NEURAL REGENERATION RESEARCH
July 2015, Volume 10, Issue 7
www.nrronline.org

RESEARCH ARTICLE

Stability of rat models of fluid percussion-induced traumatic brain injury: comparison of three different impact forces

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***doi:**10.4103/1673-5374.160100*
***http:**//www.nrronline.org/*

***Accepted:** 2015-05-07*

Abstract

Fluid percussion-induced traumatic brain injury models have been widely used in experimental research for years. In an experiment, the stability of impaction is inevitably affected by factors such as the appearance of liquid spikes. Management of impact pressure is a crucial factor that determines the stability of these models, and direction of impact control is another basic element. To improve experimental stability, we calculated a pressure curve by generating repeated impacts using a fluid percussion device at different pendulum angles. A stereotactic frame was used to control the direction of impact. We produced stable and reproducible models, including mild, moderate, and severe traumatic brain injury, using the MODEL01-B device at pendulum angles of 6°, 11° and 13°, with corresponding impact force values of 1.0 ± 0.11 atm (101.32 ± 11.16 kPa), 2.6 ± 0.16 atm (263.44 ± 16.21 kPa), and 3.6 ± 0.16 atm (364.77 ± 16.21 kPa), respectively. Behavioral tests, hematoxylin-eosin staining, and magnetic resonance imaging revealed that models for different degrees of injury were consistent with the clinical properties of mild, moderate, and severe craniocerebral injuries. Using this method, we established fluid percussion models for different degrees of injury and stabilized pathological features based on precise power and direction control.

***Key Words:** nerve regeneration; traumatic brain injury; fluid percussion; impact force; pressure curve; head fixed; impact peak; animal models; neural regeneration*

***Funding:** This work was supported by a grant from the International Science and Technology Cooperation Projects of China, No. 2011DFG33430.*

Lin YP, Jiang RC, Zhang JN (2015) Stability of rat models of fluid percussion-induced traumatic brain injury: comparison of three different impact forces. Neural Regen Res 10(7):1088-1094.

Introduction

An ideal animal model of traumatic brain injury (TBI) is considered as having good stability, accuracy, and repeatability (Chen et al., 2001; Mahmood et al., 2001; Oliva et al., 2012). The fluid percussion model is one of the most widely used models of experimentally induced TBI in small animals (Yamashita et al., 2011). However, even when advanced fluid percussion devices are used in strict accordance with the manufacturer's instructions, many factors can interfere with the stability of the fluid percussion-induced TBI model. In high-level research, we sometimes need to easily observe and compare TBI models that have different degrees of injury. Thus, improving the stability and reproducibility of the fluid percussion models is one of the most important topics in TBI-related research. In this study, we modified the traditional method of preparing fluid percussion-induced TBI by calculating a pressure cure and improving the method of head fixation, thus allowing for high-level TBI animal experiments.

Materials and Methods

Drawing the pressure curve

Fluid percussion experiments were conducted at different time points, and the pendulum angle ranged from 5° to 13.5°. Corresponding oscilloscope readings were recorded every day. Based on the collected data, we graphed the curves between oscilloscope readings and pendulum angles (**Figure 1A**). After eliminating spike readings, we recorded and graphed pressure curves at the different times (**Figure 1B**). Based on the data shown in **Figure 1A** and **B**, we calculated the mean pressure curve (**Figure 1C** and **D**). Spike features were observed and the corresponding impact effectiveness was analyzed comparatively.

Relying solely on oscilloscope readings when inducing TBI models could result in a greater drift when using a fixed pendulum, which could in turn cause spikes in the oscilloscope readings. When spikes occur, the reading does not match the degree of injury, with the actual degree of injury being less than the readings. Thus, unless the spikes can be eliminated,

using oscilloscope readings to determine the degree of injury is not accurate. In contrast, the pendulum angle has a strong reference value that avoids inaccurate readings, and can be used as a reliable fluid percussion parameter. Furthermore, in this study, excluding the random spikes minimized the drift in TBI degree.

Animals

Sixty-three clean 7-week-old male Wistar rats weighing 300–500 g were provided by the Experimental Animal Center of the PLA Academy of Military Medical Sciences, China. Rats were housed separately with free access to food and water, in a 12-hour day/night cycle. Rats were randomly divided into a control group (n = 6), a sham surgery group (n = 6), and an experimental group (n = 51). The experimental group was subdivided into mild (n = 17), moderate (n = 17), and severe (n = 17) injury groups. Animal housing and care were maintained according to the guidelines for animal care provided by the U.S.A National Institute of Health.

