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An experimental technique for performing fish cardiovascular perfusion in vivo

Yong Peng*, Ru Tian, Wei-Chao Shen, Ya-Kun Ju, Cong-Shan Guo, Xiang-Qian Zhou, Yang-Yang Su, Fan Zhang

Department of Biomedical Engineering, College of Electrical Engineering, Yanshan University, Qinhuangdao 066004, China

*Corresponding author, e-mail: PY81@sina.com

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ABSTRACT: Tissue extraction plays an important role in the study of brain structure and function. To avoid brain tissue autolysis, we propose an in vivo cardiovascular perfusion technique. In this experiment, we selected the carp as a research object and used the medicated bath method to anaesthetize it. Then two infusion needles were, respectively, inserted into the ventral aorta and total primary vein. Subsequently, the brain tissue of carp was perfused with physiological saline containing 10% formalin. Then the carp was decapitated and the brain fixed in 10% formalin solution. Finally, paraffin sections were prepared and observed under a light microscope. The results showed that this technique could effectively avoid the autolysis and thus result in relatively complete and normal brain tissue. The results suggested that the method is simple, reliable, and able to effectively fix the brain tissue.

KEYWORDS: intracardiac catheterization technique, cardiovascular perfusion technique, brain tissue fixation

INTRODUCTION

Well-fixed brain tissue specimens are necessary in some research methods, such as brain tissue slices, immunocytochemistry, pathological specimens, in situ hybridization, in situ PCR, and position mark within brain tissue. Over the years, the technology of animal brain tissue fixation has been applied mainly to terrestrial animals such as rats and rabbits[1–4]. Perfusion fixation of carp brain tissue however has not been reported up to now. The brain tissue is extracted from the fish after its death by conventional methods, but autolytic change often occurs soon after the fish death indicating that this method is not ideal. Cardiovascular perfusion in vivo is a method to fix brain tissue by means of heart perfusion under anaesthesia[4]. Because the brain tissue has been fixed before the death of the fish, the brain tissue autolysis phenomenon can be avoided to obtain relatively complete and normal brain tissue. In this study, we proposed a cardiovascular perfusion technique in vivo to fix the brain tissue of carps, which included intracardiac catheterization technique and cardiovascular perfusion technique.

MATERIALS AND METHODS

Experimental animals

Thirty healthy adult carps with an average weight of 1.0 ± 0.2 kg, and an average length of 37 ± 2 cm, were purchased from the market of Qinhuangdao aquatic product. This study was approved by the Animal Ethics Committee of Yanshan University.

Instruments

The following instruments were used: BQ50S micro-flow variable speed peristaltic pump (Baoding Lead Fluid Technology Co., Ltd.), 1 set of surgical instruments, 2 sets of infusion tube, KDM thermoregulation electric jacket (Jiangsu Jintan medical equipment factory), HH-W420 digital thermostatic Laboratory water tank (Jintan medical instruments factory), 101-2AB electrothermal blowing dry box (Tianjin Taisite Instrument Co., Ltd.), RM2235 tissue slicer (Leica Instrument Co., Ltd., Germany), TS100 inverted biological microscope (Nikon).

Reagents

Eugenol (FA-100, $C_{10}H_{12}O_2$) was obtained from Shanghai Medical Devices Co., Ltd. Dental Material Factory, China, with Lot 201103. Physiological saline, 2% potassium ferrocyanide solution, 10% formalin solution, paraffin, absolute ethyl alcohol, xylene, neutral gum, haematoxylin-eosin were provided by Beyotime Institute of Biotechnology, China.

