RESEARCH ARTICLE

Experimental study on the establishment and maintenance of brain death model with pigs

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Abstract   It remains controversial that after the transplantation of using grafts from brain-dead donors, organs injury and rejection can influence the effects of transplantation. This study sought to explore methods of establishing a stable brain death (BD) model using Bama mini pigs and to maintain the brain-dead state for a comparatively long period to provide a model for investigating changes in brain death. Sixteen anesthetized Bama mini pigs were randomized into a control group ($n = 5$) and a BD group ($n = 11$). Intracranial pressure (ICP) was increased in a modified, slow, and intermittent way to establish BD. Respiration and circulation were sustained during the brain-dead state. Hemodynamic changes were monitored during the experiment. In the BD group, 10 pigs met the requirements for brain death and 1 died of cardiopulmonary complications following an increase in ICP. Brain death was maintained for more than 48 hours with artificial life support. During the experiment, the heart rate and blood pressure showed characteristic changes due to increased ICP. Prior to BD being established, a "tic reaction" inevitably occurred. We used an improved method of increasing ICP to establish a stable BD model. The BD state could be maintained for more than 48 hours with effective respiratory and circulatory support. Disappearance of the tic reaction was considered to be one of the verified indexes for BD via encephalic pressure increase.

Keywords   brain death; bama mini pig; intracranial pressure

1   Introduction

Organ transplantation outcomes have improved considerably during recent years with progressive improvements in surgical techniques and immunosuppressive regimens. However, in China, the shortage of grafts has greatly restricted transplantation advances. A possible alternative is to use organs from brain-dead donors (BDD). This demands further studies as brain death (BD) in donors may potentially cause graft injury and may also be a risk factor for chronic graft dysfunction [1]. The use of grafts from brain-dead donors remains controversial due to the possible role of BD in organ injury and rejection after transplantation [2,3]. A stable and long-term BD model with large animals must be established. Currently, there are few reports on maintaining the BD state with large animals for longer than 24 hours [4].

It has been shown that increases in intracranial pressure (ICP) play an important role in the physiological process of BD [5]. Patients who are brain-dead inevitably expire despite effective circulatory and respiratory support. This study aims to establish a BD model using Bama mini pigs through increasing ICP and maintaining BD for a comparatively long time using artificial support to simulate clinical BD. Another aim is to consider possible routes of establishing BD, and to provide a consistent model for the study of organ injury and its mechanisms in the BD state.

2   Materials and methods

2.1   Animals

Sixteen healthy Bama mini pigs (inbred strain bred in China), all 6 months of age and weighing approximately 30 kg each were provided by the Experimental Animal Centre of the Third Military Medical University (Chongqing, China). Prior to the study, all the protocols were approved by our institution's animal welfare regulatory committee and all the protocols conform with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health 86-23 and revised in 1985.

2.2 Brain death model

The pigs were fasting for 12 hours with free access to water. Anaesthesia was accomplished with ketamine (10 mg/kg IM), benzodiazepine(10 mg IM), and atropine (0.03 mg/kg IM); 20 minutes later, thiopental sodium (10 mg/kg), pancuronium (0.15 mg/kg), and ketamine (1–2 mg/kg) injected intravenously in the auricular vein.

After the animals were anesthetized, a craniotomy was performed. Foley catheters were then placed in the skull. The pigs were randomly assigned to 2 groups: the BD group (group B, $n = 11$) (in which a BD model was established by increasing the ICP and maintaining it for 48 hours) and a control group (group A, $n = 5$) (in which the animals were anesthetized for 48 hours without increasing the ICP).

A 6.5-mm endotracheal tube was inserted, and a SIEMENS Ventilator 710 was used to provide positive end-expiratory pressure ventilation to maintain an oxygen concentration of 30%–70%. The ventilators were adjusted to the following settings: a tidal volume of 8–10 mL/kg, a respiratory frequency of 18/minute, and inspiration / expiration ratio of 1:2. Catheters were placed in the carotid aorta and the jugular vein to monitor the blood pressure, the rate of infusion, and to perform blood sampling. Arterial pH and electrolyte levels, as well as oxygen partial pressure, were measured using an IRMA blood analysis system and were maintained by adjusting the respirator and infusion. Measurement of urine volume per hour was determined using an 18F Foley catheter placed directly into the urinary bladder via a small laparotomy incision.

