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Regulation of pathological blood brain barrier for intracranial enhanced drug delivery and anti-glioblastoma therapeutics

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Abstract

The blood brain barrier (BBB) is an essential component in regulating and maintaining the homeostatic microenvironment of the central nervous system (CNS). During the occurrence and development of glioblastoma (GBM), BBB is pathologically disrupted with markedly increased permeability. Due to the obstruction imposed by the BBB, strategies currently employed for GBM therapeutics still obtain a very low success rate and lead to systemic toxicity. Moreover, chemotherapy could promote pathological BBB functional restoration, accompanied with a prominent decrease of intracerebral therapeutics transportion during GBM multiple administration, resulting in chemotherapy failure for GBM treatment. The effective delivery of therapeutics into the brain still faces with severe challenges. Regulation of pathological BBB for enhanced transporting of therapeutics across the barrier may provide new opportunities for effective and safe treatment of GBM. This article reviews the structure and function of BBB in physiological state, the mechanisms underlying BBB pathological fenestration during the development of GBM, and the therapeutic strategies of GBM based on BBB intervention and therapeutic drugs transporting across the BBB.

Keywords: blood brain barrier; physiological; pathological; glioblastoma; intervention

1. Introduction

The main cause for the failure of tumor chemotherapy is the lack of effective drug delivery in the lesion site, which is still a serious challenge for the treatment of glioblastoma (GBM) [1, 2]. The use of low specificity and high cytotoxicity therapeutic agents, their side effects and plasma concentration fluctuations increase the possibility of tumor resistance. The blood brain

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barrier (BBB) is a specialized endothelial structure that is partially covered by pericytes and almost completely surrounded by the end feet of astrocytes, which maintains the normal physiological function of brain and the balance of central nervous systems (CNS) environment [3, 4]. In addition to some special transport channels, almost 100% biological macromolecules and 98% small molecules cannot cross the BBB into the brain, severely limiting the efficacy of therapeutic agents to brain diseases, including GBM [5]. Therefore, how to effectively improve the transport efficiency of therapeutics into the brain is the key scientific problem to be urgently solved for the treatment of GBM.

Recently, extensive and important progress has been made in GBM therapy, including brain receptor-mediated targeted therapy [6], focused ultrasound opening the BBB [7] and Borneol for "orifice-opening" of the BBB [8]. Among them, the preferred strategy for GBM-targeted therapy is receptor-mediated intracerebral transport which shares characteristics of high specificity, affinity and selectivity [9, 10]. Since so many kinds of receptors, including insulin receptors (IR) [11], low density lipoprotein receptors (LDLR) [12], transferrin receptors (TfR) [13] and nicotinic acetylcholine receptors (nAChRs) [14] are overexpressed on the BBB, their ligands are usually employed to facilitate receptor-mediated intracerebral delivery of anti-GBM therapeutic drugs. However, the success rate of GBM treatment is still very low, and new therapies are still needed to effectively treat GBM. So far, studies on brain targeted drug delivery systems have mostly focused on the mutual effect between ligands and their receptors, as well as targeted cells and targeted tissues, while ignoring the functional changes of the BBB in the development and treatment periods of brain diseases. In addition, druggability of the vast majority of brain targeted drug delivery systems limited their clinical application. Therefore, there is still a long way to find safe and effective ways to treat brain diseases, and anti-GBM therapy still need the discovery of new mechanisms, the breakthrough of new technologies and the emergence of new therapies.

The pathological process of GBM is likely to be closely related to BBB, which might also play a vital role in the occurrence and treatment of GBM [15]. Under physiological conditions, BBB is a biological membrane barrier composed of brain microvascular endothelial cells and their tight junctions (TJs), basement membranes and the end feet of glial cells around capillaries [16]. TJs structure is an important morphological basis of the BBB, forming the low permeability of the barrier and its high resistance properties [17]. Under pathological conditions, the occurrences of brain diseases [18-21], including GBM, Parkinson's disease (PD), cerebral ischemia and Alzheimer's disease (AD) are usually accompanied by pathological impairment of BBB, with decreased TJs proteins and increased BBB permeability.

Studies has confirmed that BBB pathological fenestration provides an opening paracellular pathway for the transport of therapeutics across the BBB [22]. Nevertheless, during multiple dosing treatments for GBM, first administration with brain targeted nanotherapeutics would cause pathological BBB restoration, then during re-administration, the delivery of therapeutic drugs into brain is restricted, resulting in poor anti-GBM effect. Thus, reversibly re-opening function restored pathological BBB to increase intracranial delivery of therapeutics could ultimately lead to an observable and significant enhancement of anti-GBM effect [23]. Hence, Regulation of pathological BBB for enhanced therapeutics transporting across the BBB may provide new opportunities for GBM effective treatment.

2. Physiological Characteristics of the BBB

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BBB is the distinct structural difference between the peripheral and cerebral vasculatures, and the barrier achieves CNS homeostasis by strictly regulating molecules transport between brain and blood [24]. The BBB is formed by brain endothelial cells and is characterized by extensive windowless TJs, as well as cytoskeleton and adherens junctions (AJs) (Figure 1A).

