

An injectable refrigerated hydrogel for Inducing local hypothermia and neuroprotection against traumatic brain injury

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Abstract

Hypothermia is a promising therapy for Traumatic brain injury (TBI) in the clinic. However, the neuroprotective outcomes of hypothermia-treated TBI are not consistent in clinical studies due to several severe side effects. Here, an injectable refrigerated hydrogel is designed to deliver 3-iodothyronamine (T1AM) to achieve a longer period of local hypothermia for TBI treatment. The hydrogel has four advantages: (1) It can be injected into injured site after TBI, where it forms a hydrogel and avoids the side effects of whole-body cooling. (2) The hydrogel can biodegrade and be used for controlled drug release. (3) Released T1AM can bind to trace amine-associated receptor 1 (TAAR1) to produce cyclic adenosine monophosphate (cAMP), which induces hypothermia. (4) This hydrogel has an increased medical value due to its simple operation and ability to achieve timely treatment. This hydrogel is able to cool the brain to 30.25 ± 2.25 °C for 12 hours while maintaining the body temperature at 36.80 ± 1.75 °C after TBI. More importantly, the hypothermia induced by this hydrogel leads to the maintenance of blood-brain barrier (BBB) integrity, the prevention of cell death, the reduction of the inflammatory response and brain edema, and the promotion of functional recovery after TBI. This cooling method can potentially be developed as a new approach for hypothermia treatment in TBI.

Introduction

TBI is recognized as a leading cause of disability and death worldwide (1-3). Secondary TBI occurs after the initial mechanism of trauma has run its course (4, 5). If the initial damage is extensive, secondary TBI occurs, which can result in the initiation of an acute inflammatory response, edema and swelling, continuous breakdown of the BBB, local electrolyte imbalance, and persistent neurological impairment in many patients (6, 7). At present, clinical therapeutic agents for TBI are relatively scarce for preventing the secondary spread of damage beyond the initial insult.

Hypothermia has been confirmed to exert strong neuroprotective effects to prevent secondary damage after TBI (8, 9). The major mechanisms of neuroprotection by hypothermia are attenuation of BBB permeability, reduction of glutamate release, alleviation of inflammation, and the reduction of free radical generation and release (10). However, the neuroprotective outcomes of hypothermia-treated TBI are not consistent in different clinical studies (11, 12). Two issues limit the therapeutic effect of hypothermia: (1) Hypothermia is associated with several severe side effects, such as hypotension, arrhythmias, electrolyte disorders, shivering and local infections, when conventional whole-body cooling is used. In these cases, the adverse effects outweigh the neuroprotective benefits of therapeutic hypothermia (13). (2) The time of cooling initiation is a key factor that affects the neuroprotective outcomes associated with brain hypothermia. Clinical studies demonstrated that the window of opportunity appears to be 90 min or less for hypothermia therapy (14, 15). However, it is still a challenge to shorten the time between the injury and induction of hypothermia. Usually, it is impossible to predict the time spent on transporting TBI patients to the hospital. As a result, hypothermia therapy for TBI is largely limited.

To resolve the question of whole-body cooling side effects, targeted brain cooling has been proposed as a reasonable alternative. Local brain hypothermia has the advantage of decreasing the local brain temperature while maintaining whole-body normothermia, potentially achieving the neuroprotective effects of hypothermia with minimal systemic side effects (16,17). At present, many ways to achieve local hypothermia have been used clinically such as epidural placement of a cooling catheter (18), local cold fluid infusion (16), and local ice compress (19), which lead to a certain therapeutic effect. However, truly effective and rapid selective brain hypothermia is difficult to achieve because of the many problems that occur such as the difficulty and complexity of the surgery, infection of implants, expensive materials, and technical limitations (16,17, 20). In addition, it is difficult to solve the problem of time when the TBI patients begin to experience mild hypothermia. Therefore, the discovery of an effective and timely approach to achieve local brain hypothermia is still challenging.

Injectable hydrogels can be used for the local delivery of drugs and have been widely used for the treatment of brain diseases (TBI and stroke) due to their good biocompatibility, biofunctionality, and ability to form via an in situ gelling process (21, 22). The hydrogels themselves can be used as carriers to load various drugs, and the drug utilization efficiency can be improved while prolonging the action time of the drug by injecting it in situ or directly on the tissue surface (20). Moreover, these hydrogels are easy to carry and inject into wounds in a timely manner. Many studies have shown that these hydrogels are more advantageous in many diseases such as spinal injury, stroke and glioma compared with traditional therapies (23–25). SURGIFLO® (a gelatin-based hydrogel) and the Porcine Fibrin Sealant Kit (a collagen-based hydrogel) have been widely used for hemostasis and preventing cerebrospinal fluid leakage after neurosurgery (26, 27). Therefore, the clinical application of hydrogels appears promising. Inspired by these hydrogels, we speculated that a hydrogel could be developed to achieve hypothermia.