The method of establishing BD, as previously described by Pratschke and Lanza [6,7], was used and improved upon. To sum it up briefly, tracheotomized and intubated pigs were fixed on their left sides. A cranial drill was used to penetrate the skull and the lower osseous lamella of the frontal sinus along the median line of the skull. The dura mater was exposed. An 18F Foley catheter was then inserted intracranially, through the hole, pointing toward the porcine tail. The catheter was connected with a pressure transducer to record the ICP. Two electrodes were placed symmetrically on the periosteum of the porcine parietal bone and the occipital bone, respectively, and reference electrodes were attached to the ears. Brain wave changes were measured on an electroencephalogram (EEG). A brain-dead state was induced by inflating the balloon of the Foley catheter. First, the balloon was infused with 10 mL saline, at a rate of 3 mL/min, while changes in EEG, the heart rate (HR), and mean arterial pressure (MAP) were observed for 3–5 min. An additional 5 mL saline was infused at a rate of 2 mL/min to increase ICP until the EEG reading was a flat line. This second step was repeated until no effect on the pigs' blood pressure could be recorded, and they could be confirmed as brain dead.

2.3 Criteria for BD with Bama mini pigs

Criteria for BD verification, which had been previously drafted and instituted by the Health Ministry in China [8,4–7], was used to establish a porcine BD model. These criteria include: (1) a deep coma, as exhibited by a lack of facial muscular activity when thumb pressure was applied to the incision of the porcine eye socket or when the face was pricked with a needle (excluding reversible factors such as anesthesia and low body temperature); (2) an absence of the oculo-pupillary and corneal reflexes, repeated twice; (3) a loss of spontaneous breathing (oxygen saturation and oxygen partial pressure must be maintained by a respirator); (4) an absence of EEG activity for more than 30 min; (5) negative results on an atropine test (ie, with the administration of atropine [1 mg, IV], the animal's HR did not accelerate when monitored for 5–15 min [if the heart rate accelerates by 20%–40%, the test is positive]); and (6) verification of brain death after observing no changes with regard to the previous 5 criteria during 12 hours' time.

2.4 Maintenance of BD state

As changes occurred in the observed indexes, the pigs' life signs under the BD state were maintained until they met the requirements of a BDD [9], that is: MAP $\geqslant 60$ mm Hg, oxygen saturation $\geqslant 95\%$, oxygen partial pressure $\geqslant 100$ mm Hg, body temperature $\geqslant 35°C$, central venous pressure (CVP) $\leqslant 10$–12 mm Hg, and stable hemodynamics. The animals were given saline or Ringer's lactate solution, as well as glucose and colloid solution (hydroxyethyl starch) intravenously at a ratio of 1:6 to crystalloid to maintain stable hemodynamics, electrolyte levels, and acid-base equilibrium. Special circumstances were managed as follows: (1) when systolic arterial pressure was $\leqslant 90$ mm Hg, MAP was $\leqslant 60$ mm Hg, or arterial HR was $\leqslant 60$ beats per minute, and dependence on fluid replacement was unable to maintain stable hemodynamics, dopamine hydrochloride (2–10 µg/kg/min), dobutamine ($\leqslant 15$ µg/kg·min^{-1}), or adrenaline (0.1 µg/kg·min^{-1}) were administered; and (2) if diabetes insipidus ($\geqslant 200$ mL urine per hour) occurred, an intravenous injection of pituirinum ($\leqslant 5$ U/kg·min^{-1}) and noradrenaline ($\leqslant 4$ µg/min) or isoproterenol (2–20 µg/min) were administered.

During the whole experiment, changes in ICP, BP, HR, respiration, oxygen saturation, EEG, and urine volume were continually observed, and porcine body temperatures were maintained at about 38°C using the warming blanket. Data are presented as $\bar{x} \pm s$. Single-factor variance analysis and a two-sample t test were performed. Data were analyzed using SPSS software (Statistical Package for the Social Sciences, version 10.0, SSPS Inc, Chicago, Ill, USA). Values for P less than 0.05 were considered statistically significant.

