal levels approximately 72 h post endotoxemia or sepsis, supporting fetuin-A as a negative APP in animal models of lethal endotoxemia and sepsis [89]. In agreement with the capacities of early proinflammatory cytokines (TNF, IL-6, and IFN-γ) in inhibiting fetuin-A expression [88,89], we found that the genetic disruption of IFN-γ expression led to an impairment of endotoxin-mediated down-regulation of fetuin-A expression [89]. It is thus possible that early cytokines (such as TNF and IFN-γ) negatively reduce circulating fetuin-A levels during an early stage of endotoxemia or sepsis; whereas late-acting mediators (e.g., HMGB1) serve as a positive regulator to restore circulating fetuin-A levels at a late stage of these inflammatory diseases.

In patients with other inflammatory diseases such as pancreatitis [90], chronic kidney diseases [91], and rheumatoid arthritis [92], serum fetuin A levels were also decreased by 20–30%. In these patients, circulating fetuin-A levels negatively correlated with levels of cytokines (such as IL-6) [90], and associated with increased mortality rates [91]. Collectively, these observations classify fetuin-A as a negative APP during infection or other inflammatory illness.

3.2. Fetuin-A as a Positive APP in Injury

Clinically, plasma fetuin-A levels were paradoxically elevated in patients with cerebral ischemic injury (stroke) [93,94]. The magnitude of fetuin-A elevation positively correlated with an increase in LDL-cholesterol levels and risk of cardiovascular disorders [93]. Following traumatic injury, serum fetuin-A levels were increased up to 10-folds in cattle [95], suggesting fetuin-A as a positive APP in response to injury. In light of the findings that HMGB1 can be passively leaked from injured cells [96] and function as an early mediator of traumatic injury [97–101], it is plausible that HMGB1 may contribute to the up-regulation of hepatic fetuin-A expression during injury.

In an animal model of focal cerebral ischemia (i.e., permanent middle cerebral artery occlusion, MCAo), fetuin-A levels in the ischemic brain tissue were elevated in a time-dependent manner, starting between 2–6 h, peaking around 24–48 h, and returning towards baseline at 72 h post MCAo [102]. This dynamic increase in cerebral fetuin-A levels parallels with the transient elevation of the blood-brain barrier (BBB) permeability [103], suggesting that circulating fetuin-A can gain entry across the BBB into the ischemic brain tissue (Figure 2). This possibility was supported by the observation that peripherally (intravenously) administered FITC-labeled fetuin-A destined to the ischemic brain region at 24 h after MCAo [102].

4. FETUIN-A AS AN ANTI-INFLAMMATORY PROTEIN

Despite its abundance, the functions of fetuin-A remain poorly understood. A wide range of biological functions have been proposed for fetuin-A based on its structural similarities to other proteins or physical interactions with biogenic molecules. For instance, fetuin-A shares amino acid sequence homology to type II TGF-β receptors [104], and has been proposed as an inhibitor of the TGF-β signaling pathway. Similarly, fetuin-A exhibits amino acid sequence similarity to insulin receptor tyrosine kinases [105,106], and can bind to the insulin receptor, thereby inactivating (rather than activating, as in the case for insulin) the receptor tyrosine kinase [107]. This may partly explain why higher fetuin-A levels were associated with insulin resistance in some patients with type 2 diabetes [108]. As a glycoprotein, fetuin-A carries two N-linked and three O-linked oligosaccharide chains that terminate with sialic acid residues, enabling the binding of cationic Ca^{2+} ions. Accordingly, fetuin-A has been proposed as an endogenous inhibitor of pathological mineralization or calcification in soft tissues [109–111]. Specifically, fetuin-A forms protein-mineral colloids with calcium and phosphate [112–114], thereby preventing uncontrolled mineralization that may otherwise occur under pathological conditions [115].

As aforementioned, fetuin-A also functions as an opsonin for cationic spermine, and its availability to immune cells may be critical for regulating the innate immune response [81]. Indeed, levels of fetuin-A in macrophage cultures was decreased by 40% after stimulation with LPS (100 ng/ml, 2 h). Supplementation of LPS-stimulated macrophages with fetuin-A (100 μg/ml) conversely elevated cellular fetuin-A levels by 30–50% [116], confirming the notion that macrophages can ‘adopt” fetuin-A from the environment [81]. Intriguingly, exogenously administered fetuin-A was predominantly localized in LC3-containing cytoplasmic vesicles - possibly autophagosomes or amphisomes - in LPS-stimulated macrophages [116]. At higher concentrations (e.g., 3.5 mg/ml), even crude fetuin-A (> 98%) can almost completely abrogated endotoxin-induced release of IL-1 and nitric oxide in macrophage cultures [117]. Following gel filtration and ion-exchange chromatography, the highly purified fetuin-A almost completely abrogated IFN-γ- or LPS-induced HMGB1 release even when given at relative lower doses (e.g., 100 μg/ml) [89], suggesting fetuin-A as an effective anti-inflammatory APP.