T1AM is an agonist of TAAR1, a G-protein-coupled receptor activated by phenylethylamine, tyramine, methamphetamine, and its congeners. T1AM can bind to TAAR1 to produce cAMP, which induces profound hypothermia within minutes (28). T1AM has been used in animal models to protect against stroke-induced brain injury and is able to provide marked neuroprotection because TAAR1 is enriched in neurons and astrocytes in the brain (29). However, T1AM given by intraperitoneal injection is associated with some side effects, which is because TAAR1 is widely distributed in various organs such as the heart, aorta, kidney, stomach, and small intestine (30).

The advantages of hydrogels allow us to believe that we can use them as drug carriers to achieve timely and effective local hypothermia to avoid multiple problems with existing methods. In this study, we used an injectable poloxamer hydrogel to deliver T1AM (Pol/T hydrogel) to achieve timely and effective local hypothermia for TBI treatment. This Pol/T hydrogel consisted of a thermosensitive Pol hydrogel and T1AM. Poloxamer hydrogel is approved by the U.S. Food and Drug Administration (FDA) for use in humans and it demonstrates the property of thermosensitive sol-gel transition behavior in situ. Poloxamer hydrogels are liquid at 4 °C, and they transform into a gel at 37 °C (Scheme.1A). Thus, a 4 °C sol Poloxamer was injected into a wound cavity where it formed a Pol hydrogel, which resulted in a decreased temperature of the brain (Scheme.1B). After the Pol/T hydrogel was formed, the Pol hydrogel was biodegraded in the brain trauma microenvironment (BTE), which released T1AM for hypothermia treatment. The released T1AM coupled with TAAR1 distributed in neurons and astrocytes, which stimulated cAMP production to cool the temperature of the brain (Scheme.1C). Importantly, we used this hydrogel to deliver T1AM directly to the injured brain area, effectively achieving brain hypothermia and avoiding the side effects of systemic medication in other organs. Given its ease of preparation, injectability, and local drug delivery, this strategy for the Pol/T refrigerated hydrogel induced local cerebral hypothermia and showed great potential in neuroprotection against TBI.

Results

Characterization of the hydrogel. To form hydrogels, which are liquid at 4 °C and transform into gels at 37 °C, poloxamer 407 and poloxamer 188 were mixed at different mass ratios. As shown in Fig.1Aa, when the mixture was composed of 25% w/v high poloxamer 407 concentration and 5% w/v low poloxamer (Pol hydrogel), the prepared hydrogel could transform into a gel state at 37 °C, close to human body temperature. In order to clearly observe the process of Pol at 37 °C, Evans blue dye was embedded into the Pol hydrogel to form Pol/Eb with blue color. The sol Pol/Eb (4 °C) was injected into 37 °C water, and then the Pol/Eb hydrogel was quickly formed (Fig.1Ab). Furthermore, we investigated the sol-gel transition and injectable behavior of the Pol hydrogel in vivo using the TBI model of Feeney's weight-drop injury (WDI). The sol pol could transform into a gel pol after 2 min in the wound cavity of a TBI mouse (Fig.1B). The porous structure of the Pol hydrogel was observed by scanning electron microscopy (SEM) (Fig.1C). T1AM at 50 mg/mL contained within the Pol hydrogel did not affect the structure of the hydrogel (Fig.1Ca, b). To test the biodegradation property of this Pol/T hydrogel in vivo, 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindotricarbocyanine iodide (DiR) was embedded into the Pol hydrogel (Pol/DiR). As shown in Fig.1Da, b, the fluorescence signal was decayed, and the lowest fluorescence intensity was tested 12 hours after injection, which suggested that the Pol hydrogel could biodegrade in the BTE over the 12 hours period.

To further monitor the degradation behavior of the Pol hydrogel in an environment similar to the human TBI environment, we collected the cerebrospinal fluid (CSF) from clinical TBI patients, which have been confirmed to be germfree, and soaked the hydrogel in CSF to verify the biodegradation behavior of Pol in vitro (Fig.1Ea). Pol/Eb was mostly biodegraded by 12 hours (Fig.1Eb), and 89.07 ± 4.38% of T1AM was released from the Pol hydrogel during incubation in CSF within 12 hours (Fig.1F). Taken together, these results demonstrate that the Pol hydrogel could biodegrade and accomplish controlled-release T1AM in a post-traumatic environment.

The safety of materials is the primary problem in clinical application. Pol hydrogels are polymers with good biological safety and are approved by the FDA. In this study, the cytotoxicity of the Pol hydrogel was determined by MTT assay. As shown in fig.S1, the survival rate of cells was higher than 90% when they were incubated for 3, 6, and 12 hours in Pol hydrogel immersion solution, which indicates that the Pol hydrogel had high favorable safety.