Establishment of TBI models

TBI models were established in the experimental group. Anesthesia was induced by injection of 10% chloral hydrate 3.5 mL/kg, given intraperitoneally. Fur on the top of a rat's head was trimmed as close to the skin as possible. Under sterile conditions, a median incision was made to expose the parietal bone. A 3-mm diameter window in the bone was made 3 mm posterior to the bregma and 2 mm lateral to the sagittal suture, without injuring the dura mater. The rat's head was positioned in a self-developed stereotaxic alignment instrument for fluid percussion using a toothed ring and earrings (**Figure 2**). By adjusting the position, the pulse of pressure was administered vertically downward into the brain tissue. The bone window was connected to the fluid percussion device so that the pulse of pressure could be delivered until recovery of the corneal and tail-clamp reflexes. Pendulum angles were set to 6°, 11°, and 13°, which generated mild, moderate, and severe TBI, respectively. According to fluid percussion pressure curves, the corresponding impact force values were 1.0 ± 0.11 atm (101.32 ± 11.16 kPa), 2.6 ± 0.16 atm (263.44 ± 16.21 kPa), and 3.6 ± 0.16 atm (364.77 ± 16.21 kPa). The control group received no treatment. In the sham surgery group, the rats were anesthetized and a bone window was prepared.

Behavioral function detected by modified neurological severity score

Seven rats were selected in each of the three experimental subgroups. A modified neurological severity score (Mahmood et al., 2012) was used for functional evaluation at 12, 24, 48, 72, 96 and 168 hours after fluid percussion. The maximum score was 18, with higher scores indicating greater severity of injury.

Hematoxylin-eosin staining and MRI analysis

Two rats were selected from each of the experimental subgroups, anesthetized with chloride hydrate (3 mL/kg, i.p.), transcardially perfused with 4% paraformaldehyde, and then decapitated 48 hours after fluid percussion to remove the brain. Brain tissue was fixed in formalin overnight and then cut into 2.5-mm coronal blocks to obtain the center of the injured cortex. Tissue blocks were then embedded in paraffin and serially sliced into 75-μm coronal sections for coronal hematoxylin-eosin staining (Allen, 1992). Pathological changes were observed under a light microscope (Leica, Wetzlar, Germany). An additional two rats were taken from each group for nuclear magnetic resonance imaging (MRI) (GE, Fairfield, CT, USA) at 24, 48, 72, and 168 hours after fluid percussion (Shenton et al., 2012).

Morris water navigation test

A water maze (150-cm diameter, 50-cm high; DMS-2, Chinese Academy of Sciences, Beijing, China) was filled with water containing a small amount of milk powder to conceal the underwater platform location. Water temperature was maintained at 23 ± 2°C. To acclimate them to the environment, six rats from each group were placed in the water tank for 2 minutes of free swimming on the day before training. Formal training began 7 days after TBI was induced by fluid percussion, and was performed at fixed time periods. The water tank was equally divided into four quadrants: northeast, southeast, southwest and northwest. The platform was placed in the center of the southwest quadrant. The rats were randomly put into the water tank at the southeast, east, north, or northwest regions, facing the wall. Escape latency was the time from entering the water tank to reaching the underwater platform. If the rats did not find the platform within 2 minutes, they were artificially guided to the platform, where they remained for 15 seconds. In this case, escape latency was set to 120 seconds. At the end of each training session, rats were dried and caged until their next training session. This training was conducted once per day for 4 days. Escape latency and swimming distance were recorded with a computerized tracking system (Ethovision 3.0, Noldus, Wageningen, Netherlands). Higher escape latency or longer swimming distance in this test means that the rat cannot find the platform and is equated with impaired memory.

Statistical analysis

Experimental data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). All measurement data were expressed as the mean ± SD. Modified neurological severity score data were analyzed by rank sum test, and data from the Morris water-maze test were compared statistically using repeated-measure analysis of variance. A P value < 0.05 was considered to be statistically significant.