Carp anaesthesia

The eugenol was mixed with ethanol absolute and water at the proportion of 1:8:6000, and then an

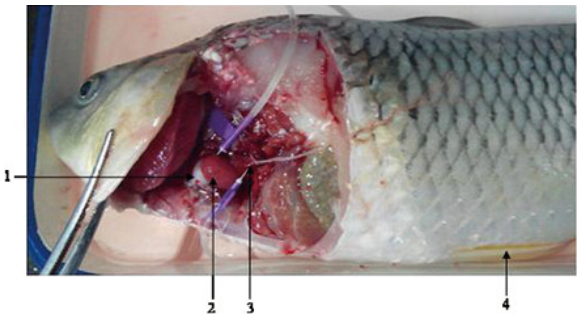

Fig. 1 Carp thoracotomy: (1) bulbus arteriosus; (2) ventricle; (3) atrium; (4) pelvic fin.

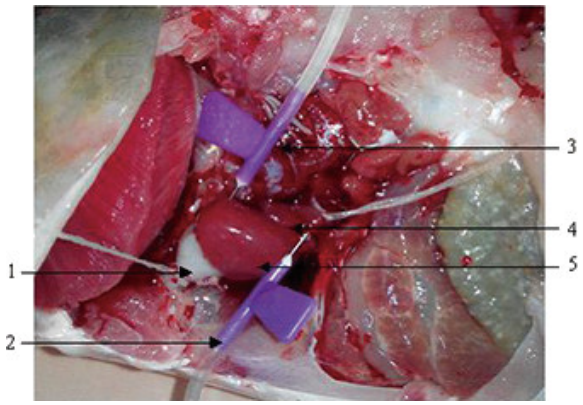

Fig. 2 Experiment of heart perfusion: (1) bulbus arteriosus; (2) total primary vein catheterization; (3) abdominal aorta catheterization; (4) atrium; (5) ventricle.

0.36 g/l eugenol solution was prepared. The carps ($n = 30$) were put into the anaesthetic solution to make them fall into anaesthesia phase A3[5].

Carp thoracotomy

Firstly, an incision was cut from carp abdomen before the pelvic fin, then the carp was cut along lateral line from the incision to the operculum. Secondly, the carp was cut along the vertical direction of the lateral line from the cloaca to the spine, then cut along the spine to the rear of the gill. Finally, the left muscle was removed to expose the ventricles, atria, abdominal aorta, main vein total (Fig. 1).

Vascular intubation

In this experiment we used cardiac endovascular catheterization. First, we connected peristaltic pump with an infusion tube, then passed it through the ventricular wall and inserted it into the ventral aorta using an infusion tube needle. Then we fixed the infusion tube needle with an operative suture through the bottom of the abdominal aorta. The peristaltic pump infused liquid into the ventricle and abdominal aorta through the infusion tube, thus realizing the perfusion of brain tissue. Secondly, we pierced the atrial wall using another infusion tube needle and slowly inserted it into the total primary vein. Then the infusion tube needle was fixed to the total primary vein with an operative suture, and the other end of infusion tube was placed into a graduated beaker, so the volume of liquid flowing out could be calculated in real time (Fig. 2). In this method, two infusion tubes and cardiovascular together constituted the one-way flow of liquid pipeline system.

Organ perfusion

In this experiment a cardiovascular perfusion technique was applied. A peristaltic pump was used for the systemic blood circulation system with constant perfusion, thus completing the brain tissue perfusion. At the beginning of the perfusion, we draw four straight lines on the skull forming a rectangle. Then open the cranial cavity along the rectangle, exposing the brain. In order to prevent blood from forming clots that may block blood vessels, physiological saline was used as a replacement fluid. The dosage of physiological saline was determined according to the colour of the liquid outflow from the atrium. Perfusion was stopped when the outflow liquid was colourless. Then 10% formalin solution was infused at a constant rate. When the brain became white and its volume augmented, the perfusion was stopped and the brain removed.

Fixing specimens

The brain of the carp was immediately fixed for 48 h with 10% formalin solution. The amount of fixed liquid was generally 20 times as much as the volume of the fish brain.