The local hypothermia induced by Pol/T hydrogel without leading to the systemic negative effects. T1AM can stimulate TAAR1-expressing cells to produce cAMP, which induces profound hypothermia within minutes (28). Neurons and astrocytes in the brain both express TAAR1 (30). Therefore, we speculated that local administration of free T1AM in the brain would achieve local hypothermia (Fig.2A). To investigate the effect of hypothermia induced by the Pol/T hydrogel, we assessed the temperature variation in the brain and body. Rectal temperature was representative of the body temperature. Intraperitoneal injection of T1AM has been reported to induce a rapid dose-dependent drop in body and brain temperature (28). The dose of free T1AM and that of T1AM (from 50 mg/kg to 100 mg/kg) was evaluated for safety by survival curve of the mice through intraperitoneal injection. As shown in fig.S2, no mice died with the T1AM concentration of 50 mg/kg (consistent with reporting by Thomas S Scanlan), and some mice died with the rise of T1AM concentration beyond 50 mg/kg. When the dose of T1AM reached 100 mg/kg, the mice became inactive, assumed a slightly hunched-back posture and developed ptosis (drooping eyelids), and some mice even died within 1 hour of the intraperitoneal injection (see Movie S1 in supporting information). Nevertheless, when we used Pol as a T1AM carrier to deliver 100 mg/kg T1AM directly to the brain, the behavior of the treated mice was similar to that of normal mice, and the side effects of whole-body cooling were avoided (see Movie S2 in supporting information). According to our experiments and data reported by Thomas S Scanlan (28), 50 mg/kg T1AM was used for all subsequent experiments.

As shown in Fig.2B, the TBI mice injected intraperitoneally with free T1AM had a body and brain temperature 4–7 °C lower than normal, and the low temperature was only maintained for approximately 4 hours in both the brain and body. The brain temperature decreased 2 °C from the normal brain temperature for less than 2 hours, while the body temperature was unchanged only in the Pol hydrogel injection group, which suggests that the transition of a 4 °C sol to a gel led to a cooler brain temperature. Compared with the other treatments, the brain temperature of the TBI mice treated with the Pol/T hydrogel could be maintained at 30.25±2.25 °C for 12 hours and the body temperature did not noticeably change (36.80±1.75 °C), which suggests that T1AM embedded in the Pol hydrogel was released slowly over time to maintain a cooler brain temperature.

The negative systemic effect of whole-body hypothermia limits its clinical application, but local hypothermia can effectively avoid this negative effect. In this study, heart rate, systolic blood pressure, respiratory rate and oxygen saturation were monitored for 12 hours after the trauma. As shown in Fig.2C, each vital sign showed a similar trend in temperature variation. Free T1AM has already been confirmed to have a negative effect on the heart and causes a decline in cardiac output (28) and thus we observed a significant decline in heart rate and systolic blood pressure in the free T1AM-treated group. As the temperature returned to normal, the heart rate and systolic blood pressure suddenly increased and even exhibited a rebound effect that increased these two vital signs beyond the normal level. Similarly, the respiratory rate and oxygen saturation were significantly reduced with a sharp drop in body temperature as a result of free T1AM. However, when we used the Pol/T hydrogel to induce local hypothermia in the brain, these vital signs did not fluctuate appreciably (Fig.2Ca, b, c, d). These phenomena showed that the Pol/T hydrogel even induced a very low temperature of 28.9±0.9 °C in the brain, but this did not result in

any negative behavior in the mice. The above results demonstrated that the Pol/T hydrogel could induce effective and timely local hypothermia after TBI without the side effects.

Effect of the local hypothermia induced by Pol/T hydrogel on trauma-induced neuronal injury. Many studies have reported that therapeutic hypothermia exhibits a long-term neuroprotective effect after TBI (31, 32). Our aim was to test whether the Pol/T hydrogel could lead to local hypothermia and protect neurons from damage induced by TBI. H&E and Nissl staining were performed to investigate damaged neurons in the ipsilateral cortex surrounding the injury site 7 days after TBI (Fig.3A a, b). Nissl staining and H&E staining revealed the lesion volume after TBI (Fig.3B). The contusion volume was 14.00±2.67 mm3 in the TBI control group. In TBI mice that received free T1AM treatment, the contusion volume was reduced to 9.29±2.14 mm3. The contusion volume of TBI mice was reduced to 12.82±1.32 mm3 in the Pol hydrogel treatment group. More importantly, the TBI mice treatment with the Pol/T hydrogel had the smallest contusion volume (6.57±1.14 mm3) of all the treatments. From Fig3.Ab, evident damage was shown in the TBI control group, with loss of Nissl intensity and a decrease in neuron cell number, which resulted from shrinkage necrosis. The neuronal cell number was less in the Pol hydrogel group because it only cooled the brain to 33.75 ± 0.65 °C for 2 hours, which resulted in less of a neuroprotective effect after TBI. Compared with the Pol hydrogel group, the severity of neuronal degeneration was alleviated in the free T1AM group, which suggests that T1AM-induced hypothermia protected the neurons after TBI injury. Moreover, the strongest neuroprotective effect was observed in the Pol/T hydrogel group, which demonstrated that local hypothermia lasted for more than 12 hours and effectively improved the protective effect on neurons.

To further validate the neuroprotective effect of the Pol/T hydrogel, apoptosis and necrosis of neurons following TBI were tested. As shown in Fig.3C, D, more TUNEL-positive cells were found in the TBI control group, which demonstrated substantial overall cell death post trauma. We observed that the Pol/T hydrogel group reduced the overall number of TUNEL-positive cells more effectively than free T1AM because the Pol/T hydrogel induced local hypothermia for 12 hours.