TJs is distributed at the apex of cerebral endothelia cells, and consist of a series of paralleled and interconnected chains of transmembrane and cytoplasmic proteins that resemble an intricate network [25]. It is often thought that the closed endothelial fissures of the TJs allow the continued formation of vascular structures. The molecular biology of the TJs is quite complicated, and there are mainly three proteins in TJs including Claudins, Occludin and ZO proteins. Claudins homotypically bind to other claudin molecules in adjacent cerebral endothelial cells, generating the primary seal of TJs [26, 27]. Of Claudins, Claudin-5 is usually considered as a key marker of TJs breakdown and BBB pathological fenestration. The growth of GBM reduces expression and alters distribution of Claudin-5, which in turn lead to BBB functional disruption [28]. Occludin is not necessary in TJs formation, and its function presents mainly to be an additional support structure for TJs regulation as indicated in the knockdown and knockout experiments [29, 30]. The normal expression and localization of other junctional proteins, such as vascular endothelial cadherin, are well compensated for the loss of Occludin [31]. Submembranous TJ-related proteins, also known as peripheral occlusive domain proteins, like ZO proteins, are another component of TJs. They might be in involved in forming scaffolds that connect TJs and cytoskeleton [32, 33]. Moreover, other junctional adhesion molecules present in brain endothelial cells, including JAM-A, JAM-B and JAM-C, are also involved in the formation and maintenance of TJs [17, 34], as well as in the establishment of cell polarity and leukocytes migrating across endothelial junctions [16]. In addition, the brain endothelial cytoskeleton, composing of three critical elements: actin microfilaments, intermediate filaments and microtubules, is also vitally important in the establishment of interendothelial junctional integrity [35].

AJs, are distributed beneath the TJs in the basal region of the lateral plasma membrane, consist of transmembrane glycoproteins connecting cytoplasmic proteins to cytoskeleton, and exerts additional contractile structures between endothelial cells adjacent to the BBB [32]. In addition, AJs are also involved in mediating brain endothelial cells interactions, initiating cell polarity and the regulating paracellular permeability [36].

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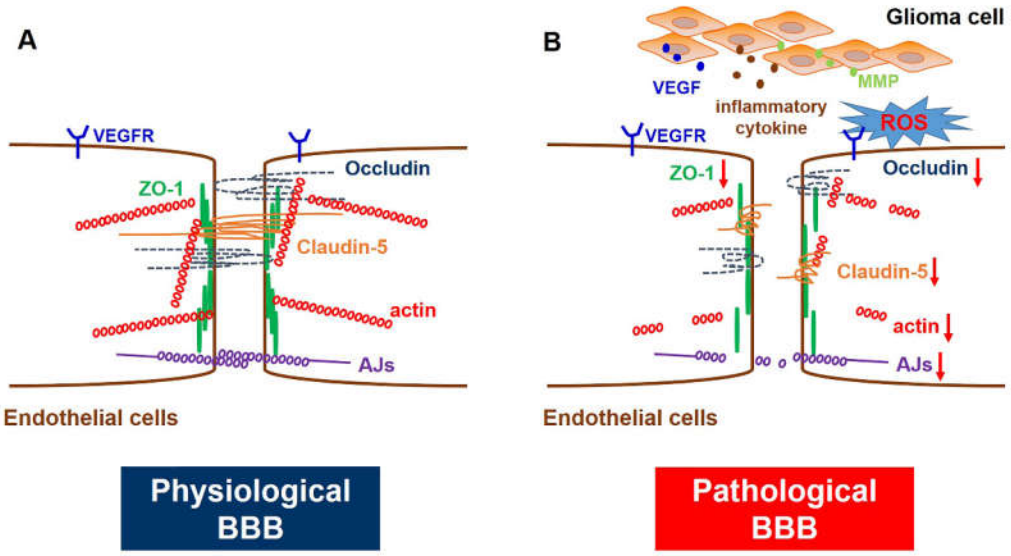

Figure 1. BBB structure under physiological and pathological conditions. (A) Under physiological condition, the BBB is formed by specialized endothelial cells that are characterized by no fenestration and extensive TJs, transmembrane proteins of TJs mainly include Claudins, Occludin and ZO proteins, and AJs are normally linked to the actin cytoskeleton. (B) GBM growth accompanies with abnormal increase of VEGF, inflammatory cytokine, ROS and MMPs, which seriously cause BBB functional disruption and pathological fenestration, with down-regulation of TJs and AJs proteins, and damage of cytoskeleton.

3. Mechanisms of BBB Pathological Disruption in GBM

BBB breakdown is generally considered detrimental in most cases because it lead to an influx of partial blood components, including leukocytes, potentially neuroactive compounds, and water (edema). The leakage of harmful substances into the brain would also accelerate the deterioration of the disease, and finally leading to poor prognosis of the diseases. Abnormal increase of vascular endothelial growth factor (VEGF), reactive oxygen species (ROS), inflammatory cytokines and matrix metalloproteinases (MMPs) during the growth period of GBM, which seriously leads to the disruption of BBB, accompanied with down-regulation of TJs and AJs proteins, as well as the damage of cytoskeleton (Figure 1B).