3 Results

3.1 Establishment of BD model with Bama mini pigs

In group B, 10 of 11 pigs were considered to have met the BD model according to the previously mentioned criteria. One

pig developed a sudden ventricular arrhythmia, ventricular fibrillation and cardiac arrest resulting in death. In group A, all the five pigs were kept anesthetized for 48 hours, revived within 1 hour of anesthetic removal, and then killed. For animals in group A, during the anesthesia period, the oculo-pupillary and corneal reflexes remained intact, the results of the EEG were almost normal, and the results of the atropine test were positive.

3.2 Changes in ICP and hemodynamics

Time 1–9 is defined as follows: after successful anesthesia and before any increase in ICP (time 1), during ICP increase (time 2), first verification of BD (time 3), 3, 6, 12, 18, 24, and 48 hours after the first verification of BD (time 4, 5, 6, 7, 8, and 9). No significant changes were present in BP and HR in the animals in group A. Changes in BP and HR in groups B and A are shown in Tables 1 and 2.

After increasing the ICP, the hemodynamics in animals in group B changed significantly. First, after slowly and intermittently increasing ICP via the Foley balloon catheter, ICP increase was prompt and later exceeded the arterial pressure then remained in the range of 282.81–392.81 mm Hg. Three minutes after an intracranial pressure increase (accomplished by adding 10 mL saline into the balloon of the Foley catheter), the Cushing reflex (i.e., an increase in blood pressure and a lowering of the heart rate—a hypothalamic response to ischemia) appeared, and respiration gradually deepened and slowed. BP and HR patterns appeared wavy, ascending and descending as the ICP increased. The BP and HR patterns, on the whole, showed an ascending trend. 17–40 minutes after the intermittent ICP increases, 18–26 mL saline was added to the balloon of the Foley catheter. Peak MAP values (296.40 ± 10.60 mm Hg) and peak HR values (296.40 ± 14.71 beats/min) were attained. During the period of increasing ICP, the mean MAP value was 183.10 ± 11.52 mm Hg, and mean HR was 183.10 ± 11.52 beats per minute. A significant difference was found between MAP and HR levels prior to ICP increase and after MAP was (117.60 ± 13.75 mm Hg) and HR was (94.50 ± 9.20) beats per minute; $P < 0.05$. Second, after verifying BD, spontaneous breathing stopped. BP and HR

decreased instead of increasing; and ICP suddenly decreased to 118.28–143.05 mm Hg, which was higher than the arterial pressure the whole time. The arterial pressure continued to drop until MAP was $\leqslant 60$ mm Hg or the systolic arterial pressure was $\leqslant 90$ mm Hg, at which point, cardiovascular self-regulation could no longer maintain blood pressure stability. Lastly, 3 hours after the initial verification of BD, MAP levels in the animals in group B dropped significantly compared with those in group A ($P < 0.05$); 6 hours later, there were no significant differences in HR between the animals in group B and the animals in group A ($P > 0.05$). EEG, MAP, and ICP changes are shown in Fig. 1.

3.3 Maintenance of the BD model

In group B, life signs as mentioned above (ie, MAP $\geqslant 60$ mm Hg, oxygen saturation $\geqslant 95\%$, oxygen partial pressure $\geqslant 100$ mm Hg, body temperature $\geqslant 35°C$, CVP $\leqslant 10–12$ mm Hg, and stable hemodynamics) fit the requirements of the brain dead donor. Brain death was maintained for more than 48 hours. After that, the respirator was removed, the infusion was stopped, and all brain-dead pigs were considered cardiopulmonary dead" within a short time.

3.4 Peculiar signs during establishment of BD model

(1) Spontaneous revival: two pigs in groups B demonstrated EEG activity in the occipital region 1–2 hours after verification of BD. At this time, an MAP of 90–120 mm Hg and an HR of 90–120 beats per minute were recorded and the ICP dropped to about 150 mm Hg. Also within this period, encephalic pressure was continuously increased via the Foley catheter, and BD was verified again; however, no "spontaneous revival" occurred, and BD was verified after 12 hours' observation.