It has been shown that Bcl-2 and Bax are a pair of antagonistic factors, where the former has an antiapoptotic effect, while the latter can promote apoptosis. Bcl-2/Bax regulates mitochondrial function and the release of apoptosis-related proteins (33). We used immunohistochemistry (IHC) to determine the expression of Bcl-2 and Bax on the edge of the injured area. IHC staining showed that the expression of Bcl-2 in mice given the Pol/T hydrogel treatment was obviously greater than that in mice that were given other treatments, but the expression of Bax was obviously weaker than that in mice given other treatments (Fig.3Ea, b). The ratio of anti-apoptosis-related Bcl-2/Bax in the brain tissue of the mice was increased under Pol/T hydrogel treatment (Fig.3F). The larger the ratio, the better the cell survival, and the smaller the ratio, the more likely apoptosis will be induced. This result indicates that the Pol/T hydrogel could inhibit the expression of Bax and upregulate the expression of Bcl-2 after TBI, thereby reducing apoptosis and acting as a neuroprotective agent. Altogether, these results suggest that the Pol/T hydrogel induced local hypothermia and effectively protected neurons from damage caused by TBI. *Effect of the local hypothermia induced by Pol/T hydrogel on BBB permeability and brain edema after TBI.* After TBI, brain tissue exhibits a massive disruption in the BBB, which is followed by brain edema and exacerbates the devastating consequences of the final outcome of TBI (34,35). Hypothermia reduces the extent of BBB disruption and reduces the volume of brain edema (10). Therefore, to further explore whether the Pol/T hydrogel that induces local hypothermia has a positive effect on the destruction of the BBB, the BBB permeability following TBI was estimated by the extravasation of Evans blue dye (36). Compared with the TBI-control group, BBB permeability was significantly reduced in the ipsilateral hemisphere in the Pol hydrogel, free T1AM and Pol/T hydrogel groups (Fig.4A, B). Moreover, Pol/T hydrogel treatment obviously inhibited Evans blue leakage and restricted the leakage area to the primary lesion site, which suggests that the Pol/T hydrogel induced local hypothermia and effectively maintained BBB integrity.

Matrix metalloproteinases (MMPs) are also associated with increased BBB permeability, which is indicative the hemorrhagic potential and the extent of brain edema (31). MMP-9 levels were investigated in cerebral cortex (Fig.4C, D). In the sham group, the number of MMP-9-positive cells was very small, but the TBI mice showed a gradually increasing expression level. Compared with the TBI control group, MMP-9 expression was decreased in the Pol hydrogel, free T1AM and Pol/T hydrogel groups. The lowest expression of MMP-9 was observed in the Pol/T hydrogel group, which demonstrated that the Pol/T hydrogel effectively induced local hypothermia and prevented brain impairment after TBI injury.

To further explore the ability of the Pol/T hydrogel to effectively reduce the spread of secondary damage, brain edema was detected by T2-weighted and diffusion-weighted imaging (DWI) of MRI at 12 hours after percussion injury. As shown in Fig.4E, F, brain edema centered around the percussion site was evident in the TBI control group. Compared with the TBI control group, the high-intensity area was restricted to a relatively small region in the Pol hydrogel, free T1AM and Pol/T hydrogel groups. The hyperintensity volume was 13.39 \pm 2.91 mm3 in the TBI control group. In TBI mice that received free T1AM treatment, the hyperintensity volume was reduced to 8.9 \pm 1.55 mm3. The contusion volume in the TBI mice was reduced to 11.96 \pm 1.60 mm3 in the Pol hydrogel treatment group. This phenomenon suggests that the Pol hydrogel and T1AM showed different degrees of neuroprotection. Most importantly, the volume of edema surrounding the damage was the lowest (5.94 \pm 1.70 mm3) in the Pol/T hydrogel group. We also assessed brain edema in different groups after 12 hours by measuring brain water content and these results were consistent with the result of T2-weighted MR (fig.S3).

The dispersion of water molecules on the surface of the cerebral cortex was further evaluated by measuring the apparent diffusion coefficient (ADC) values of DWI to determine the edema of brain tissue, as ADC values can reflect the variation trend of edema with high sensitivity (37). The reduction of ADC values reflected the cytotoxic edema gradually occurred and aggravated as the release of neurotoxic substances.(38) The ADC values of mice treated with the Pol hydrogel ($0.53 \pm 0.10 \times 10-3 \text{ mm2/sec}$) and free T1AM ($0.57 \pm 0.11 \times 10-3 \text{ mm2/sec}$) were all higher than those in the TBI control group ($0.43 \pm 0.15 \times 10-3 \text{ mm2/sec}$). Consistent with the trend of hyperintensity volume in T2-weighted images, the

ADC values were higher ($0.72 \pm 0.10 \times 10-3 \text{ mm2/sec}$) in mice treated with the Pol/T hydrogel (Fig.4G). Collectively, the Pol/T hydrogel effectively reduced brain edema and protected BBB integrity.