3.1. Vascular Endothelial Growth Factor

Recent data indicated that VEGF, an angiogenic growth factor known to be produced at an abnormally elevated level in GBM, promoted the down-regulation of TJs proteins, resulting in disruption of BBB function and increased endothelial permeability [37, 38]. Moreover, exogenous VEGF treatment may also induces TJs structural fracture and Occludin trafficking, accompanied with Occludin phosphorylation, finally resulting in vascular impairment by TJs trafficking [39].Yang and colleagues indicated that VEGF can increase the permeability of BBB in GBM by a paracellular pathway (down-regulation of Claudin-5 and Occludin) and a transcellular pathway (up-regulation of caveolin-1 and caveolin-2) [40]. Inhibition of VEGF/VEGFR signaling pathway promotes restoration of pathological BBB, accompanied by increased expression of TJs, including Claudin-5 and Occludin, as well as cytoskeletal remodeling [23, 41].

3.2. Oxidative Stress

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Proinflammatory status, causing oxidative stress and up-regulated endothelial expression of cell adhesion molecules. ROS are generally overexpressed in tumor cells, including GBM cells, when compared to healthy cells. Studies have shown that the VEGF secreted by GBM can activate its downstream signaling pathway, with an increased intric oxide (NO) release and production of ROS, which finally leads to the pathological disruption of BBB and the development of brain edema [42, 43]. Elizabeth and colleagues proposed that the generation of ROS could enhance the tyrosine phosphorylation of VE-cadherin and β-catenin, which ultimately influences AJs integrity [44]. Gerty and colleagues further demonstrated that ROS regulates BBB integrity, accompanied by rearrangement of cytoskeleton and disappearance of TJs proteins. And this process involves specific signaling pathways, including RhoA, PI3K kinase and PKB signaling [45].

3.3. Inflammation

In the regulation of BBB permeability, inflammatory mediators are known modulators. As such, in most neuroinflammatory disease states, including GBM, compromised TJs is an obvious hallmark [46]. Magnetic resonance imaging (MRI) has fully verified that BBB disruption is associated with the development of vasogenic edema in the patients with intracranial tumor [47]. Consistently, studies based on GBM-bearing mice model indicate a down-expressed of TJs proteins including Claudin-5, Occludin and ZO-1, and these proteins that may be lost in the microvasculature mediated by cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), interferon-γ and NF-κB [48, 49].

3.4. Matrix Metalloproteinases

As previously mentioned, increased levels of MMPs have also been observed in GBM, accompanied with disrupted basal lamina proteins and degraded TJs complexes. Zinc-containing enzymes MMPs mediates the degradation of protein substrates mainly via Zn^{2+}-mediated activation of site-bound water molecule. The accumulation of zinc in microvessels of GBM could activate MMP-2 and MMP-9, producing the remodeling and degradation of TJs [50, 51]. Suppression of MMP-2/9 using SB-3CT could partially alleviate the disruption of TJs [52].

In conclusion, the pathological process of GBM causes pathological fenestration of BBB, which is rooted in the destruction of TJs structure. Therefore, signaling pathways in which TJs proteins (Occludin, Claudins and ZO) involved may be potential targets for BBB intervention in the treatment of GBM.

4. Targeting Pathological BBB for GBM Therapy

4.1. Active Targeting

In general, water-soluble molecules are transported across the BBB by simple diffusion through the paracellular pathway [53]. Small-molecule of lipid-soluble substances such as steroids and ethanol are transported into the brain through the transcellular diffusion pathway. In addition, the transport of substances across the BBB also includes absorptive-mediated transport (AMT), carrier-mediated transport (CMT), receptor-mediated transport (RMT), active efflux transport, and cell-mediated transport [54, 55]. Among them, AMT has poor selectivity and cationic protein can bind to any negatively charged cell membrane component. In addition, the use of cationic proteins may produce potential toxicity and immunogenicity. The transport of CMT is substrate selective, and only drug molecules structurally similar to endogenous carrier substrates can be captured and transported into the brain. Previous studies have confirmed that in anti-GBM therapy, RMT is considered to be one of the most mature

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strategies for intracerebral drug delivery [56-58]. Brain endothelial cells express a large number of receptors, including TfR, IR, LDLR, scavenger receptor, acetylcholine receptor, leptin receptor, etc. Structural modification with corresponding ligands (such as transferrin, monoclonal antibody OX26, CDX, RVG29, TGN, angiopep-2, etc.) [59-61] can promote the transport of therapeutics across the BBB via RMT pathway, offering the possibility for GBM-targeted therapy.