Results

Modified neurological severity scores for rat models of fluid percussion-induced traumatic brain injury

We found significant differences between modified neurological severity scores for mild, moderate, and severe injury groups (**Table 1**). Scores were significantly higher in the moderate and severe injury groups than in the mild injury

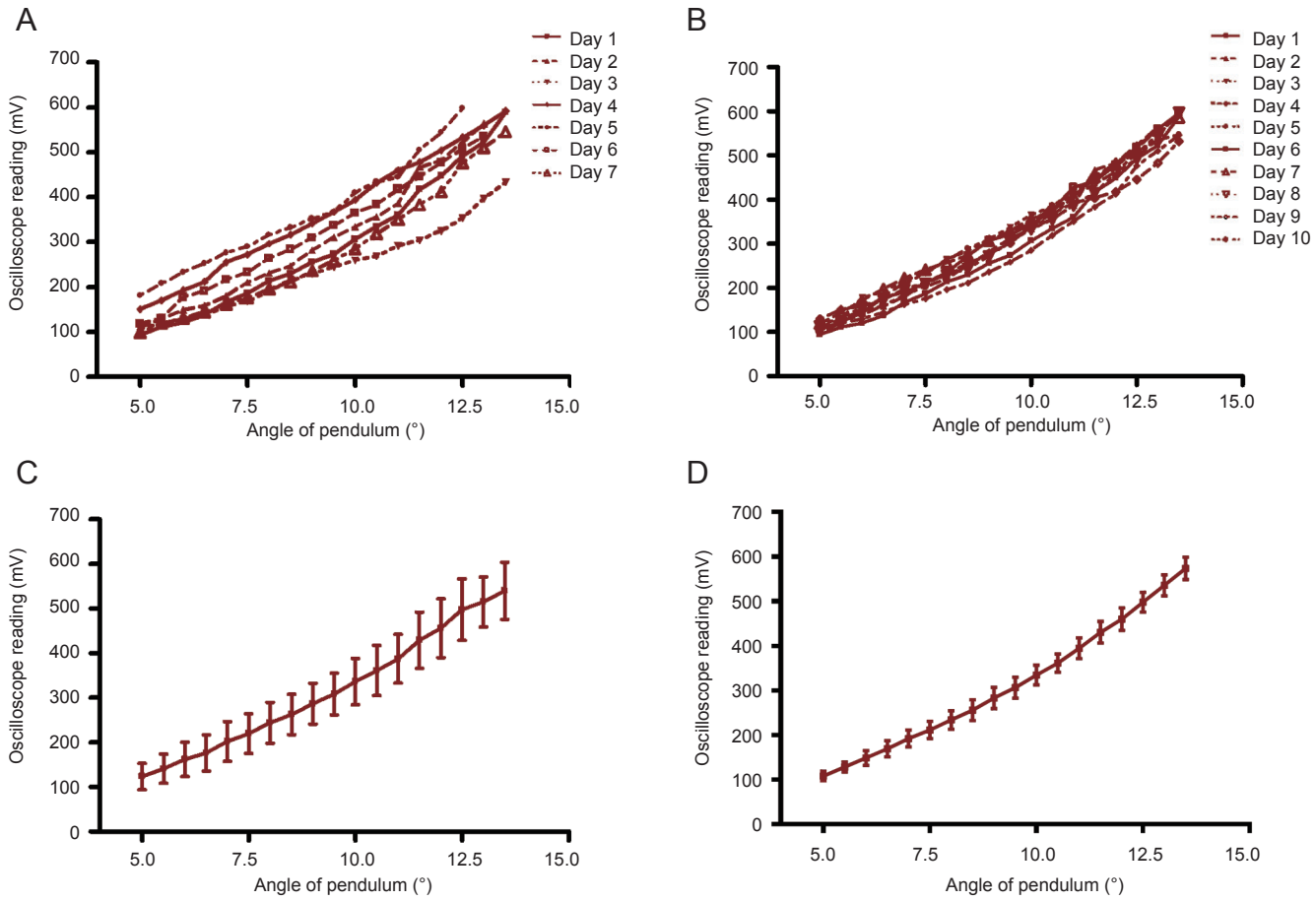

Figure 1 Establishment of fluid-percussion pressure curves.
The oscilloscope readings recorded at different time points were used to determine the curve between the pendulum angle and oscilloscope readings (A), and the mean pressure curve was drawn (C). After eliminating the spikes, new pressure curves (B) and their mean curve (D) were obtained.