Anti-inflammatory effect of the local hypothermia induced by Pol/T hydrogel. The inflammatory response plays important roles in secondary damage after TBI injury (39). The astrocytes and microglia are quickly activated and the cell bodies are enlarged and branched after brain tissue is damaged. Ionized calcium binding adapter molecule (lba-1) is the indicator of activated microglia and glial fibrillary acidic protein (GFAP) is the indicator of the activity of the astrocytes (46). Their expressions were detected by IHC. As shown in Fig.5Aa, b, c, d, we observed that the expression of lba-1 and GFAP was significantly increased on the edge of the injured area in mice of the TBI control group, which was consistent with the results described above. The expression of lba-1 and GFAP expression surrounding the TBI injury site was the lowest in the Pol/T hydrogel group compared with the other treatment groups. The results show that local hypothermia induced by the Pol/T hydrogel inhibited the chronic secondary astrogliosis inflammatory response at the TBI-injured site.

TBI induced an acute increase in proinflammatory cytokine (TNFα, IL-1β and IL-6) and chemokine (CXCL1) production throughout the brain (Fig.5Ba, b, c, d) (39). According to enzyme-linked immunosorbent assay (ELISA) analysis of protein levels in the brain tissue 12 hours post trauma, durable and stable local hypothermia induced by Pol/T hydrogel could more significantly decrease inflammatory factor expression than transitory whole-body hypothermia via free T1AM. Taken together, the method of hypothermia induced by the Pol/T hydrogel effectively inhibited the inflammatory response after TBI.

Promotion in functional recovery after TBI. The behavioral tests were performed after the hypothermia treatment with the Pol/T hydrogel on 21 days after TBI. The Morris water maze test was used to assess the long-term learning and memory abilities of TBI mice (Fig.6A-G). As shown in Fig.6B, C, during first 5 days of training, the mice treated with the Pol/T hydrogel exhibited comparable improvement in learning capacity, as confirmed by decreasing the searching time for the platform and distance to the platform. On the last day, the platform was removed and the memory capacity of each mouse was evaluated. The mice treated with the Pol/T hydrogel used the shortest distance to the original position of platform (Fig.6D). Correspondingly, the time spent in the target quadrant, the duration time in the platform area and the platform crossing frequency were increased in the Pol/T hydrogel-treated TBI mice (Fig.6E-G). The modified neurological severity score (mNSS) was adopted to evaluate motor and sensory function as well as reflex and balance after TBI. A high score indicated more serious damage in the TBI mice (40). Compared with the TBI and Pol groups, the mNSS scores were lower than in the free T1AM- and Pol/T hydrogel-treated TBI mice (Fig.6H). The Pol/T hydrogel-treated mice had the lowest mNSS score compared with the other groups except the sham group, which demonstrates that the Pol/T hydrogeltreated TBI mice showed enhanced neurological recovery. The hanging-wire-grip test was used to assess motor strength after Pol/T hydrogel treatment. As shown in Fig.6I, Pol/T hydrogel-treated TBI mice exhibited maximum endurance compared with mice in the other treatment groups, which indicates that the motor strength of TBI mice was enhanced after Pol/T hydrogel treatment. These results suggest that

injection of the Pol/T hydrogel into TBI mice could efficiently enhance the behavioral ability of these animals

Discussion

TBI accounts for millions of deaths each year worldwide and is one of the leading causes of death and disability in young people (1). Researches have shown that secondary brain injury is a significant risk factor leading to an acute inflammatory response, which inducing the breakdown of the BBB, edema formation and swelling, local electrolyte imbalance, and persistent neurological impairment. As the primary injury is irreversible, preventing secondary injury has its significance in the clinical practice (4, 5, 41). Therapeutic hypothermia can reduce intracranial hypertension and regard as a neuroprotective agent to prevent secondary brain injury in TBI patients (8, 9). Current therapies of hypothermia, however, have many negative effects and technical barriers, which limit its application in the clinic. In this project, we developed an injectable refrigerated hydrogel which could induce local hypothermia and neuroprotection against TBI (13–15). The refrigerated hydrogel has some unique advantages features which induce timely, effective, and persistent local hypothermia while avoiding the systemic side effects. The results show that the hydrogel can effectively protect BBB integrity, prevent cell death, reduce the inflammatory response and brain edema, and moreover promote functional recovery after TBI.

Due to the systemic negative effects of the whole-body hypothermia and the low operability of local hypothermia, hypothermia is greatly limited in the clinic (42). The advantages of hydrogel allow us to believe that we can use it as a drug carrier to achieve timely and effective local hypothermia. This new way can avoid multiple problems in existing methods. We used Pol/T hydrogel to treat the TBI mice and monitored the vital signs closely. T1AM can stimulate TAAR1-expressing cells to produce cAMP, which induces profound hypothermia within minutes. Neurons and astrocytes in the brain both express TAAR1. The TAAR1, however, is widely distributed in various organs, and the negative heart effect is especially significant, which even causes death at the high dose of T1AM in animal models. The local hypothermia caused by the Pol/T hydrogel could last 12 hours because this hydrogel could load a high dose of T1AM, which could induce death in experimental animals by traditional administration. We observed that the brain temperature markedly decreased to a low level, but the body temperature maintained at the normal level. The cooling time was longer than traditional pharmacologically induced hypothermia, and the rewarming process was gradual without the dramatic change. Importantly, the vital signs and behavioral expression of TBI mice which received the Pol/T hydrogel remained steadier than the mice treated with the T1AM in the whole process. These results demonstrated that the Pol/T hydrogel induced prolonged and effective selective brain hypothermia and maintained stable of the whole body by avoiding the systemic negative effects around the cooling time.