(2) "Tic reaction": As the ICP increased, all 10 pigs in group B developed a tic reaction (which ultimately proved to be only temporary) prior to the first verification of BD. At this time, HR and BP rose sharply, and a disorderly brain wave was observed. When the tic reaction ceased, BD was confirmed. Eight of ten pigs developed a tic reaction before

Table 1 Changes in BP in both groups (unit: mm Hg, $\bar{x} \pm s$)

Group	n	Time 1	Time 2*	Time 3	Time 4*	Time 5*	Time 6*	Time 7*	Time 8*	Time 9*
B	10	118.40±9.24	199.60±35.03	103.60±41.21	93.40±28.80	78.60±20.85	69.00±6.20	67.20±5.22	68.80±6.14	63.80±5.54
A	5	115.80±10.66	115.80±10.66	098.00±8.54	115.80±12.48	114.20±10.31	119.80±4.02	110.60±11.01	105.80±6.06	103.60±4.04

Notes: Measurements given are in mm Hg and were determined by chi-square analyses.
*$P < 0.05$

Table 2 Changes of HR in both groups (unit: beat per minute, $\bar{x} \pm s$)

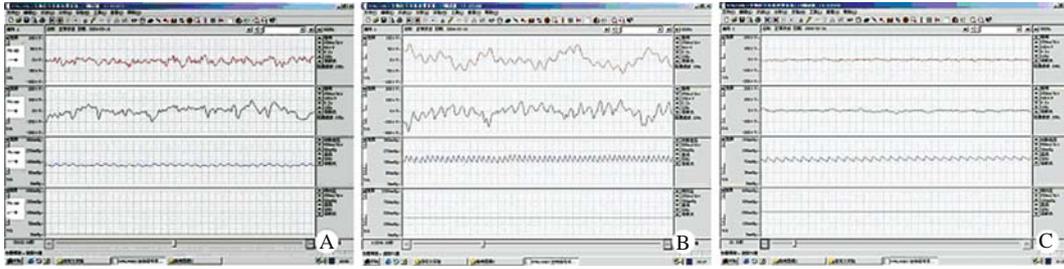| Group	n	Time 1*	Time 2	Time 3*	Time 4	Time 5	Time 6	Time 7	Time 8	Time 9
B	10	100.80±9.55	100.80±9.55	086.80±4.44	096.60±8.62	099.60±6.19	104.00±9.30	098.80±8.81	094.60±5.46	094.00±2.55
A	5	096.60±7.10	197.00±22.69	171.80±34.38	160.40±19.21	142.20±17.14	122.20±25.53	111.40±23.10	105.40±32.51	98.60±225.02

*$P < 0.05$

Fig. 1

A: BP and EEG in group B after successful anesthesia

Note: strips 1 and 2 show changes in the EEG; strip 3 shows changes in MAP; and strip 4 shows changes in ICP.

B: Changes of BP and EEG during ICP

Note: strips 1 and 2 show changes in the EEG; strip 3 shows changes in MAP; and strip 4 shows changes in ICP. ICP and MAP increased sharply, the EEG amplitude increased, and the frequency slowed.

C: BP and EEG at the first verification of brain death

Note: strips 1 and 2 show changes in the EEG; strip 3 shows changes in MAP; and strip 4 shows changes in ICP. Electroencephalogram tended to be straight, MAP dropped, and ICP was higher than MAP.

the first verification of BD, and 2 accorded with porcine BD verification criteria (1), (2), (3), (4), and (5); however, none of the above phenomena happened 1–2 hours after the pigs were spontaneously resuscitated and after ICP was increased a second time. After 12 hours' observation, all EEG activity disappeared, the tic reaction occurred, and BD was verified.