The application of fish in vivo cardiovascular perfusion technique

In the control group, 10 carps were used to make tissue sections with conventional histological method and paraffin section with haematoxylin-eosin (HE) staining. The carp brains were embedded, stained, and changes in morphology observed at a light microscopy without perfusion fixation.

In the experimental group, carps with successful intubation were randomly divided into two groups. One group was perfused and fixed with 10% formalin solution. The other was used to mark positioning sites within the brain tissue by using anode current.

A stainless steel electrode of 40 μm in tip diameter was implanted into specific sites of the brain tissue, then 9 V DC was delivered for 20 s. The brain perfusion was carried out with 2% potassium ferrocyanide and 10% formalin solution by means of cardiovascular perfusion technique. The brain excised from the carp was fixed with 2% potassium ferrocyanide and 10% formalin mixture for 48 h. Then the sections of fixed brain were studied, after HE staining. Localization sites of brain tissue were observed with a light microscope.

RESULTS

Vascular intubation

Intracardiac catheterization technique was carried out in this experiment, which requires two infusion tubes to be inserted into the ventral aorta and common cardinal vein, respectively. The results showed once successful intubation in 14 of 20 cases (70%), twice successful intubation in 4 of 20 cases (20%), three times successful intubation only in 1 of 20 cases (5%). During the process of intubation, one case had a failed intubation because of artery damage, thus the success rate of vascular intubation was 95%.

Organ perfusion

After successful intubation, the carps in the control group were filled with 10% formalin while the carps in the experimental group were filled with 2% potassium ferrocyanide and 10% formalin mixture using a peristaltic pump with constant rate. The results showed each group had 1 case of perfusion-related death, in which the outflow of liquid was more than other successful case. The success rate of organ perfusion was 90%.

Specimens fixing

Brains were taken off from the 17 carps which were successful in vascular intubation and organ perfusion. After being fixed in 10% formalin solution for 48 h, the brain specimens had a normal shape, complete structures, and were in good condition.

Slice contrast

Brain tissue sections are shown in Fig. 3. In the brain tissue slice that was made without heart perfusion the structures were neither complete nor clear, with some damage, and cells appeared necrotic (Fig. 3a). The brain tissue slice prepared after heart perfusion showed complete and clear structures, without obvious structural damage or cell necrosis

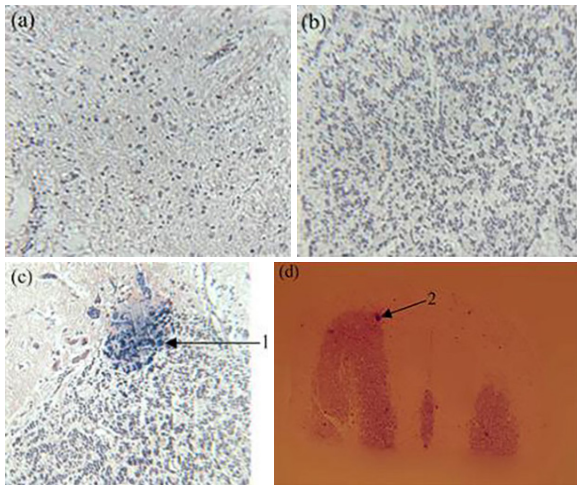

Fig. 3 Tissue slices of carp brain: (a) brain tissue slice made without heart perfusion; (b) brain tissue slice made by heart perfusion; (c) brain tissue slice made with dot mark by heart perfusion; (d) the position of the blue dot in fish brain. The arrow marks the position of the blue dot.

(Fig. 3b). The brain tissue slice c was made with dot mark by heart perfusion. The tissue structure was complete and clear, without significant structural damage or cell necrosis. The brain tissue labelled with blue point was clearly and visible (Fig. 3d).