We found that this local hypothermia had great protective effect on neurons from damage induced by TBI. Neuro apoptosis is an inevitable process and the main form of neuro death in the acute phase of secondary brain injury when cerebral cortex takes a hit. In the TBI mice model, it showed that the lesion volume and apoptosis-restraining protein Bcl-2 reduced in the normothermic group and increased in the

hypothermic group (33). This phenomenon was consistent with our observation that the persistent local hypothermia induced by Pol/T hydrogel suppressed pro-apoptosis genes and increasing anti-apoptosis genes. Some studies have shown that the level of Bcl-2 genes of TBI mice which treated with hypothermia therapy in the first 3 hours was higher than the mice which was delayed by 3 hours after TBI (31). These results showed that the time window was the key of hypothermia therapy, and the earlier therapy started, the better effect was. The timely local hypothermia induced by this hydrogel is perfectly consistent with this treatment concept.

The destruction of BBB is the essential basis of the brain edema after TBI (41).Hypothermia can protect the BBB, associated with the concomitant decrease in BBB permeability, structural changes in capillary blood vessels, upregulation of MMP-9 activity (10). We showed that this local hypothermia induced by Pol/T hydrogel enhanced the integrity of the BBB and decreased brain edema. In our study, MMP-9 was upregulated after TBI and this increase was significantly inhibited by Pol/T hydrogel-induced local hypothermia. As a result, Pol/T hydrogel treatment inhibited Evans blue leakage and restricted the leakage area to the primary lesion site, which suggested that the Pol/T hydrogel induced local hypothermia and effectively maintained BBB integrity. T2-weighted and DWI of MRI was used to detect the severity of brain edema. In our study, the T1AM group and the Pol/T group all reduced the level of brain edema after TBI. However, the local hypothermia showed more effective protection because the longer time of hypothermia and the miler rewarming process.

The inflammatory response plays an important role in secondary damage after TBI injury (39). Hypothermia can decrease inflammatory production of different pro-inflammatory factors and the activation of microglia and astrocyte (43). The astrocytes and microglia are quickly activated, and the cell bodies are enlarged and branched after brain tissue is damaged. Iba-1 is the indicator of GFAP is the indicator of the activity of the astrocytes. Our studies showed that the local hypothermia induced Pol/T hydrogel attenuated expression of pro-inflammatory factors and restrained the activation of microglia and astrocyte.

Clinic studies show that the neurologic function deficit is the most severe sequela caused by TBI (1, 3). It is essential to promote recovery of neurologic function and enhance the quality of life. In animal studies, the hypothermia therapy has great protective effect of sensorimotor function in hypoxia-ischemia mice. The local hypothermia induced Pol/T hydrogel effective recovered the long-term learning and memory abilities of TBI mice. The sensitive of reaction and muscular strength was also improved by Pol/T hydrogel. This results suggested that the long-term functional benefits could be achieved after local hypothermia induced by Pol/T hydrogel.

In summary, injectable and biodegradable refrigerated hydrogels (Pol/T hydrogels) were developed to induce local hypothermia for TBI treatment. These hydrogels were composed of Pol and were embedded with T1AM. The Pol/T was in solution at 4 °C and formed a gel at body temperature. The sol Pol/T was directly injected into the surgical cavity after TBI to form a Pol/T hydrogel, which biodegraded to release T1AM. T1AM then acted on TAAR1-expressing neurons and astrocytes to achieve local hypothermia in

the brain. The temperature of the brain was cooled to 30.25±2.25 °C for 12 hours, and the body temperature was not decreased after treatment with the Pol/T hydrogels. Moreover, no severe side effects were observed after treatment with Pol/T hydrogels. More importantly, the Pol/T hydrogels effectively protected BBB integrity, prevented cell death, reduced the inflammatory response and brain edema, and promoted functional recovery after TBI. This Pol/T hydrogel is easily transported on ice and can be used in TBI patients in a timely manner. Of course, there are still many problems to be solved in clinical application. Overall, these findings demonstrate that the Pol/T hydrogel provided a potential local hypothermia approach for preventing secondary injury after TBI.

Methods

Preparation of Pol/T hydrogel

A solution was prepared by dissolving a high poloxamer 407 concentration (25% w/v) and low poloxamer 188 (5% w/v) in distilled water containing 5% Dimethyl sulfoxide(DMSO)which dissolved appropriate T1AM. The solution was kept at 4 °C overnight until a clear solution was formed.