3.5　Pathological changes in the brain

After the animals in group B had been killed, examination revealed that the cerebral parenchyma adjacent to the Foley catheter balloon had liquefied and necrotized; blood could be seen all along brain surface and cerebral herniation was noted. The brain stem had been distorted. The cerebellar tissues at the dorsal margin of the occipital region stuck into the medulla, while at the central side, the inferior part of the medulla dropped into the spinal cord and the tissues were soft, showing pallor and ischemia (Fig. 2). None of the above changes were observed in group A.

Fig. 2　Skull anatomy of the animals after establishing brain death model

Note: Necrosis of the brain parenchyma around the Foley catheter, blood around the brain tissues, with occipital foramen magnum herniation.

4　Discussion

By increasing ICP, a lesion in the intracranial space was artificially induced. ICP was higher than arterial pressure. This resulted in brain herniation, disappearance of brain stem reflex, and a flat line response on an EEG. These signs indicate brain dysfunction. At the same time, the stability of the porcine circulation and respiration function was artificially sustained in group A for more than 48 hours; however, Fig. 1A and B shows a tendency toward circulatory failure. The preceding indicates that without regulation of the central nerve system, heart function may be sustained for a comparatively long time; however, failure is inevitable. In this study, using Bama mini pigs, we created a model that simulated clinical BD and ultimate heart failure.

There have been reports about establishing a BD model [10]. However, those results vary owing to differences in the way the model was established; therefore, it has been hard to reach a uniform and scientific understanding to the physiological changes of BD [2,3]. A report by Wijdicks and coworkers has shown that increasing ICP plays an important role in the physiological process of BD [5]. The current experiment established a model of BD by increasing ICP and showed that after increasing the ICP in the outer cavity of dura mater using a Foley catheter, the ICP rose rapidly and was higher than the arterial pressure the whole time. The blood pressure (BP) fluctuated rapidly. During the early stage, hypertension was noted, which was a compensatory reaction to brain ischemia when the ICP was increased. During the later stages, the pigs were either normotensive or hypotensive. The reaction to the rising ICP disappeared, which suggests that the self-regulation function of blood flow in the brain was on the brink of failing as the pig was close to being brain dead. Certainly, at present, the understanding of how an organism changes after BD remains limited [11], and therefore, the physiological changes of an organism in a state of BD as well as the influence of BD on a donor's organ requires further study.

It has been reported that the longest time for BD maintenance was more than 20 days in a clinical setting [12]; however, few experiments have reported BD maintenance of more than 24 hours [4]. A stable BD model that could be maintained for a longer period is needed to observe and study the organ state and function changes during BD and to understand its potential effects on transplantation.

In this study, we used a modified method of slowly and intermittently increasing ICP, and measured EEG, BP to determine BD. The results showed a higher success rate (91%) (10/11) when compared with earlier studies [13,14] (67.5% and 82.4%, respectively). In addition, the time it took to establish a BD model was shortened.

Usually, this process of increasing ICP is self-limiting and reversible. Nonreversible arrhythmia should be prevented (the incidence in the current study was 9.1% [1/11]). When animals develop paroxysmal ventricular tachycardia or frequent ventricular arrythmia, the increase in ICP should be slowed down or stopped. If there is no effect, an intravenous injection of lidocaine should be administered, and repeated as necessary. If acute left heart failure occurs, an intravenous injection of Furosemide and Cedilanid is preferred.

In an earlier experiment [15], 2 pigs were used to establish a BD model without sustaining respiration and circulation. The Cushing reflex was apparent, and BD occurred immediately after the increase in ICP. With no external intervention, experimental animals usually die from cardiopulmonary complications within 2 hours of verifying BD. In group B of the current study, without respiratory or fluid support, the same results occurred 48 hours later. Therefore, sustaining respiration and circulation is important to the stability of the donor's homeostasis. Our experience was to use a respirator as early as possible to sustain effective ventilation and to provide fluid infusion to prevent non-reversible pulmonary infarction or heart failure.