5. THERAPEUTIC POTENTIAL OF ANTI-INFLAMMATORY AGENTS

Given the anti-inflammatory properties of spermine and fetuin-A, we have evaluated their therapeutic potential in several animal models of local and systemic inflammation.

5.1 Carrageenan-induced Paw Edema

Local administration of spermine directly into the carrageenan-injected paw dose-dependently inhibited the development of edema, with a maximally reduction of footpad swelling by ~50% [76]. Similarly, intraperitoneal administration of fetuin-A (5 to 500 mg/kg) dose-dependently attenuated the development of paw edema [118]. When the sialic acid residues were removed by neuraminidase, the resultant asialofetuin-A failed to potentiate the anti-inflammatory activities of spermine [80], and similarly failed to attenuate carrageenan-induced TNF production *in vivo* [118], suggesting the requirement of sialic acid moieties for its anti-inflammatory activities. In contrast, administration of fetuin-A-neutralizing antibodies abolished fetuin-A-mediated inhibition of paw edema, indicating an essential role of fetuin-A in counter-regulating inflammatory responses.

5.2 Cerebral Ischemic Injury

During cerebral ischemic injury, spermine may be protective by inhibiting the expression of proinflammatory cytokines [28,76,77,79,119,120] and scavenging cytotoxic free radicals

(Figure 3) [121,122]. At higher (millimolar) concentrations, spermine is also neuroprotective by directly binding and blocking the NMDA receptor [123,124]. On the other hand, spermine can be enzymatically converted by polyamine oxidases into cytotoxic metabolites (e.g., 3-aminopropanal) [125], which readily spreads and mediates direct cytotoxicities [125]. Furthermore, at low (micromolar) concentrations, spermine activates the NMDA receptor [124,126], thereby augmenting glutamate-mediated neurotoxicity by overactivating Ca^{2+} fluxes and disturbances of the calcium homeostasis (Figure 3). During cerebral ischemia, brain spermine levels are decreased [127], owing largely to an accompanying increase in the enzymatic activity of brain polyamine oxidase [125]. The loss of spermine consequently tilts the balance towards neurotoxicity through activating the NMDA receptor, and increasing susceptibility to oxidative stress as well as excessive inflammatory response.

As mentioned earlier, cerebral ischemia induces a transient (5–24 h) elevation of the BBB permeability [103], which allows temporal entry of circulating fetuin-A across the BBB into the ischemic brain tissue. Consistently, peripheral administration of fetuin-A promoted a short-term protection against cerebral ischemic injury [102]. Given the aforementioned pathogenic roles of Ca^{2+} and spermine in cerebral ischemia, as well as the capacity of fetuin-A in binding Ca^{2+} and spermine [80,112], it is also possible that fetuin-A confers protection by caging these toxic cationic molecules [51,125], thereby depriving them from damaging enzymes (such as Ca^{2+}-dependent proteases and polyamine oxidase). Furthermore, the fetuin-A-mediated protection is associated with a reduction of ischemia-elicited HMGB1 leakage from the ischemic core, and an inhibition of expression of proinflammatory cytokines (e.g., TNF) in the penumbra (Figure 2) [102], suggesting that fetuin-A confers protection partly by attenuating early inflammatory responses. The fetuin-A-mediated neuroprotection was not long-lasting, and gradually diminished at a later stage (e.g., 7 days post MCAo). It is possible that the restore of BBB function at a late stage (3 days after MCAo) limits subsequent fetuin-A extravasation, thereby diminishing fetuin-A-mediated long-lasting protective effects.

5.3 Endotoxemia and Sepsis

Despite the anti-inflammatory activities of spermine *in vitro* [76,128], spermine did not confer protection against lethal endotoxemia. However, it promoted a dose-dependent protection against lethal sepsis when given at relative lower doses (1 – 10 mg/kg). This protection was associated with a significant attenuation of systemic accumulation of HMGB1 and other cytokines (e.g., IL-6, KC, MCP-1, MIP-2, TIMP-1, sTNFRI and sTNFRII) [128]. At a higher dose (100 mg/kg), however, spermine decreased animal survival rates from 58% to 38% at 48 h post CLP, and further decreasing it to 0% at 72 h post CLP. It is not yet known whether spermine is enzymatically converted by polyamine oxidases into cytotoxic metabolites (e.g., 3-aminopropanal), thereby exerting these potentially toxic effects.