4.2. Cell Penetrating Peptide

Cell penetrating peptides (CPPs) are a class of short peptides with amino acids less than 30, they are extracted from insect, viral and mammalian proteins that can induce translocation of bioactive macromolecules cross cell membranes without specifically interacting with receptors [62]. They are generally classified into three categories based on their physical and chemical properties: cationic, amphiphilic, and hydrophobic. Of these, cationic CPPs are mainly composed of positively charged amino acids, such as arginine and lysine, which can interact with the negatively charged plasma membrane [63]. The apical surface of brain capillaries is densely covered with negatively charged polysaccharide protein complexes, making positively charged CPPs to be effective drug carrier for brain diseases passing through the BBB. However, the low tissue specificity, instability, and cytotoxicity of CPPs *in vivo* limit their application.

4.3. Membrane-Mediated Targeting

Recent years, a new class of targeted biomimetic drug delivery system coated with biological membrane has attracted wide attention of researchers due to its immune escape and homotypic binding capacities. Various membranes could be taken for coating such as erythrocyte [64], platelets [65], stem cells [66], tumor cells [67], bacteria [68], etc. Translocation of membrane onto the surface of drug delivery system can integrate various advantages of the molecular proteins on membrane surface and chemical properties of synthetic drug delivery system, forming a membrane-coated drug delivery system with good biocompatibility, low immunogenicity and homologous targeting function at lesion sites [69, 70]. Gboxin-loaded mesoporous silicon nanoparticles coated with a mixed membrane composed of erythrocyte membrane and tumor cell membrane can effectively target glioma cells and inhibit tumor cell proliferation [71].

In addition, exosome-based technology has also been extensively studied as a means delivering therapeutic drugs into the brain. As a kind of secretory vesicles, exosomes inherit some receptors on the surface of cell membrane, possess the targeting ability of parent cells, and can cross a variety of biological barriers [72]. Using tumor-associated macrophages exosomes as carrier to wrap thermosensitive liposomes could effectively target glioma cells, and photothermal combined with chemotherapy could finally reverse drug resistance of glioma [73]. Functionalized modification of exosomes with angiopep-2 to wrap the anti-tumor drug docetaxel can significantly enhance the intracerebral drug delivery and improve its anti-glioma efficacy [74].

As a collection of natural and synthetic entities, membrane carriers show strong therapeutic advantages and biocompatibility, which is a boon for researchers. However, each membrane has its own advantages and limitations. For example, erythrocyte membrane can prevent phagocytosis by reticuloendothelial system (RES) and prolong the circulation time *in vivo*, but it lacks active targeting ability; leukocytes have homologous targeting capabilities, but

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are specific for certain tumors; platelets can target tumor and injured areas, but can also activate the immune system; cancer cells have the ability to attach homologously and aggressively, but have a short circulation time in the blood; stem cells have the ability to target tumors, but with low specificity; bacterial membranes has immune-inducing properties, but the process of membrane extraction is complicated due to the need to remove peptidoglycan [75-77]. Therefore, fusion of two membranes can be effectively carried out to form a hybrid membrane with therapeutic benefits of both cells. Although great achievements have been made in the research of membrane-coated nanoparticles, there are still some challenges to be solved that the level of translation from laboratory to clinical trials has not yet been fully developed, and more practical explorations are needed in the future.

4.4. EPR Effect

The pathological process of GBM is also accompanied by the generation of EPR effect, and the drug delivery system with appropriate particle size enters the lesion region through the tumor microvascular endothelial space [78]. However, multiple studies have shown that the EPR effect is largely dependent on the type and location of the tumor, and the presence of BBB further limits the effective accumulation of therapeutic drugs at GBM lesion area. Caro and colleagues found that only nanoparticles with a particle size of about 50 nm could distribute into the stroma of glioma by EPR effect. Due to the presence of BBB, particles larger than 50 nm cannot accumulate in the tumor, and only a very few nanoparticles with particle size of 30-50 nm can accumualte near the large blood vessels of the tumor [79]. Therefore, EPR effect is an extremely inefficient strategy for brain tumor targeting, and active targeted therapy strategy is particularly important in the treatment of GBM.

5. Opening Pathological BBB for GBM Therapy

BBB permeability also exhibits high variability in different areas of the same tumor, as BBB integrity often exhibits high heterogeneity within tumor tissue. In the core of the tumor, the permeability of BBB is generally the strongest, while the barrier structure remains relatively intact at peripheral regions of the tumor. Moreover, the BBB of the peripheral GBM still exhibits highly functional. The intact BBB of tumor-infiltrating regions severely limits the effective delivery of chemotherapeutic drugs to GBM cells, and is a critical factor leading to the inefficiency of GBM treatment and high recurrence rate. Therefore, further increasing the permeability of BBB and promoting the intracerebral delivery of therapeutic drugs are important strategies to achieve efficient treatment of GBM. Moreover, BBB pathological fenestration in GBM could provide an additional paracellular pathway for active targeting therapeutics transporting into the brain (Figure 2B) [80-82], while chemotherapy could promote the functional recovery of pathological BBB. Seriously, in the course of GBM multiple administration, the restoration of pathological BBB further limits the transport of intracerebral therapeutics, making anti-GBM therapy failed (Figure 2C). Therefore, there has been much interest in seeking methods to further enhance endothelial permeability for biomedical and (nano-) pharmaceutics acrossing the BBB in anti-GBM therapy (Figure 2D).