Micromorphology of Pol/T hydrogel

The Pol/T hydrogels were frozen to solid state in liquid nitrogen and then the samples were critical dried via vacuum freeze dryer for 48 hours. The freeze dried samples were fixed on aluminum plate. After the powder was sputter-coated with gold, the surface morphology was observed by a scanning electron microscope.

In vitro drug-release of Pol/T hydrogel

To measure the release of T1AM from Pol/T hydrogels, 500 µL of Pol/T hydrogels were transferred in 4 mL centrifugal tube and suspended in 3 mL CSF. The samples were incubated at 37 °C and T1AM released from Pol/T hydrogels was quantified at different time intervals by high performance liquid chromatography. The column was an advanced Hypersil C18 250 × 4.6 mm and the 1 mL/min mobile phase consisted of methanol: water (45: 55) with 0.01% trifluoroacetic acid.

TBI animal models

All experiment procedures were approved by Xuzhou Medical University. Male ICR mice 5 weeks old were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). Experimental TBI model was constructed with modification of Feeney's weight-drop method. A 5 mm craniotomy (3 mm posterior and 3 mm lateral from bregma) was performed on the right parietal. A weight-drop hitting device (ZH-ZYQ, Anhui Zhenghua biological instrument equipment Co., Ltd, Huaibei, China) with a 4.0 mm diameter footplate was used to induce injury. Forces of impact was produced by a 40 g weight dropping from a height of 7.5 cm. The TBIs mice model were randomized into 4 groups (Control, Pol hydrogel, T1AM, Pol/T hydrogel). The Pol hydrogel group and Pol/T hydrogel group was injected with 50 µL hydrogel and the T1AM group was injected intraperitoneally with 50 mg/kg of T1AM. The control group did not get any therapy. The sham group just completed right parietal craniotomy, but did not receive any injury.

Degradation studies in vivo

For the assessment of degradation in vivo, the Pol hydrogels, which were loaded with 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindotricarbocyanine iodide (DiR) were injected into the injured part of brain after trauma. The TBI models were imaged by the Xenogen IVIS Sepcturm optical device (Caliper, USA) on the 3 hours, 6 hours, and 12 hours.

Temperature and vital sign measurement

Body and brain temperatures were measured by the probes (Omega Engineering, China). Rectal temperature was measured to represent body temperature. Brain temperature was monitored using the implantable probe placed on the surfaced of the cerebral cortex. These data were shown by the temperature acquisition system controlled by an instrument (Omega Engineering, China). Vital sign, including blood pressure, heart rate, respiratory rate and oxygen saturation were continuously recorded by monitor (Anhui Zhenghua biological instrument equipment co. LTD, Huaibei, China) during and for 1 hours following the cooling procedure.

Evans blue extravasation study

BBB permeability was mesured by evaluating the extravasation of Evans blue (EB) at 12 hours post trauma. EB dye (2% 2 mL/kg) was administered intravenously at 9 hours after TBI and then allowed to circulate for 3 hours to ensure that the EB dye was fully circulating through the blood system. After anaesthesia, the 100 mL phosphate buffer saline was perfused through the left ventricle of the heart and removed the brains. The ipsilateral percussion side was separated and weighted. Then the tissue was placed in 4 mL potassium hydroxide (1 M) and homogenized. The 1 mL mixture was blending in 5 mL 0.2 M phosphoric acid and acetone (5: 12) and centrifuged at 3000 rpm for 30 min. The supernatant was transferred and detected the absorbance at 620 nm.

Measurement of lesion volume and histochemical staining

Brain sections were stained with hematoxylin-eosin (H&E staining) and cresyl violet (Nissl staining). The sections were digitized by using Image J software and lesion volume was evaluated according to the Cavalieri's method. Immunohistochemistry was performed as described previously.

Magnetic resonance imaging examination

T2-weighted sequence and Diffusion-weighted imaging (DWI) was evaluated by small animal-specific 7T Micro-MR (Bruker, Bio Spin MRI Pharma Scan 7.0 T). The main acquisition parameters were: repetition time (TR): 2500 ms, echo time (TE): 36 ms, slice thickness: 1 mm, field of view (FOV): 20 ×20 mm, image matrix: 256 × 256.

Enzyme-linked immunosorbent assay (ELISA)

The mice were narcotized and removed the brains at 12 hours after injury. Then the ipsilateral percussion side was separated and weighted. The tissue was fully homogenized in lysis solution and centrifuged at 12000 rpm for 10 minutes. The supernatant was assessed by ELISA kits (Boster Biological Technology, Wu han, China) according to instructions.

Neurologic function assessment

Morris water maze experiment was used to evaluate the recovery of spatial memory and learning ability in mice. All mice were tested for 6 days from 21st day to 26th day after TBI. During the first 5 days, mice were randomly put into water from a certain quadrant and these mice could swim freely in the pool until finding the platform to rest. On the 6th day, the platform was removed and the quadrant was randomly selected. All the data during the test was recorded and analyzed by the video tracking system (Anhui Zhenghua biological instrument equipment co. LTD, Huaibei, China).The residual motor dysfunction was evaluated by the improved neurological severity score (mNSS) and the wire hanging test at 21 days after TBI. The mNSS included rotation, cylinder, corner, and beam tests. In the course of the wire hanging test, the mice were put on metallic wire and inverted the wire. The latency when the mice fall was recorded.

