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Characterization of the withdrawal phase in a swine controlled intestinal donation after circulatory death model

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Abstract

Purpose: Transplantation of donation after cardiac death (DCD) intestine has higher rates of organ failure and complications. Fortunately, this is less grievous in a subclass of DCD called controlled (CDCD), those with irreversible but incomplete brain injury. The aim of the paper is to establish a CDCD porcine model which is closely mimicking human CDCD scenario, and investigate the physiologic changes from withdrawal of ventilatory support to circulatory arrest.

Method: Ten domestic crossbred pigs were anesthetized and ventilated with room air. Once all baseline data was taken, atracurium besilate (0.9 mg/kg, 3×ED95) was administered and the ventilator was discontinued while the animal was under deep anesthesia to establish the porcine CDCD model. Meanwhile, heparin (150~200 U/kg) was administered after discontinuation of the ventilator. The time to death and the changes of arterial blood gases and hemodynamic parameters were monitored every 5 minutes until circulatory arrest. In addition, histopathology, ultrastructures (via electron microscope) and expression of tight junction proteins of intestinal mucosa were observed at the baseline and the time of death.

Result: The mean time to death was approximately (21.8±3.12 min. Within 5 minutes of removal of the ventilator, there was a hyperdynamic period. Systolic blood pressure and heart rate quickly increased to 118.5±10.4 mmHg and 108.2±4.94 bpm, respectively. Blood pressure and heart rate then reduced rapidly until circulatory arrest. Moreover, the PaO₂ quickly dropped to 17.4±3.13 mmHg, the blood gases throughout the apneic time showed a rapid hypercapnia and acidosis. In addition, warm ischemia damaged intestinal mucosa and reduced TJ proteins expression.

Conclusion: A new swine CDCD model, simulating three stages of “withdrawal of ventilation, systemic anticoagulation and determination of death”, which closely mimics the human DCD scenario and can thus be used in studies related to organ transplantation, was successfully established.

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Manuscript submitted 28th May, 2014

Manuscript accepted 21st September, 2014

Clin Invest Med 2014; 37 (5): E331-E337.

Over the past two decades, advances in organ preservation, surgical transplantation techniques, immunosuppressive strategies and postoperative management have brought small bowel transplantation from an experimental technique to the preferred treatment option for intestinal failure [1, 2]. Treatment success has led to an increased demand for intestinal donors whilst the number of suitable grafts remained static; therefore, researchers have been trying to seek new sources of donors to alleviate this pressure [3].

Currently, donation after cardiac death (DCD), which would increase a small but significant percentage of the current organ donor pool, is considered to be the most promising donor mechanism. According to the European Maastricht Criteria [4], the type 3 donor that serves as the controlled DCD (CDCD) has major but not lethal brain damage, is on life-support treatment (usually mechanical ventilation), and is electively withdrawn from all treatments, with the expectation of cardiac death. The intestinal mucosa is extremely vulnerable to ischemic injury, which has a significant effect on the quality of the intestinal graft and its barrier function [5]; therefore, the intestinal mucosa from typical class 3 donor, with specific warm ischemia time and good maneuverability, has the potential to be the best alternative of intestinal transplantation.

To further investigate the potential use of CDCD for intestinal transplantation, a reliable and clinically-relevant model of CDCD must be established. Although several techniques have been used to induce cardiac death in previous studies, most of the methods, such as hyperkalemia, electrical stimulation and ketamine overdose, have been used to induce cardiac arrest [6-8]. In contrast, a model that uses asphyxia to induce cardiac death could more accurately mimic the human CDCD scenario with the three stages of "withdrawal of ventilation, systemic anticoagulation and determination of death".

To investigate the DCD physiologic events during the time after life-support treatment is withdrawn but before death is declared, a large animal model of intestine donation was developed, which more accurately mimics the human DCD scenario. Using this model, physiological changes during the withdrawal phase can be better characterized and preliminary histological evidence for the effects of cardiac death on the intestinal mucosa after warm ischemia can be provided.