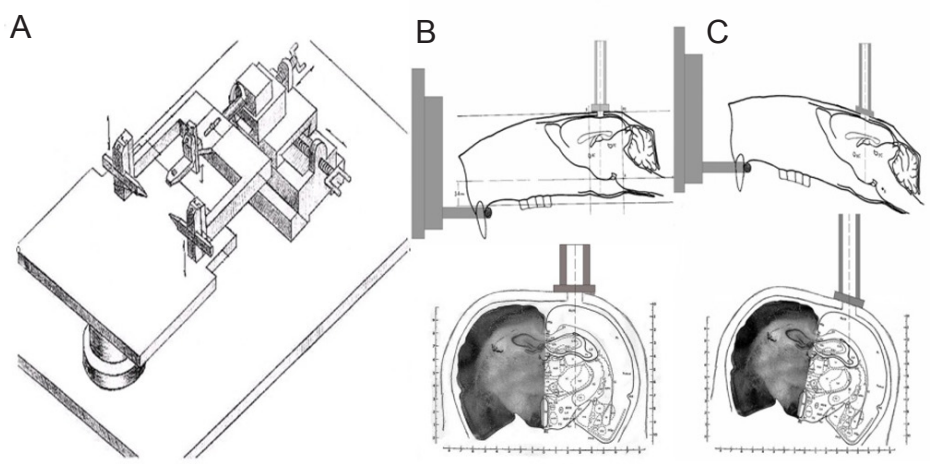

Figure 2 Self-developed stereotactic fluid-percussion device and direction control.
(A) The direction of impact was controlled by adjusting the stereotactic fluid-percussion device, which contributed to rat head fixation, positioning accuracy, and balancing the impact effectiveness. (B) Correct fixation of rats; (C) incorrect fixation of rats.

group at several time points ($P < 0.05$). Moreover, scores were significantly higher in the severe injury group than in the moderate injury group at 24 hours after model induction ($P < 0.05$; **Table 1**).

Pathomorphological changes in rat models of fluid percussion-induced TBI

Forty-eight hours after establishing the models, hematoxylin-eosin staining showed that brain contusion in the mild injury group was mild, small, limited, and did not penetrate the cortex. Peripheral edema was not serious, only a few inflammatory cell infiltrations were seen at the injury site, and there was no substantial hematoma. In the moderate injury group, brain contusion was large and penetrated the cortex. Subarachnoid hemorrhage was visible and spread into the ipsilateral ventricle, edema and inflammatory cell infiltration