5.1. Biological Stimuli

Virus is a kind of biostimulant material which can open TJs via regulation of chemokines as precursors for inflammatory cell infiltration in the CNS [83]. Verma and colleagues indicated that West Nile virus exhibited the ability to across the BBB via the “Trojan horse” mechanism by enhancing the expression of cell adhesion molecules, allowing macrophages to migrate

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through the BBB without disrupting the BBB. Foust and colleagues also verified that adeno-associated virus (AAV9) exhibit effective ability to target CNS cells, including neurons and adult astrocytes, without disrupting BBB integrity [84, 85]. Rabies virus (RABV) is a highly neurotropic virus, the RABV glycoprotein expressed in RABV can specifically bind to nAchR on the surface of neuron cells to realize the intracerebral transport of the virus [70, 86, 87]. The unique properties of virus crossing the BBB have led to a wide range of targeted therapies for brain diseases. Zhang and colleagues used recombinant adeno-associated virus rAAV as a carrier to introduce the single-chain antibody scFV targeting abnormal phosphorylated Tau protein (P-Tau) into the brain, inducing the continuous expression of scFV *in vivo* for AD immunotherapy [88]. Qiao and colleagues used RABV as a biological carrier to wrap virus-like nanoparticles, which could effectively overcome BBB, finally realizing GBM targeting and safe treatment [89].

Although biological viruses can reach the brain in a reasonable and precise way, but there are still some inherent security in the use of virus-mediated drug delivery system. In addition, the existing virus-like biomimetic nanocarriers mostly focuse on the simulation of specific viral proteins, while ignoring the physical and chemical properties of viruses, leading to poor drug delivery and therapeutic effect. Therefore, the construction of biomimetic nanocarriers that highly similar in structure, shape and function to virus may be one of the most effective ways to precisely and targeted delivery therapeutics for brain diseases treatment in the future.

5.2. Chemical Stimuli

5.2.1. Mannitol

Mannitol, an osmotic agent commonly explored, could shrink the endothelial cells lining CNS capillaries with resultant separation of the endothelial TJs, finally promoting passive diffusion of large molecules transport through the BBB. It has been used in combination with peptides, nanoparticles and gene delivery. Nevertheless, mannitol therapy exhibits its beneficial effects on BBB opening, there are still some risk factors, including brain damage, altered glucose uptake, passage of plasma proteins and abnormal neuronal function [90, 91]. Notablely, mannitol produces non-selective disruption of the BBB, and only a 25% increase in the permeability of tumor microvasculature.

5.2.2. Bradykinin

Bradykinin is a 9-peptide substance with cardioprotective effect by reducing the infarct area of acute ischemia-reperfusion myocardium. Although the endogenous bradykinin may achieve some advances in regulating the permeability of BBB in animal studies, some drawbacks limit its application as a BBB permeability regulator, such as the short half-life (only several seconds), potent vasoactive metabolites [92], weak opening BBB effect. Therefore, the selective B2 bradykinin agonist Cereport (labradimil or RMP-7) have also been designed and investigated as potential inducers for BBB transient disruption. RMP-7 can cause intracellular Ca^{2+} levels influx by selectively binding to B2 receptors on the membrane of cerebral endothelial cells, which triggers a series of signal transmission reactions including phosphatidylinositol, endothelial NOS and cGMP, increase the transport of endocytic vesicles or open the tight junctions of endothelial cells to temporarily open the BBB [93, 94]. And it exhibits specific time-dependent and dose-dependent actions on human cerebral microvascular endothelial cells. Either intracarotid or i.v. administration with RMP-7 could produce the effect on BBB opening. Since RMP-7 has no ability to suppress the P-gp efflux pump, RMP-7 stimulation shows no

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effect on drugs which are P-gp substrates (such as paclitaxel) transporting across the BBB.

5.2.3. Alkylglycerols

Alkylglycerols can also be employed for BBB opening, and the extent of disruption in the BBB mainly depends on the number and length of glycerols and alky groups presented in the chemical structure, respectively. Hulper and colleagues have indicated that 1-O-pentylglycerol and 2-O-hexyldiglycerol exhibited the ability to reversibly increase the permeability of BBB, without any influence on the TJs strand complexity[95, 96]. The phenomena could be due to proteins rearrangement and alterations in the cells shape, as well as the cytoskeleton remodeling under the invention of alkylglycerols.