In contrast to the limited efficacy of spermine, fetuin-A exhibits a greater therapeutic potential in animal models of lethal systemic inflammatory diseases. Administration of fetuin-A (20–100 mg/kg) provided a dose-dependent protection against lethal endotoxemia. Furthermore, delayed administration of fetuin-A (20 – 100 mg/kg), beginning 24 h *after* the onset of sepsis and followed by an additional dose at 48 h post CLP, significantly increased long-term animal survival rates from 45% to 90% [89]. The integral role of fetuin-A in host defense against lethal systemic inflammation was supported by the observations that fetuin-A-deficient C57BL/6J mice were more susceptible to lethal endotoxemic or septic insult than sex- and body-matched (male, 27–29 g) wild-type C57BL/6J mice [102].

It now appears that fetuin-A serves as a negative regulator of HMGB1 release during lethal endotoxemia or sepsis (Figure 1). On one hand, the time-dependent decrease of circulating

fetuin-A levels was paralleled by the contrast increase of serum HMGB1 levels in animal models of endotoxemia [7] or sepsis [29]. On the other hand, disruption of fetuin-A expression led to greater elevation of serum HMGB1 levels [89]; whereas supplementation of fetuin-A resulted in significant protection by reducing circulating HMGB1 levels [89]. The mechanisms underlying fetuin-A-mediated suppression of HMGB1 release remains poorly understood. At the concentrations (100 μg/ml) that fetuin-A attenuated LPS-induced HMGB1 release, fetuin-A stimulated autophagy and impaired LPS-induced elevation of cytoplasmic and nuclear HMGB1 levels [89]. It is not yet known whether fetuin-A, like other HMGB1 inhibitors (such as Green tea epigallocatechin gallate, EGCG) [129], reduces cytoplasmic HMGB1 levels by stimulating its degradation in an autophagy-dependent fashion. In addition, fetuin-A may confer these protective effects through other alternative mechanisms. For instance, fetuin-A may be capable of binding bacteria [130,131], thereby affecting macrophage-mediated pathogen elimination. Furthermore, fetuin-A may facilitate macrophage-mediated ingestion and elimination of apoptotic neutrophils [132,133], thereby preventing secondary necrosis and passive leakage of injurious molecules (e.g., proteases, reactive oxygen species, and HMGB1) [134].

6. CONCLUSIONS

The hepatic fetuin-A expression may be divergently regulated by different proinflammatory mediators – inhibited by TNF and IFN-γ, but stimulated by HMGB1. As a positive or negative APP, fetuin-A counter-regulates both injury- and infection-elicited inflammatory responses. Consistent with the transient changes of the blood-brain barrier permeability, fetuin-A gains temporal entry into the ischemic brain tissue, and confers a short-term neuroprotective effects. In contrast, administration of fetuin-A confers a dose-dependent and long-lasting protection against lethal systemic inflammatory diseases. It is thus important to further explore its therapeutic potential for the clinical management of sepsis and other inflammatory diseases.

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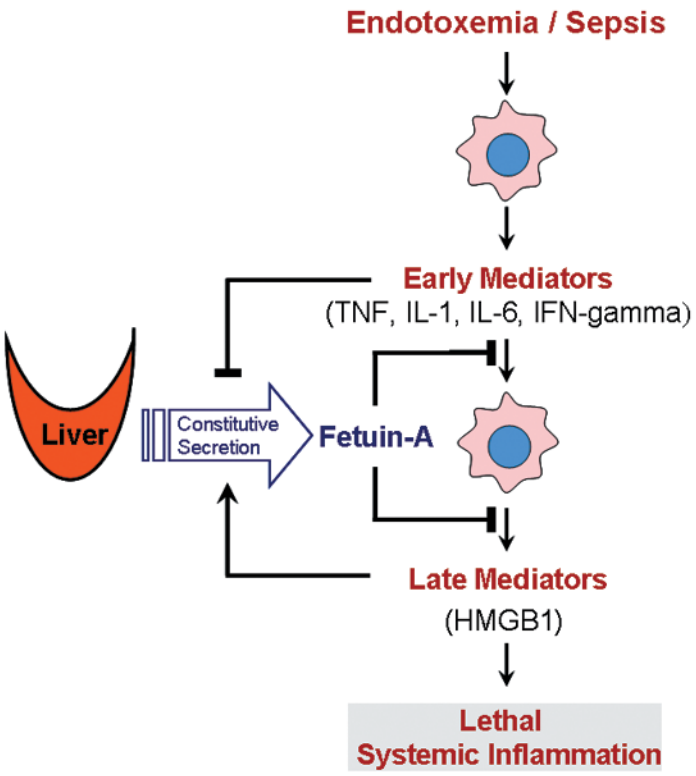

Figure 1. Protective roles of fetuin-A in endotoxemia and sepsis

In response to lethal endotoxemia or sepsis, innate immune cells (such as macrophages) sequentially release early (e.g., TNF and IFN-γ) and late (e.g., HMGB1) proinflammatory mediators. Early proinflammatory cytokines participate in the down-regulation of hepatic fetuin-A expression, allowing propagation of a rigorous inflammatory response manifested by excess accumulation of late proinflammatory mediators (such as HMGB1). On the other hand, HMGB1 stimulates hepatic fetuin-A expression, thereby restoring circulating fetuin-A levels during a late stage of lethal endotoxemia and sepsis. Fetuin-A functions as a negative regulator of the innate immune response by inhibiting LPS- or IFN-γ-induced HMGB1 release in macrophages. Adapted from doi:10.1371/journal.pone.0016945.g006 with granted permission from the publisher.