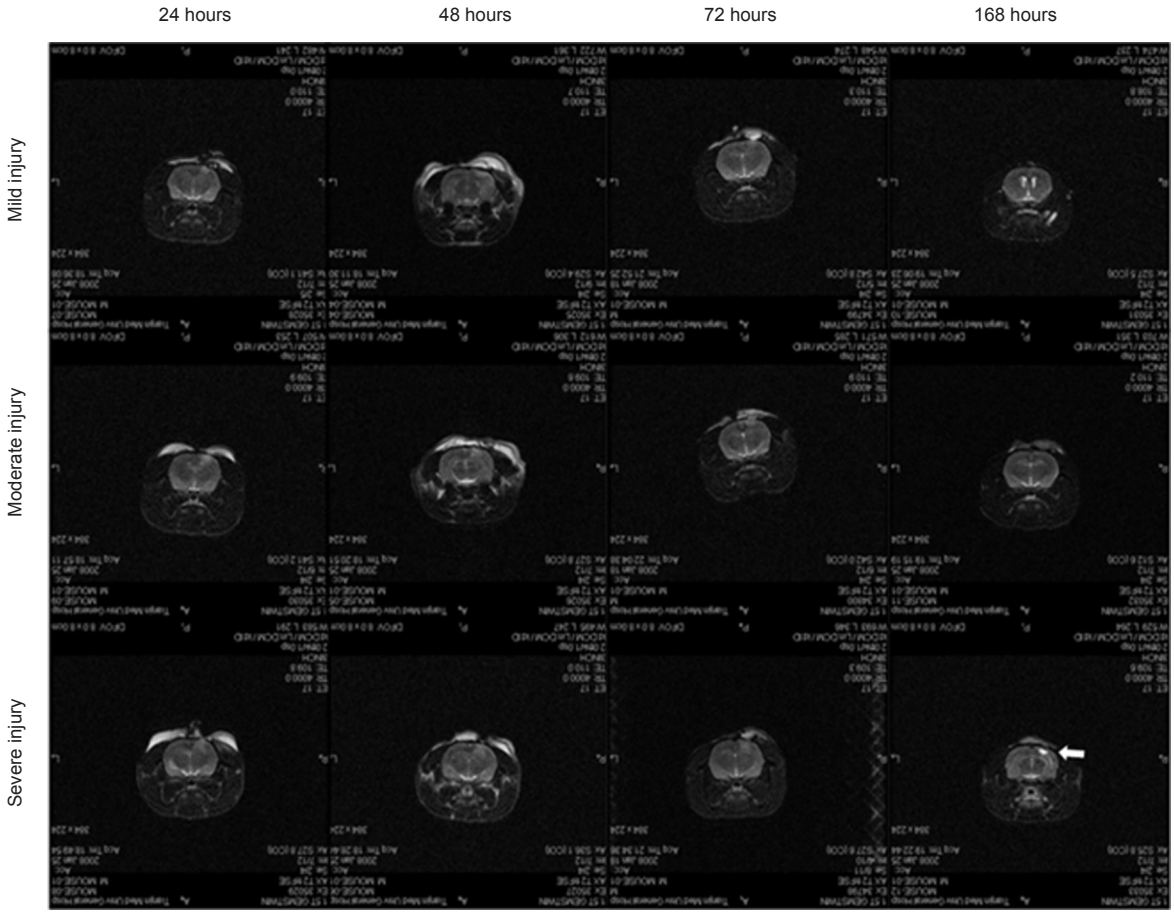

Figure 4 Magnetic resonance imaging in rat models of different degrees of fluid percussion-induced traumatic brain injury.
Significant brain edema was observed after fluid percussion. With increased severity of traumatic brain injury, the area of brain edema became larger. Significant brain necrosis (white arrow) was obvious in the severe injury group at 168 hours after traumatic brain injury. Necrotic voids are clearly visible on the images.

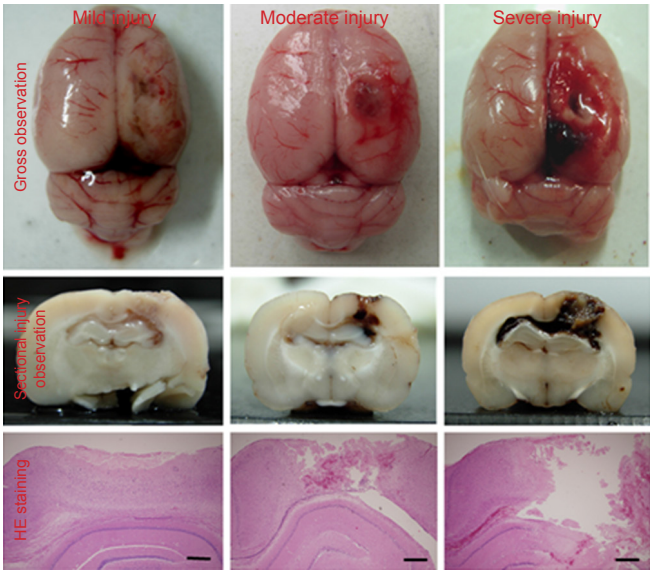

Figure 3 Pathomorphological changes in rat models of different degrees of traumatic brain injury induced by fluid percussion.
Forty-eight hours after the fluid percussion experiment, gross observation, sectional injury observation, and hematoxylin-eosin (HE) staining displayed obvious differences in the degree of injury between the different experimental groups. Scale bars: 500 μm.

significantly expanded and involved the sensorimotor area of the frontal and parietal lobes, and although no significant damage to the hippocampus was found, a small amount of degenerated and necrotic hippocampal pyramidal cells were seen. Further, marked parenchymal hematoma had formed. In the severe injury group, the largest range of brain contusions was accompanied by a wide range of subarachnoid hemorrhage that entered the bilateral ventricles. Injuries involved bilateral cerebral hemispheres, obvious parenchymal hematoma and even multiple hematomas were commonly seen, and peripheral edema was conspicuous (**Figure 3**). Brain necrosis was obvious in the severe injury group, and after 7 days, necrotic voids were clearly visible on MRI, and expanded further within a few weeks (**Figure 4**).