Materials and Methods

Animals

Ten domestic crossbred pigs of either sex, weighing 20 to 25 kg (age 2~3 months), were used in the study after a 5- to 7-d acclimatization. Food and water were provided *ad libitum*. Ani-

mals were treated humanely by use of protocols that were approved by the Institutional Animal Use and Care Committee of Shandong University, China.

Experimental Preparation

After 24-hr fast with water *ad libitum*, all animals were pre-medicated with ketamine (20 mg/kg), diazepam (8 mg/kg) and atropine (0.06 mg/kg), intramuscularly, prior to being intubated and anesthetized with propofol (150 mg/kg/hr) and fentanyl (3-6 µg/kg/hr), intravenously. After endotracheal intubation, the animals were ventilated with room air and the ventilator settings were adjusted to maintain PaCO₂ between 35 and 45 mmHg with a peak inspiratory pressure of less than 25 cm H₂O at any time. Internal jugular vein and carotid arterial catheters were aseptically placed for intravenous access, hemodynamic monitoring and sample collection. In addition, a 12-lead electrocardiogram (ECG) recorded cardiac activity in these animals.

Experimental Protocol

Once all baseline data was taken, atracurium besylate (0.9 mg/kg, 3×ED₉₅, Jiangsu Hengrui Medicine, Lianyungang, China) was administered intravenously as a paralytic, and the ventilator was discontinued while the animal was under deep anesthesia. Respiration rate and heart rate were used as markers for depth of anesthesia. Heparin (150-200 U/kg) was administered intravenously after withdrawal of ventilatory support.

The time of circulatory arrest was determined when a pulse pressure of zero, determined by arterial line, was observed after a 5-min "no-touch" period. Declaration of death was determined by an absence of electrical activity on ECG monitor for 5 min. The time to death was calculated as the time from withdrawal of life support to the time circulatory arrest. Warm ischemia time (WIT) was calculated as the time from withdrawal of life support to the time death was declared.

Arterial blood gases (PaCO₂, PaO₂), pH, lactic acid and hemodynamic parameters, including heart rate (HR), systolic, diastolic pressures and mean arterial pressure (MAP), were recorded (PiCCO₂, PULSION, Munich, Germany) at baseline and every 5 minutes from withdrawal of life support to cardiac arrest (not including the "no-touch" period). The distal intestinal biopsies were taken through a midline laparotomy at baseline and the time death was declared.

Histopathological Evaluation

Intestinal biopsies were fixed by 10% buffered formaldehyde and embedded in paraffin. Histopathology tissue sections,

stained with hematoxylin and eosin (H&E), were evaluated by light microscopy. Histological injury of the intestinal samples was quantitatively evaluated according to the scoring system of Chiu *et al.* in a blinded manner, which is recommended as a standard scoring scale for histological evaluation of intestinal damage [9]. The grades are as follows: 0, normal mucosa; 1, subepithelial Gruenhagen's space (edema) at the apex of villi; 2, extension of subepithelial space with moderate epithelial lifting; 3, large subepithelial space and extensive epithelial lifting with occasional denuded villi tips; 4, denuded villi with dilated capillaries; and 5, lamina propria disintegration, hemorrhage, and ulceration.

Transmission Electron Microscopy

Pieces of small bowel, 2 mm×2 mm, were washed and fixed with 4% glutaraldehyde for 2 h and then post-fixed with 1% osmium tetroxide. Tissues were embedded in Epon 812. Thin sections were cut and stained with uranyl acetate and lead citrate and then examined with an H-600 transmission electron microscope (Hitachi, Tokyo, Japan).

Western Blotting of Tight Junction Proteins

Intestinal mucosal biopsies were frozen in liquid nitrogen until further use. Proteins were extracted from intestinal tissues, separated by SDS-PAGE and then subsequently transferred onto PVDF membranes. The membranes were incubated with primary antibody rabbit anti-ZO-1 (1:50, Abcam, Cambridge, MA, USA), rabbit anti-occludin (1:250, abcam), or rabbit anti-GAPDH (1:500, Beijing ZSGB-BIO, Beijing China) over-night at 4 °C, followed by exposure to secondary antibody (HRP- anti -rabbit IgG, 1:1000, GenScript, Piscataway, NJ, USA) for 2 h at room temperature. The proteins were visualized with an enhanced chemiluminescent detection system (ThermoFisher, Waltham, MA, USA) and exposed to X-ray film. Densitometry of the blots was performed using the Quantity One 1-D analysis software (Bio-Rad, Hercules, CA, USA).