Statistical analyses

Statistical analyses were performed using SPSS version 16.0 and analyzed using one-way ANOVA. The experimental results were given in the format of mean, mean \pm SEM or mean \pm SD in the figures (*P < 0.05, **P < 0.01, ***P < 0.001).

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Declarations

Competing interests. The authors declare no competing interests.

Figures



Preparation and characteristics of the Pol/T hydrogel. (A) Gelation and syringe ability of the Pol/T hydrogel at 37 °C in vitro (a and b). (B) The Pol/T hydrogels were injected into the postsurgical cavity in TBI (a) and the Pol/T hydrogels formed a hydrogel in vivo (b). (C) SEM images of the Pol hydrogels and the Pol/T hydrogels. (D) In vivo release kinetics of DiR from the Pol/DiR hydrogel in response to the post-traumatic environment (a) and quantification of the DiR fluorescence signal (b). (E) CSF was collected from clinical TBI patients by lumbar puncture (a) and the Pol/EB hydrogels were degraded in the CSF from TBI patients (b). (F) In vitro release kinetics of T1AM from the Pol/T hydrogels in the CSF from a TBI patient.



Figure 2

The Pol/T hydrogel provided effective and long-term local hypothermia and avoided systemic side effects that occur as a result of whole-body hypothermia. (A) The mechanism of local hypothermia via the Pol/T hydrogels. (B) The temperature variation in the body (a) and brain (b) of TBI mice along with the time that the mice received different treatments. (C) The variation in heart rate (a), systolic pressure (b), respiratory rate (c), and oxygen saturation (d) with time in the different groups.



Figure 3

The Pol/T hydrogel-induced hypothermia reduced tissue damage. (A) Representative H&E brain sections of TBI mice that received different treatments (a). Representative Nissl staining in the brains of TBI mice that received different treatments (b). (B) Lesion volumes in the different groups. (C) Representative fields of cells positive for TUNEL stain in the different groups. (D) Quantified bar graph of TUNEL+ cell death. (E) Representative fields of Bcl-2 staining around the injury site in different groups (a). Representative fields of Bax staining around the injury site in different groups (b). (F) The ratio of Bcl-2/Bax-positive

cells/field. Asterisks indicate significant differences (*P 0.05 ***P 0.001) between the T1AM group and the Pol/T group.



Figure 4

Local hypothermia induced by the Pol/T hydrogel protected the integrity of the BBB and educed brain edema in the TBI mouse model. (A) Images of Evans blue leakage from brain capillary vessels in the right brains of mice in each group at 12 hours (n = 5). (B) Results are expressed as the mean \pm standard error

of the mean of 5 rats. (C) Expression of MMP-9 in the injured tissue at 12 hours. (D) Number of MMP-9-positive cells/field. (E) Representative images of T2-weighted and DWI in each group at 12 hours. (F) Quantification of the volume of hyperintensity around the injured tissue at 12 hours. (G) Quantification of the ADC signal around the injured tissue 12 hours. Asterisks indicate significant differences (*P 0.05, **P 0.01) between the free T1AM group and the Pol/T group.



Figure 5

Analysis of neuroinflammation in response to different treatments. (A) Expression of Iba-1 in microglial cells in injured tissue on 7 days after TBI (a). Number of Iba-1-positive cells/field (b). Expression of GFAP in astrocytes in injured tissue at 7 days (c). Number of GFAP-positive cells/field (d). (B) Levels of TNF- α (a), IL-1 β (b), IL-6 (c) and CXCL1 (d) in injured tissue by ELISA at 12 hours after TBI. Asterisks indicate significant differences (*P 0.05, **P 0.01) between the T1AM group and the Pol/T group.

Α



Figure 6

Functional recovery assessments by Morris water maze test, mNSS score and wire hanging test on 21 days after TBI. (A) Computer printouts of the swimming trajectories during the learning phase and memory phase. (B) Swimming distance to the platform and (C) searching time for the platform distance at the last trial each day during 5 days of training. (D) Swimming distance to the platform, (E) target quadrant time, (F) duration time on the platform and (G) frequency on the platform. (H) mNSS score was examined. (I) Motor function was evaluated by the wire hanging test. Asterisks indicate significant differences (*P 0.05, **P 0.01) between the free T1AM group and the Pol/T group.



Figure 7

Scheme 1. Formation and mechanism of the Pol/T hydrogel in TBI. (A) Schematic illustration of Pol/T hydrogel preparation procedures and sol-gel transition properties at 37 °C. (B) In situ postsurgical injection of the Pol/T sol into the brain after TBI; the Pol/T hydrogel was formed and biodegraded in the BTE to release T1AM to achieve brain hypothermia. (C) The mechanism of the Pol/T hydrogel for hypothermia treatment. The released T1AM bound to TAAR1 distributed in neurons and astrocytes in the brain, which stimulated cAMP production and led to hypothermia in the brain.

Supplementary Files

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