5.2.4. 'Orifice-opening' Agents

There are a series of traditional Chinese medicine (TCM) specifically used for resuscitation purpose to restore consciousness in certain emergency conditions, such as coma, stroke, heart attack and brain related traumatic brain injury etc. TCM resuscitation agents are mostly derived from aromatic minerals and animal materials, and are known as aromatic 'orifice-opening' agents. Borneol is a bicyclic monoterpene with fragrant odor, pungent and highly hydrophobic, which is a representative resuscitation agent in TCM that has been used in clinical practice for more than 1500 years. Increasing evidences prove that borneol is an effective adjuvant that can modulate the BBB permeability and promote drug delivery into the brain. The enhanced permeability induced by borneol is closely associated with activating adenosine receptors, the down-regulation of TJs, the increase of void structures between the endothelial cells and the suppression of efflux protein function [97, 98].Moreover, systemic borneol was found to modulate the vasodilatory neurotransmitters such as histamine and serotonin in the hypothalamus [99]. The activation of these neurotransmitters can induce cerebral vasodilation and enhance the permeability of BBB through nitric oxide and receptors distributed on endothelial cells and astrocytes. However, current studies mainly include cell lines or rodent-based models, there is still a lack of research data on permeation enhancing effect of borneol in higher animal models such as primates and pigs. And the efficacy and safety of borneol still need to be further studied.

5.2.5. Adenosine

Adenosine, a purine nucleoside produced by many organs (heart, lung, gut, brain) and immune cells throughout the body, acts as a cellular metabolic distress signal. Its main function is to reduce tissue damage and promote repair through diverse receptor-mediated mechanisms, including increasing oxygen supply ratio to demand, protect against ischemic damage, anti-inflammatory effects and stimulating angiogenesis [100, 101].The combination of adenosine and A2A adenosine receptor (AR) expressed on the BBB could temporarily and transiently increase intercellular spaces between the brain endothelial cells, with an up-regulation of BBB permeability [102]. Kim and colleagues indicated that the activation of AR with Lexiscan increases the permeability of an *in vitro* primary human BBB with an increased passage of chemotherapeutic drugs and T cells. And the phenomenon was mainly due to the morphological changes in actin-cytoskeletal reorganization induced by RhoA signaling and a potent down-regulated expression of VE-cadherin and Claudin-5 [103]. Gao and colleagues developed a series of nanoagonists (NAs) for AR activation, and NAs increased permeability of the endothelial cell monolayer through TJs disruption. *In vivo* imaging studies further indicated that the intravenous injection of NAs could remarkably increase brain uptake of

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model drug and the BBB opening of TJs lasted in a range of 0.5-2.0 h. And the temporary opening of the BBB induced by NAs shows the promise to reduce potential risks like overdosage and uncontrollable BBB leakage [104].

5.2.6. 5-phosphodiesterase Inhibitors

5-phosphodiesterase inhibitors are the selective inhibitors of type 5 PDE (PDE5) such as sildenafil, vardenafil, and tadalafil, which can prevent phosphodiesterases (PDE) from reducing intracellular cGMP levels down to 5'-GMP. cGMP is a crucial intracellular second messenger, which could activate cGMP-dependent protein kinase (PKG) and stimulated K_{Ca} channels to increase vesicle trafficking in brain tumor microvasculature [105, 106]. Thus, as an oral treatment for erectile dysfunction in men approved by FDA, oral 5-phosphodiesterase inhibitor cannot regulate tight junction integrity in tumor capillary endothelium, but can increase transendothelial vesicular density to enhance the penetration of chemotherapy into brain [107]. In addition, 5-phosphodiesterase inhibitors may reduce the chaperone glucose-regulated protein (GRP78) and P-glycoprotein involved in chemoresistance in glioblastoma mice and inhibited microglial activation induced by lipopolysaccharide (LPS), which indicated they were excellent drugs that can be combined with chemotherapy drugs to treat brain tumors [108, 109].

5.2.7. NOS-3 Activators and Nitric Oxide (NO) Donors

The nitric oxide synthase-3, (also called endothelial constitutive nitric oxide synthase, eNOS or ecNOS) was mainly expressed in endothelial cells and neurons. The principal physiological function of NOS-3 was to catalyze L-arginine to L-citrulline and produce nitric oxide (NO) with the participation of many cofactors, such as tetrahydrobiopterin (BH4) and calmodulin. Nitric oxide, as a biological messenger, was first identified as an endothelial cell relaxing factor and also closely involved in mediating regulation of the integrity of the BBB. For one hand, NO activated soluble guanylate cyclase, resulted in increased intracellular cyclic GMP levels and further regulated the macromolecular transport into the brain microvessels [94]. For another, NO has an unpaired electron and easily react with H_2O_2 or proteins to form highly toxic peroxynitrite ($ONOO^-$) and the S-nitrosylation proteins in some degenerative diseases or tumors [110]. Excessive $ONOO^-$ and the S-nitrosylation of proteins severely damaged target cells and inactivated target enzymes, which further enhanced the opening of the pathological blood-brain barrier [110, 111].