Statistics

All values were expressed as means $\pm$ SD. Student t test was used for statistical analysis by using SPSS 16.0 (IBM, Chicago, IL, USA) Software. A *P* value < 0.05 was considered statistically significant.

Results

General Characteristics

All data was collected every 5 minutes from withdrawal of life support to cardiac arrest during the experiment. The mean time

to death after ventilator removal was 21.8 ± 3.12 minutes (range of 17~27 minutes). There is a sharp drop in surviving animals between 15 and 25 minutes and 80% of animals died during this period (Figure 1).

Systemic Hemodynamic Parameters

As shown in Figure 2, within 5 minutes of removal of the ventilator, there was a hyperdynamic period induced by apnea that affected all hemodynamics. The systolic pressures increased from 113.9 ± 11.52 mmHg to 118.5 ± 10.4 mmHg. And the heart rate increased from 108.2 ± 4.94 bpm to 154.9 ± 7.06 bpm. Then the hyperdynamic reflex response was followed by hypotension and bradycardia and, finally, asystole.

Blood Gases Analysis

As shown in Figure 3, within 5 minutes of removal of the ventilator, the blood gases analysis showed a precipitous decline in PaO₂ (from 123.8 ± 6.76 to 17.4 ± 3.13 mmHg) and a gradual increase in PaCO₂ (from 37.64 ± 1.44 to 77.03 ± 5.32 mmHg) resulting in hypercapnia. Henceforth, acidosis tends to get pro-

FIGURE 1. The Kaplan-Meier curve of survival.

TABLE 1. Average Data at the Time of Death (n=10)

Main Values at the Time of Death	Mean	SD	Range
Time to death (min)	21.8	3.12	17-27
pH	7.09	0.02	7.068-7.124
Lactic acid (mmol/L)	9.00	0.24	8.5-9.25
PaCO ₂ (mmHg)	90.37	1.34	87.6-92.1
PaO ₂ (mmHg)	5.59	0.96	4.5-7.3

FIGURE 2. Hemodynamic parameters from the time of withdrawal of ventilatory support to circulatory arrest. A, Blood pressures; B, Heart rate.

FIGURE 3. Arterial blood gases analysis from the time of withdrawal of ventilatory support to circulatory arrest. A, PaO_2 ; B, PaCO_2 ; C, Lactic acid; D, pH.

FIGURE 4. Morphologic changes of small bowel mucosa (HE×100) and intestinal injury scores. A, Normal; B, Time of death; C, Intestinal injury scores. * $P<0.05$

gressively worse until cardiac arrest, with a correlating decrease in arterial pH (Table 1).

Histopathological Evaluation

Figure 4A revealed integrated villi and compactly arrayed epithelium at baseline. In contrast, when cardiac death was declared, 1) slight edema was evident, 2) an infiltration of necrotic epithelial cells was found in the intestinal villi and 3) the gap between epithelial cells slightly increased (Figure 4B). Based on the Chiu's scoring system, controlled cardiac death was found to promote intestinal mucosal damage induced by ischemic injury (Figure 4C). This result indicated that warm ischemia may intensify intestinal mucosa injury.

Transmission Electron Microscopy

Tight junctions (TJs) are belt-shaped and expand around the apex of epithelial cells. At baseline, the TJs appeared as typical membrane fusions with intact TJ structures and desmosomes (Figure 5A); however, TJ ultrastructures were altered after warm ischemic injury. TJs were discontinuous, with few membrane fusions apparent in intestinal tissues, indicating disruption in TJs morphology. The microvilli were sparse, with irregular length and arrangement (Figure 5B).

Western Blotting of ZO-1 and Occludin Protein

Western blot analysis showed that there was a statistically significant decrease in Occludin and ZO-1 expression post-

FIGURE 5. Electron micrograph of intestinal mucosa tight junction. A, Normal; B, Time of death. Arrows, desmosome; arrow heads, TJ.