DISCUSSION

Over the years, fixation technology of animal brain tissue has been used mainly in the research of terrestrial animals such as rats and rabbits[1–4], and no fixation of fish brain tissue has been reported. A new method for fish brain tissue front-fixation is therefore proposed in this paper. The traditional method consists in extracting the brain after the death of the fish, which is a post-fixation method of brain tissue. This fixed method is prone to cause tissue autolysis, therefore it is difficult to obtain brain tissue samples with complete structure in good condition. We put forward a new method which is the heart perfusion technique in vivo, it rinses the blood quickly and fixes the tissues before the animal dies. The heart perfusion technique presented in this article is to fix brain in situ through a cardiovascular perfusion method, which belongs to a former fixing tissue method. This front-fixation method is effective in preventing tissue from autolysis, so it is easier to obtain tissue specimens with integral structures, normal morphology, and the tissue is in good condition. Endovascular heart catheteriza-

tion and cardiovascular perfusion techniques cannot only be good for the fixation of the carp's brain, but also provide a research idea and experimental techniques for the front-fixation of a variety of fish.

In this article, the technique of endovascular heart catheterization was proposed. We use one infusion tube needle inserted into the ventral aorta through the ventricle, while another infusion tube needle is inserted into common cardinal vein through the atrium. Because the carp has only one ventricle and one atrium, blood circulation comprises a single cycle and there is no distinction between systemic circulation and pulmonary circulation. So the perfusate flows out according to the single line in this type of intubation. In this method, it is crucial to insert two infusion tube needles into the ventral aorta and the common cardinal vein, respectively. The results of this study showed that the method is easy to realize with high reliability and easy to operate after practice.

In perfusion experiment, the results showed the perfusion and outflow in a failed case were more than that of the successful cases. It is possible that excessive perfusion lead to brain oedema. In order to achieve a better perfusion results during the operation, it is necessary that (1) the amount of stationary liquid is generally 1.5 times the weight of the animal, (2) the amount of perfusate should be as less as possible, and perfusion time should be short. Excessive perfusion fluid and long perfusion time can lead to tissue oedema, instead of diluting the fixation liquid[6]. In the process of operation, the fluency and integrity of blood circulation is first assured.

During the process of tissue fixation, stationary liquid cannot only avoid tissue autolysis and protect tissues from microbial invasion, but also increase the hardness of tissues and therefore prevent tissue deformation. Currently, paraformaldehyde solution prepared with neutral buffer solution was used to fix specimens. This study shows that although cardiovascular perfusion using 10% formalin solution as a fixative agent gave satisfactory results, the carp had a tremor phenomenon during perfusion. This is an issue that remains to be examined and resolved.

Fish cardiovascular perfusion technology can be used in several fields. We only applied and tested the method in brain tissue positioning mark. The brain tissue localization site was marked using anode current labelling method. The stainless steel electrodes were implanted into specific sites in the brain tissue of the fish. Electrolytic reaction occurred in the presence of applied direct electric current. Fe^{3+} which is the result of electrolysis reacted with Cl^- in tissue fluid, and formed $FeCl_3$. After the iron ions were deposited, the fish brain tissue was perfused with 2% potassium ferrocyanide and 10% formalin solution for fixation according to the cardiovascular perfusion technique. The $FeCl_3$ reacted with $K_4Fe(CN)_6$ in the perfusion solution and formed Prussian blue $K[Fe^{3+}(CN)_6Fe^{2+}]$. The blue spots caused by Prussian blue are the locating points. In this trial, we successfully observed the localized sites in the brain tissue (Fig. 3c). The results showed that the front-fixed technology of brain tissue can be used to mark locating brain sites without damage to brain tissue, providing a new experimental method for the study of brain structure and function.

In our research, the total success rate of intracardiac vascular catheterization was 90%. It is therefore believed that the fish heart cardiovascular perfusion technique may be effective and easy in front-fixation of brain tissue in situ, which can be widely used for a variety of animal brain tissue fixation in research areas such as tissue sections, pathological specimens, immunohistochemistry, in situ hybridization, in situ PCR and brain tissue localization sites markings.

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