5.2.8. VEGF Signaling Pathway

VEGF-A is described as VPF (vascular permeability factor), a key regulator of normal and pathological blood vessel growth of VEGF family, and a potent protein that rapidly and instantaneously enhances the vascular permeability of an intact BBB [112]. It is widely acknowledged that VEGF-A possesses two major biological activities, one is to stimulate the proliferation of vascular endothelial cells, the other is to increase vascular permeability [113]. The increase in vascular permeability induced by VEGF-A is common in pathological angiogenesis areas, such as solid tumors, wounds, and chronic inflammation. Notably, although some inflammatory cytokines, such as histamine and bradykinin also induced increase vascular permeability, experimental evidences showed that vascular leakage caused by VEGF and inflammatory mediators occur through different molecular processes [114]. Microvascular walls exposure to VEGF induces permeability with abnormally rapid kinetics and 50,000 times higher than histamine [115].

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The mechanism of VEGF-A in regulation of vascular permeability is mainly involved with several complex transmembrane transport processes [115]. VEGF-A induced fenestrations for small solutes leakage. VEGF-A also induced formation of caveolae, which assembed into vesiculovacuolar organelles (VVOs) for proteins transmembrane transport. Leakage of larger proteins and extravasation of erythrocytes depend on the release of vascular endothelial (VE), which mediates adhesions by cadherin. Furthermore, VEGF-A phosphorylates VEGFR2 and activates downstream signaling pathways involved in the regulation of the permeability process. VEGF mediated production of endothelial nitric oxide synthetase (eNOS) by PI3K/AKT. Furthermore, the activation of VEGFR2 stimulated phospholipase C-γ(PLC), produced diacylglycerol (DAG) and PKC, which activated possibly the nonselective cation channels(e.g. TRPC6 or TRPC3) and induced calcium influx [116].

5.3. Physical Stimuli

5.3.1. Ultrasound-Facilitated Opening

Focused ultrasound (FU) is emerging as a new strategy for localized, reversible, and safe BBB disruption to enhance therapeutics delivery to the brain [117]. However, due to the complex structure of the brain, FU is easily reflected by the skull, which greatly attenuates the ultrasonic energy, which brings new challenges to the use of ultrasound to open the BBB for the treatment of brain diseases. Therefore, FU is typically used in combination with prefabricated microbubbles (FU-MBs) consisting of albumin or lipid core encapsulated by a semi-solid gas (perfluorocarbon) confined in the vasculature, which can realize the non-invasive, targeted and reversible opening of BBB at a low sound pressure, with good repeatability and no permanent damage to brain tissue. At present, FU-MBs has been widely used in delivering nano drug delivery system, gene drugs and other small molecule drugs across the BBB [59, 118, 119]. Transcytosis, partial opening of interendothelial clefts and TJs, transendothelial channel formation and fenestion, and free passage through the damaged endothelium may be involved in the mechanism for molecules transporting across the BBB induced by FU-MBs [120, 121]. However, the use of FU-MBs may accompanied with the temperature increase of local target tissues, resulting in microvascular rupture and cerebral edema. In addition, the type and concentration of MBs would seriously affect the degree and duration of ultrasonic opening the BBB. Therefore, optimizing ultrasound equipment, controlling ultrasound parameters (sound pressure, frequency, irradiation mode, etc.) and screening appropriate MBs doses have important guiding significance for FU-MBs mediated BBB opening in the treatment of brain diseases and clinical translation.

5.3.2. Microwave-Facilitated Opening

Microwave is another physical stimuli to open BBB for enhanced drug delivery into the brain. Wang and colleagues found that microwave exposure could cause VEGF/FlK-1-ERK pathway activation and Occludin phosphorylation, accompanied with Occludin down-regulation and its interaction with ZO-1 [122]. In addition, the resultant brain temperature increase induced by sufficiently strong microwave energy may also produce increased BBB permeability. Indeed, it was reported that the rate head exposed to microwave frequencies (2.5-3.2 GHz) could produce brain temperature rise with an enhanced permeability of HRP, Evans blue and sodium fluorescein to BBB, and the temperature was measured above 40 °C. When brain temperature was cooled down below 40 °C, BBB opening effect is ignorable, suggesting that the mechanism of BBB opening is also related to the thermal effect of microwaves [123,

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124]. Notably, the thermal effect induced by microwaves may also increase brain infections. Furthermore, long-term exposure to microwave radiation can easily cause damage to brain tissues, resulting in symptoms such as memory loss, insomnia, headaches and dreams, the mechanisms still remain unclear, possibly including neuronal degeneration, apoptosis and necrosis, mitochondria swelling and cavitation, reduction of Nissl bodies, BBB damage, synaptic structure and function of plastic damage and calcium overload [125]. For safety concerns, there are no relevant reports on the application of microwave opening BBB in the treatment of brain diseases.

5.3.3. Electromagnetic Field

The use of electromagnetic field (EMF) pulses can also increase BBB permeability. Qiu and colleagues reported that EMF pulses could regulate protein kinase C signaling and ZO-1 translocation, inducing BBB permeability increased [18]. Experimental data *in vitro* further showed an enhanced transportation of antiviral drug acrossing BBB with EMF stimuli, and the wave shape, frequency and amplitude of EMF directly affect the level of enhancement [126]. Do and colleagues also confirmed that the application of an external EMF could significantly enhance the rate of nanoparticles across the BBB [127]. Nano-carrier combined with EMF strongly facilitates intracerebral delivery of therapeutic drugs and could have potential clinical application in the treatment of other brain-related disease and brain tumors.