FIGURE 6. The expression of ZO-1 and Occludin proteins in the small bowel demonstrated by western blot analysis. Levels of the indicated protein in the normal (N) and the time of cardiac death (D).

cardiac death when compared with baseline ($P < 0.01$) (Figure 6)

Discussion

Clinically, the typical class 3 Maastricht classified donor (CDCD) requires the withdrawal of life-sustaining treatment and the confirmation of death prior to initiation of any organ

procurement or recovery efforts. This process, known as the warm ischemia phase, induces a slow reduction of oxygen delivery to the organ of interest before death is declared and has untoward effects on graft viability, in both clinical and animal research [10-12]. No one has previously addressed the physiologic changes of intestinal mucosa induced by warm ischemia; therefore, an important first step towards the study of the pathophysiologic mechanisms is the development of a relevant, reliable and reproducible model of cardiac death in DCD.

By analyzing 914 DCDs reported to the Scientific Report of Transplantation Recipient (SRTR) from 2002 to 2007, the most common etiologies of donor death were 42% trauma, 29% anoxia, 23% cerebrovascular accident and 6% other [13]. In addition, the typical class 3 Maastricht classified donor usually requires ventilatory support during life-sustaining therapies. In previous studies, cardiac death was usually induced either by electrical stimulation or by administering high concentrations of potassium intravenously [6-8] and warm ischemia was then simulated by waiting for a period of time before organ procurement. It is regrettable that the pathophysiologic characteristics of these models are a far cry from the clinical scenario. In comparison, a model that uses asphyxia to induce cardiac death would serve as a more accurate model for CDCD. Although some studies have developed models of asphyxial cardiac death with extensive clinical applications, no studies are available for small bowel transplantation using an asphyxial cardiac death donor model [14-17].

The current protocol of a slow reduction of oxygen delivery by use of a muscle relaxant ($3 \times ED_{95}$) and withdrawal of ventilation, followed by treatment with anticoagulant was developed to closely resemble the clinical scenario for CDCD. In this model, the mean time to death (21.8 ± 3.12 minutes; range of 17-27 minutes) is comparable to that reported in a retrospective study of patients removed life-support (17 ± 15 minutes; range of 2~50 minutes) [18]. This further indicates that this is a reliable, stable, and clinically relevant swine model of apnea-induced cardiac death model.

A range of physiologic changes were evoked by systemic hypoxia. Our results revealed that within 5 minutes of removal of the ventilator, there was a hyperdynamic period induced by apnea that affected all hemodynamics and was accompanied by rapid hypercapnia and acidosis, which was followed by hypotension and bradycardia and, finally, asystole. These results indicate that the pig was in a state of hypoperfusion before circulatory arrest, indicating that total WIT of organ may in fact be longer than previously appreciated. The results of examination of morphology and western blotting also demonstrated

that the warm ischemia intensified intestinal mucosa injury, and reduced TJ proteins expression.

Although this model has proved useful in the study of the physiologic response in this apnea-induced cardiac death, there are several limitations and shortcomings compared with the clinical scenario. Firstly, the sample size needs to be expanded to improve the reliability of data. Secondly, the relevance of direct comparison between our experimental animals and the clinical setting may be limited, as our experimental animals were healthy pigs whereas the CDCD patients usually have major, but not lethal, brain damage. When removed from the ventilator, a patient may be able to breathe spontaneously for a time before apneic death; therefore, the period of time when circulation (with hypoxia) is still ongoing may be shortened or lengthened, thereby influencing the quality of organs procured. With a few modifications, the model should prove useful for studying all of the events influencing the outcome of CDCD intestinal transplantation.

Our next step is to further evaluate the effect of using extracorporeal membrane oxygenation to restart circulation of DCD and subsequently improve the physiological status of the small bowel before procurement, with the aim of increasing the organ donor pool and closing the gap between available organs and those in need of an intestinal transplantation.

Abbreviations

DCD	donation after cardiac death
CDCD	controlled DCD
WIT	warm ischemia time
HR	heart rate
MAP	mean arterial pressure
TJs	tight junction
SRTR	Scientific Report of Transplantation Recipient
H&E	hematoxylin and eosin
CA	cardiac arrest

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