Changes in learning and memory abilities in rat models of fluid percussion-induced TBI

Latency and swimming distance in the Morris water maze did not differ between the control group and the sham surgery or mild injury groups ($P > 0.05$). However, they were significantly higher in the moderate and severe injury groups ($P < 0.05$). Latency was longer in the severe injury group at 3 and 4 days than it was in the mild injury group ($P < 0.05$).

Table 1 mNSS score in rat models of traumatic brain injury

	Hours after injury					
Group	12	24	48	72	96	168
Mild injury	8.7±1.6	6.3±1.9	3.6±1.2	2.0±0.8	1.1±0.7	0.6±0.5
Moderate injury	11.9±2.0†	9.3±1.8†	7.6±1.8†	6.4±1.9†	4.0±1.7†	2.3±1.4†
Severe injury	13.3±1.7†	11.9±1.8†§	9.9±1.9†§	8.4±1.5†§	5.4±1.3†§	3.7±1.6†§

High mNSS score presents severe neurologic impairment. Data are expressed as the mean ± SD, with seven rats in each group. mNSS data were analyzed by rank sum test. †$P < 0.05$, *vs.* mild injury group; §$P < 0.05$, *vs.* moderate injury group. mNSS: Modified neurological severity score.

Table 3 Swimming distance (cm) on the Morris water maze for rat models of fluid percussion-induced traumatic brain injury

Group	Day 1	Day 2	Day 3	Day 4
Control	1,153.2±326.4	733.6±514.4	531.3±234.1	443.5±256.2
Sham surgery	1,107.4±426.8	773.9±347.2	569.0±282.6	322.0±146.8
Mild injury	1,047.4±429.4	1,131.1±485.3	706.9±260.6	399.2±85.4
Moderate injury	1,204.6±343.3	1,143.1±202.4	1,022.8±273.3$^{*\#}$	710.8±207.3$^{*\#\dagger}$
Severe injury	1,212.4±359.6	1,289.6±563.2*	1,164.4±434.2$^{*\#\dagger}$	1,130.8±272.0$^{*\#\dagger\S}$

Long swimming distance represents poor learning and memory ability. Data are expressed as the mean ± SD, with six rats in each group. Results of the Morris water-maze test were compared statistically using repeated-measure analysis of variance. *$P < 0.05$, *vs.* control group; #$P < 0.05$, *vs.* sham surgery group; †$P < 0.05$, *vs.* mild injury group; §$P < 0.05$, *vs.* moderate injury group.

Table 2 Latency (second) on the Morris water maze for rat models of fluid percussion-induced traumatic brain injury

Group	Day 1	Day 2	Day 3	Day 4
Control	55.3±15.7	32.2±18.4	22.0±8.2	17.8±10.8
Sham surgery	56.2±23.8	39.1±16.2	28.2±13.8	16.9±7.9
Mild injury	53.5±18.8	46.2±21.5	32.4±9.6	21.2±4.6
Moderate injury	59.5±18.5	48.3±11.2	43.5±8.5$^{*\#}$	30.9±11.2$^{*\#}$
Severe injury	73.4±17.9	64.3±20.9$^{*\#}$	52.4±15.4$^{*\#\dagger}$	49.9±12.2$^{*\#\dagger\S}$

Long latency represents poor learning and memory ability. Data are expressed as the mean ± SD, with six rats in each group. Results of the Morris water-maze test were compared statistically using repeated-measure analysis of variance. *$P < 0.05$, *vs.* control group; #$P < 0.05$, *vs.* sham surgery group; †$P < 0.05$, *vs.* mild injury group; §$P < 0.05$, *vs.* moderate injury group.

Swimming distance was longer in the moderate injury group at 4 days and in the severe injury group at 3 and 4 days than it was in the mild injury group ($P < 0.05$). At the same time, latency and swimming distance were longer in the severe injury group than in the moderate injury group at 4 days ($P < 0.05$; **Tables 2**, **3**).