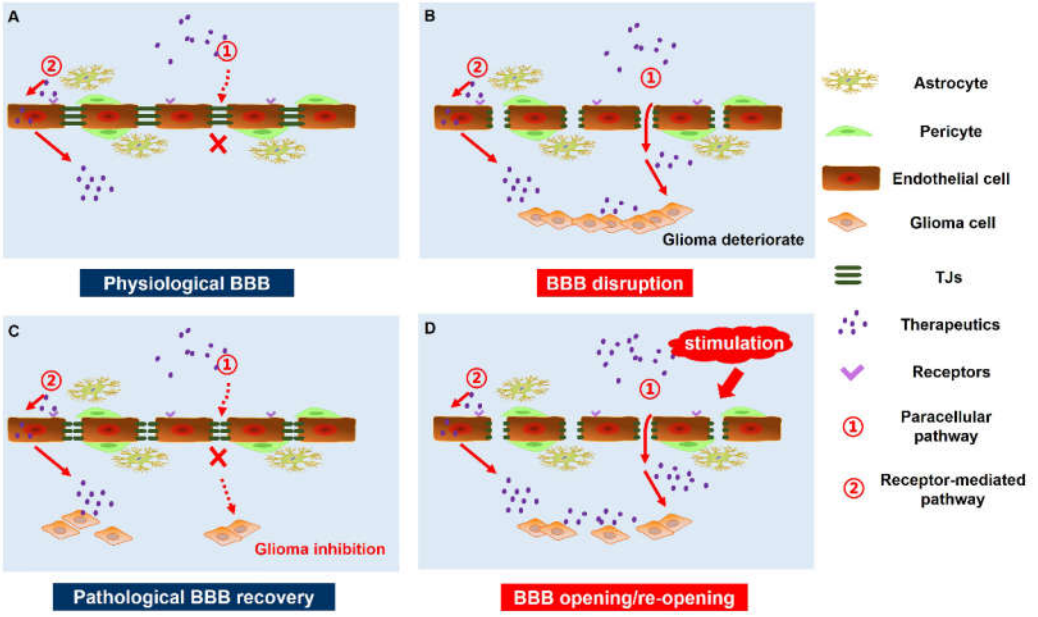

Figure 2. GBM therapy under pathological state of the BBB. (A) Under physiological condition, targeted therapeutics cross the BBB mainly by receptor-mediated pathway. (B) GBM growth causes BBB disruption and pathological fenestration, which in turn provides an extra paracellular pathway for therapeutics transporting into the brain. (C) Chemotherapy promotes pathological BBB functional recovery, which induces a significant decrease of targeted therapeutics transport across the BBB. (D) Opening of pathological BBB or re-opening of functional recovering pathological BBB via external stimulation, including biological stimuli, chemical stimuli, physical stimuli and signaling pathway regulators, could obviously increase the intracranial delivery of targeted therapeutics for enhanced GBM therapy.

6. Conclusions and Prospects

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So far, there are no effective treatments for brain diseases (AD, PD, stroke, etc.) including GBM. In addition to the complexity of these diseases, the main reason is the presence of the BBB. For decades, the delivery of drugs to the brain and spinal cord has been an exciting but exhausting research area due to the extremely complex structure of the BBB. With the development of pharmaceutical technology, therapeutic pharmaceutics and methods aiming at the BBB are constantly being discovered. Controlly regulating BBB opening provides the possibility for the transport of therapeutic drugs into the brain, various drug transport systems (AMT, CMT, RMT) also show great potential in the transport of biological macromolecules and small molecules across the BBB. The emergence of biomimetic therapeutics makes the treatment of brain diseases safer and more efficient.

BBB dysfunction, often referred to as "BBB opening", is likely to be the common feature of the progression of brain diseases, including GBM. It can be effectively and rationally utilized differences of the BBB under physiological conditions and pathological conditions, study the molecular mechanism regulating BBB permeability, then design novel drug delivery system for effective intracerebral therapeutics delivery, finally realizing GBM effective therapy. Importantly, the brain is an important organ that governs all the life activities of human body, it is also necessary to evaluate the safety, risk and benefit to patients of the new technologies that increase BBB permeability and brain targeted systems, especially for some brain diseases requiring long-term drug treatment. Overall, in the design of intracerebral drug delivery system, especially to increase the penetration of drug in the brain, it is necessary to consider the selectivity of drugs for the lesion site, minimizing the distribution in non-target tissues.

Author Contributions: Conceptualization, writing and editing were done K.W., F.Z. and C.W. All authors have read and agree to the published version of the manuscript.

Conflicts of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Funding: The research was supported by the National Natural Science Foundation of China (NSFC Nos. 82104101), Jiangxi Provincial Natural Science Foundation, China (Grant No. 20212BAB216003), the Project of Gannan Medical University (No. ZD201903) and PhD Start-up Fund of Gannan Medical University (QD201908).

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