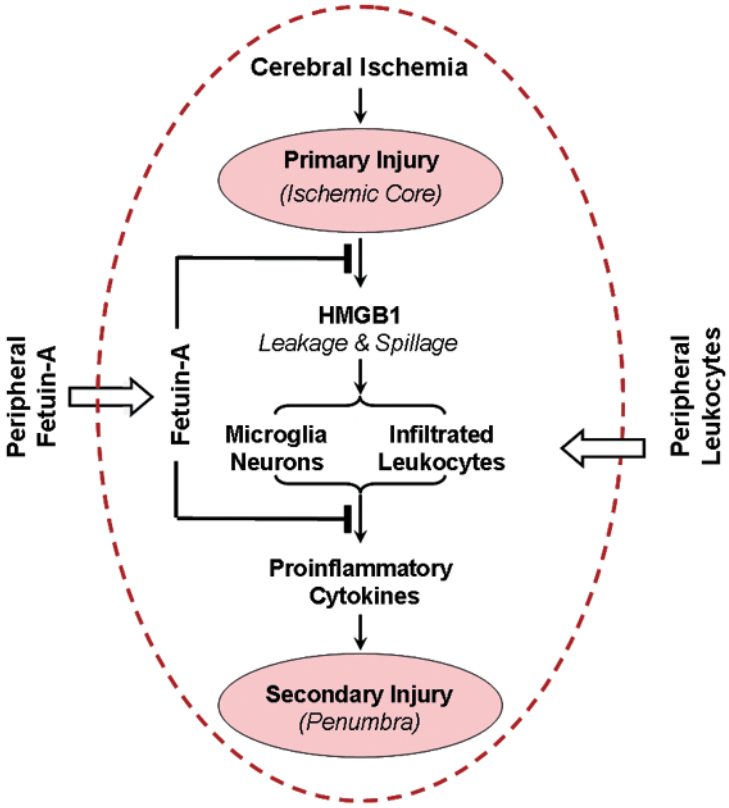

Figure 2. Protective roles of fetuin-A in cerebral ischemic injury

Cerebral ischemia causes rapid primary injury in the ischemic core, leading to HMGB1 release/leakage. Extracellular HMGB1 then diffuses into the periphery region, where it orchestrates a rigorous inflammatory response driven both by the centrally- and peripherally-derived cells. In parallel, cerebral ischemia induces transient increase in blood-brain barrier permeability, allowing entry of circulating proteins (e.g., fetuin-A) and peripheral immune cells (such as macrophage/monocytes). Peripheral administration of fetuin-A attenuates ischemia-elicited HMGB1 release and subsequent cytokine expression, thereby conferring a temporal protection against cerebral ischemic injury.

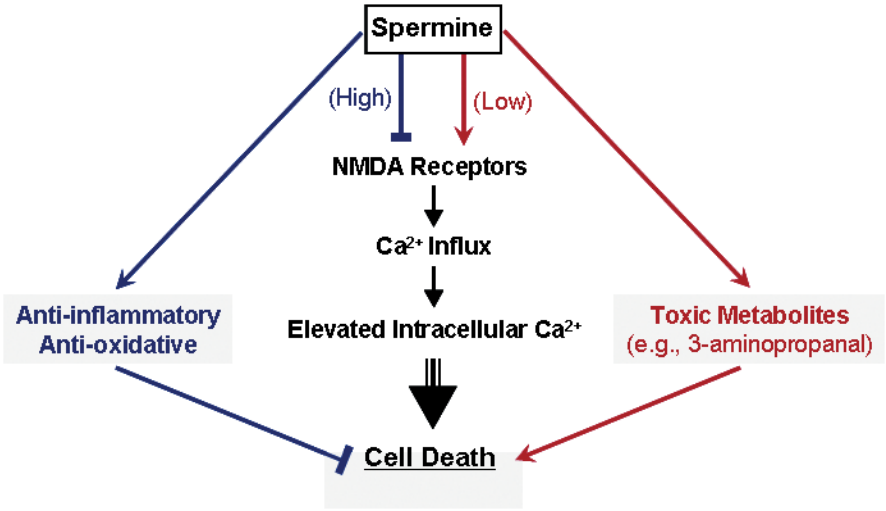

Figure 3. Divergent roles of spermine in cerebral ischemic injury