Discussion

The fluid percussion-induced TBI model was first established by Lindgren et al. (Lindgren and Rinder, 1967; Lindgren and Kinder, 1969), and improved by Dixon et al. (Dixon et al., 1987; Lyeth et al., 1988). It is currently the most widely used model of experimentally induced TBI. In the past, fluid percussion models have been used to copy different types (Schwartzkroin et al., 2010; Cao et al., 2012; Yan et al., 2012; Hawkins et al., 2013) and levels (Niogi et al., 2008; Ohsumi et al., 2010; Lusardi et al., 2012; Griesbach et al., 2013) of TBI. Common observations after TBI such as brain contusion combined with brain parenchymal damage and subarachnoid hemorrhage can be simulated in a fluid percussion model (McIntosh et al., 1989; Graham et al., 2000; Mukherjee et al., 2013). Although the device is advertised to have impact stability, controlled model preparation and repeatability, non-standardized operation and lack of an effective way to control instability in the fluctuant can directly lead to inaccurate experimental results. The benefits of the fluid percussion device can be severely limited unless these limitations are addressed. Therefore, researching improvements in impact-platform stability has been necessary in recent years.

Many factors can cause impact instability. We summarize these factors according to our experience as follows: First, attention should be paid to the specifications for installing the fluid percussion device to avoid poor sealing and impact instability that can directly affect impact effectiveness. A regulated power supply for the oscilloscope is necessary to avoid voltage instability that contaminates the true data, and interferes with the waveforms and reading accuracy of the oscilloscope. Second, when preparing the animal model, the location and size of the bone window should be consistent. In this study, the bone window was positioned to the side of the midline, which can effectively avoid direct pressure on the brain stem and hypothalamus, and reduce mortality (Thompson et al., 2005; Raible et al., 2012). Third, attention should be paid to maintaining the integrity of the dura mater when cutting out the bone window because rupturing the dura mater can directly affect the local brain surface and significantly increase brain contusion and injuries. Fourth, the fluid percussion process should be standardized. Water was added to the water tank after each impact so that the piston would remain in a fixed position and the pendulum would always fall the same distance. When adjusting the piston position, the gas within the fluid percussion device should be excluded to maintain a uniform pressure-transmission medium, stabilize the impact platform, and enhance the quality of the experiments.

In previous experiments, we found that even when strictly adhering to these guidelines, the consistency of models made by fluid percussion was still unable to meet the needs of high-level research. We conducted a more in-depth analysis and found that relying solely on oscilloscope readings can cause errors that affect the stability of the fluid percussion device. This is largely because spikes appear in the oscilloscope waveform during model preparation. Peak readings often did not match the actual degree of injury; the effectiveness of impact was overrated, and this meant that accurately evaluating impact pressure was not possible. We further found that eliminating sharp waves and only recording peak values of sinusoidal waves were effective in reducing the erroneous oscilloscope readings. This also indicates that sharp waves are a destabilizing factor that can influence the assessment of injury degree. When using the MODEL01-B fluid percussion device, we found that spikes appeared randomly, erratically, and unpredictably, showing no significant relationship with the height of the pendulum or other system-regulating factors. After repeated experiments and consultations with experts in related fields, we found that oscilloscopes are extremely sensitive and able to accurately visualize responses to small changes in pressure, but they are also susceptible to interference. The appearance of spikes may be related to design flaws in the flui dpercussion device. Pressure generated instantaneously is inhomogeneously transmitted across the fluid percussion system, in particular when passing through the "L"-shaped outlet end (the location of the pressure sensor). Additionally, this short-term fluctuation captured by the oscilloscope produces a liquid spike. In the present study, gross observation, hematoxylin-eosin staining, and modified neurological severity score showed that when spikes appeared, the actual degree of injury was milder than what the oscilloscope readings indicated. These liquid spikes can add tens of millivolts to the oscilloscope readings, but are short-lived and too small to pass through the "L"-shaped outlet and thus do not effectively impart pressure on the rat brain. Thus, obtaining oscilloscope readings that exactly reflect impact power is difficult when these spikes are generated.

As effective methods for preventing the emergence of spikes are lacking, we have developed a flui dpercussion pressure curve for this type of fluid percussion device, which can effectively estimate and revise the size of impact power at different angles of the pendulum, thereby increasing the stability of the device. Pendulum impact is the initiating factor for generating TBI. Using the pendulum angle as a measure that reflects the degree of injury has a high reference value, which avoids random distortion of oscilloscope readings and ensures impact effectiveness. We blocked the impact end, sequentially repeated the impacts at different pendulum angles, calculated the impact powers, eliminated the sharp wave values or inaccurate values, and obtained a curve that describes the relationship between pendulum angle and oscilloscope readings (the fluid percussion pressure curve). Because the flui dpercussion pressure curve represents the mean value of multiple impacts, it reflects the true range of fluid percussion with high reliability. Based on our findings, this method is able to overcome error in the oscilloscope readings after fluid percussion procedure.