After BD, the following phenomena were unavoidable: electrolyte imbalance, tissue edema, reduction of effective circulating blood volume, central diabetes insipidus, and internal environment imbalance (which could accelerate the damage of key viscera functions [16]). In the current experiment, we found that diabetes insipidus occurred in all experimental animals (100%) within 6 hours of first verifying BD. Vasoactive drugs such as Pituitrin ($\leqslant 5$ U/kg·min^{-1}) should be used as early as possible to improve microcirculation and sustain effective blood perfusion. At the same time, appropriate fluid replacement must be given, and electrolyte and acid-base balances must be sustained. A respirator should be used to sustain oxygen partial pressure and oxygen saturation and to improve tissue anoxia.

Some special circumstances were noted during the experiment. Spontaneously resuscitation and tic reactions were noted and as of yet have not been reported in related researches.

We observed that the spontaneously resuscitated bama mini pigs accompanied with lowered ICP (less than 100 mmHg) remained in BD state only after the ICP was continuously increased. The reason may be that an acute increase in ICP results in a temporary loss of brain functions and may appear as a BD state. As time goes by, however, encephalic pressure drops via compensatory actions (such as cerebrospinal fluid circumfluence), which brings about partial recovery of brain functions in the brain stem and pallidum. Therefore, at the first verification of BD, physicians should persistently monitor changes in life signs such as EEG and arterial pressure to determine an animal's level of brain activity. In this experiment, on further study, we determined the optimal observation time to be 6–12 hours after the first verification of BD.

Studies have shown that BD is a gradual process, and that encephalic neurons die successively. Usually, the cells die in the following order: brain stem, pallidum, hypothalamus, hippocampus [17], and we suppose the reason for the tic reaction may be similar to that of "decortical rigidity." This tic reaction soon disappeared, which might be related to hypothalamic failure in a brain-dead state. The tic reaction has not been reported until now, and its mechanism requires further study. Based on our results, however, we believe that disappearance of the tic reaction may be considered as one of the first indexes for verifying the porcine BD model established via ICP increase.

Brain death is relevant in many fields such as clinical medicine, law and ethics, and the diagnostic criteria for clinical BD are controversial. At present, the commonly recognized diagnostic criteria are: (1) a cessation of spontaneous breathing (not caused by central nervous system suppressive drugs) with the patient having no signs of respiration resumption for 3–5 minutes after the removal of an artificial respirator; (2) deep coma state, in which patients have no spontaneous activities; (3) dispersed and fixed pupils, disappearance of the light reflex, and immobilization of the eyeballs; (4) disappearance of all brain stem reflexes; (5) a sharp decrease in BP, which must be sustained with drugs that elevate the BP; (6) the existence of spinal cord, segmental spinal cord, and abdominal wall reflexes in some cases. After 6–12 hours' observation, the clinician should check again, and if there is no improvement or change in the above criteria, then BD may be confirmed by a flat line on the EEG, disappearance of vestibular function, and the absence of a tachycardic reaction on an atropine test [18].

Using the above criteria to verify clinical BD, we used the following criteria to establish BD in a porcine model: (1) a deep coma, as exhibited by a lack of facial muscular activity when thumb pressure was applied to the incision of the porcine eye socket or when the face was pricked with a needle (excluding reversible factors such as anesthesia and low body temperature); (2) an absence of the oculo-pupillary and corneal reflexes, repeated twice; (3) a loss of spontaneous breathing (oxygen saturation and oxygen partial pressure must be maintained by a respirator); (4) an absence of EEG activity for more than 30 minutes; (5) negative results on an atropine test (ie, with the administration of atropine [1 mg, IV], the animal's HR did not accelerate when monitored

for 5–15 minutes (if the heart rate accelerates by 20%–40%, the test is positive); and (6), verification of brain death after observing no changes with regard to the previous 5 criteria during 12 hours' time. These criteria are close to those of clinical verification, have been used in the practice of brain-dead animal experiments, and therefore, were adaptable to BD models that used various other methods of induction [19,20].

In conclusion, this experiment used an improved method of slow and intermittent intracranial pressure increase to establish a porcine BD model, which effectively simulated a clinical BD state. The porcine BD state could be maintained stably for more than 48 hours by effectively sustaining respiration and circulation, and may be useful in studying the organ state and functional changes while under a brain-dead state. A tic reaction appeared in all the pigs before BD, and disappearance of this phenomenon may be considered as a verification of BD in a porcine model.

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