Based on previous experiments, we found that although there is a certain degree of drift, the impact values of 1.0 ± 0.11 atm, 2.6 ± 0.16 atm, and 3.6 ± 0.16 atm were generated by pendulum angles of 6°, 11°, and 13°, which produced mild, moderate, and severe TBI models, respectively. A certain degree of bias is known to exist between different types of fluid percussion devices, and even between different devices of the same type. When using the fluid percussion pressure curve to guide oscilloscope readings, we recommend that before initiating any TBI models, each laboratory should generate their own fluid percussion pressure curve based on their own equipment. We note that the fluid percussion pressure curve needs to be remade because of slight drifts in the curves that happen along with changes in the environment.

Directional control is also critical, but it is often neglected. Although previous experiments have paid attention to the position and size of the bone window, impact power with uncontrolled direction can cause a divergence in the injury track resulting in injury instability. If the fluid percussion direction is partial to the midline, there can be damage to the thalamus, brainstem, and other important structures that increase mortality. In our study, the self-developed stereotactic fluid percussion device largely solved this problem. This device can be used to firmly fix and adjust the rat head in the sagittal and coronal axis through incisor rings and bilateral earrings, which can easily control the direction and also expand the scope of application for fluid percussion devices. In conventional impact experiments, the direction is always fixed vertically downward by adjusting the incisor rings and bilateral earrings to improve reproducibility of model preparation. By avoiding the brainstem, thalamus, and other important structures, we can increase the upper limit of impact power, enlarge the impact range, and elevate and diversify impact effectiveness, all with an insignificant increase in mortality (Vink et al., 2001; Floyd et al., 2002).

Following these improvements, we created models for different degrees of TBI and evaluated the stability of the model preparation. Experimental results show that the range and degree of injury increased with the enlargement of the pendulum angle, which was confirmed by hematoxylin-eosin staining, MRI observation, and modified neurological severity score evaluation. A statistically significant difference existed between different groups. Morris water-maze training began at 7 days after making the models. Variation in spatial learning ability was used to evaluate the stability of the fluid percussion device. We found that escape latency did not differ between the mild injury and control groups, and the mild injury group showed limited brain contusion, and no evident changes in structure and function of the hippocampus and hypothalamus. Compared with the control group, the moderate and severe injury groups exhibited significant differences in escape latency. Further, the escape latency significantly differed between the moderate and severe

injury groups. These results paralleled the varying damage to the subcortical hippocampus and size of the damaged region. Thus, our modifications to the fluid percussion device significantly improved the stability of the device and can easily be used to create fluid percussion-induced TBI models of different extents, which can be distinguished by morphological and behavioral features.

In summary, we performed an in-depth analysis of the causes underlying drifts in injury degree and reproducibility of fluid percussion injury, and are the first to note the significance of using a standardized process to generate a fluid percussion pressure curve based on individual laboratory conditions. Additionally, our self-developed stereoscopic fluid percussion device for small animals can precisely control the impact direction by adjusting incisor rings and earrings, further stabilizing the fluid percussion platform, and thus improving the reliability and reproducibility of fluid percussion model preparation. Finally, we determined a scale of impact power ranges that can be used for producing models of mild, moderate, and severe injuries. Such a scale improves the efficiency of experimentally preparing models, thereby providing a foundation for further experimental studies. Meanwhile, we recommend that for high-level studies, the fluid percussion pressure curve should be regenerated periodically according to the type of fluid percussion device and the laboratory environment.

Acknowledgments: *We are very grateful to Tong-ling An, Dong Wang, and Lei Zhou from the Department of Neurosurgery, Tianjin Medical University General Hospital in China for their assistance with the technical support of experiments.*

Author contributions: *YPL wrote the paper. All authors were responsible for designing, implementing, evaluating experiments, and approved the final version of the paper.*

Conflicts of interest: *None declared.*

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Copyedited by Phillips A, Frenchman B, Yu J, Qiu Y, Li CH, Song LP, Zhao M