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Establishment of a Visualization Model for Regeneration of Damaged Heart in *Xenopus*

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Abstract

Objectives: An animal model of cardiac injury and regeneration is of great significance for deciphering the molecular mechanisms of heart regeneration. Typically, heart regeneration can be surgically induced by removing heart tissue from a lower vertebrate whose heart is capable of complete regeneration. Successful establishment of a visualization model monitoring cardiac injury and regeneration of the Xenopus will provide a feasible method to observe the dynamic process of cardiac wound healing in Xenopus. This model will also facilitate our understanding of the molecular mechanism of cardiac regeneration and help us to develop novel therapeutic strategy to regenerate the damaged heart in mammals. Methods: The cadiac injury model of Xenopus laevis albino was established by excising about 10% of the ventricule. A visualization model monitoring cardiac injury and regeneration of the Xenopus was estabilised by suturing a transparent film in the front of the chest cavity. A stereo microscope was applied to directly observe the dynamic process of the regeneration and repair of the heart in Xenopus after cadiac injury. Results: We established a visualization model of cardiac wound healing in Xenopus laevis albino, and the survival rate of the Xenopus in this model was more than 80%. This method is rapid, low-cost, highly reproducible, easy to manipulate, and not labor-intensive. Combined with Tricaine and hypothermia methods, this model successfully achieved the dynamic observation for more than 10 days of the repiar and regeneration of injured heart in Xenopus under stereo microscope. We found the wound area of the injured heart was filled with the blood scab, and there was no bleeding in the injured heart with normal heart beating. The color of the blood scab in the wound area gradually became lighter with time, and the area of the blood scab continued to decrease. Conclusions: We have successfully established a visualization model which can monitor the dynamic process of cardiac wound healing and regeneration in Xenopus.

Keywords

***Xenopus*; Wound Healing; Heart Regeneration; Visualization Model.**

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1. Introduction

Previous research has shown that the adult mammals, including humans, have very limited cardiac regeneration abilities, the injured hearts can only be repaired with scar tissues [1]. In contrast, lower vertebrates like zebrafish or salamanders, can achieve scar-free regeneration after heart injury [2,3]. Our recent study has established a cardiac regeneration model in adult *Xenopus tropicalis* after resection of heart apex. We found that the heart of adult *Xenopus tropicalis* could regenerate in a nearly scar-free manner in about 30 days after apical resection of around 10% of the ventricle tissue [4]. In addition, recent reports showed that the *Xenopus laevis* injured heart only can be repaired in scar formation manner [5]. Mammalian heart has four chambers, two atria and two ventricles, while fish heart only has two chambers, one atrium and one ventricle. The *Xenopus* possesses a three-chambered heart (two atria and one ventricle), is evolutionarily placed between these two organisms [6]. Previous research showed that the main disease-causing genes are similar between *Xenopus* and human [7-10]. Thus, *Xenopus* has been used as a very important and effective animal model for biomedical and regenerative medicine research.

The myocardium of mammalian including human hearts has a dense myocardial cell structure, which has coronary artery and its branches for blood supply to maintain its normal structure and function. However, the myocardium of the *Xenopus* (including *Xenopus tropicalis* and *Xenopus laevis*) heart is composed of numerous trabecular structures composed of cardiomyocytes. Due to the lack of coronary artery and its branches in the myocardium, the *Xenopus* uses the blood between the trabecular structures as the blood supply. Taking the advantage of this unique structure of *Xenopus* heart, we only need to use a low anesthetic to anesthetize it and put it on the ice, the heart apical is able to be resected in the state of heart beating and without a ventilator. After surgery, the bleeding could be stopped by applying pressure at the wound for around 10-30 seconds. Followed by suturing the chest cavity and skin, the entire surgical process could be completed in about 10 minutes. This established protocol has an over 90% survival rate [4]. *Xenopus* is easy to stop bleeding after heart injury, and can achieve long-term survival, repair and regeneration within a certain range of heart resection, which is very helpful for us to establish a visualization model which can monitor the repair and regeneration process of injured heart *in vivo*. In this study, we used the *Xenopus laevis albino*. On the basis of our previous heart apical resection injury model in *Xenopus tropicalis*, after 10% of the ventricle tissue from the cardiac apex was resected, we put a polyethylene transparent film in front of the chest cavity to construct a closed chest window (about 1cm in diameter) by surgical suture. Thus, we can monitor the process of repair and regeneration of the injured heart *in vivo* while maintaining the survival of the *Xenopus*. Our research shows that this modeling is feasible and can achieve the visual observation of the cardiac wound healing and regeneration process *in vivo*.

2. Methods

2.1 Experimental animals

Xenopus laevis albino (7-8 months old) were applied and maintained in a freshwater tank at 26°C under a 12/12 light cycle. All the experimental protocol related with *Xenopus* was approved by the Jinan University Animal Care Committee.

2.2 Reagents

The main reagents used in this study include: ice particles; clean aerated water (clean water, aerated at least 12 hr); iodine tincture (2.2% iodine, 50% alcohol, can be stored at room temperature for a few months); 1% tricaine solution (weigh 5 g Tricaine and dissolve it in 500ml ddH_2O).

2.3 Materials, equipments and main instruments

The main materials used in this study include: plastic box (25cm × 18cm × 18cm); absorbent cellulose tissue; plastic culture dish (diameter 8cm); absorbent gauze; surgical tape; absorbent cotton; sterile gloves.

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The main equipments and instruments used in this study are shown in Table 1 and Figure 1.

Table 1. Instruments used in this study

Name	Company
Fully motorized fluorescence stereo microscope	Leica
Fine surgical scissors	World Precision Instruments
Small vessel scissors	World Precision Instruments
Dumont tweezers	World Precision Instruments
Scissors, superfine vannas	World Precision Instruments

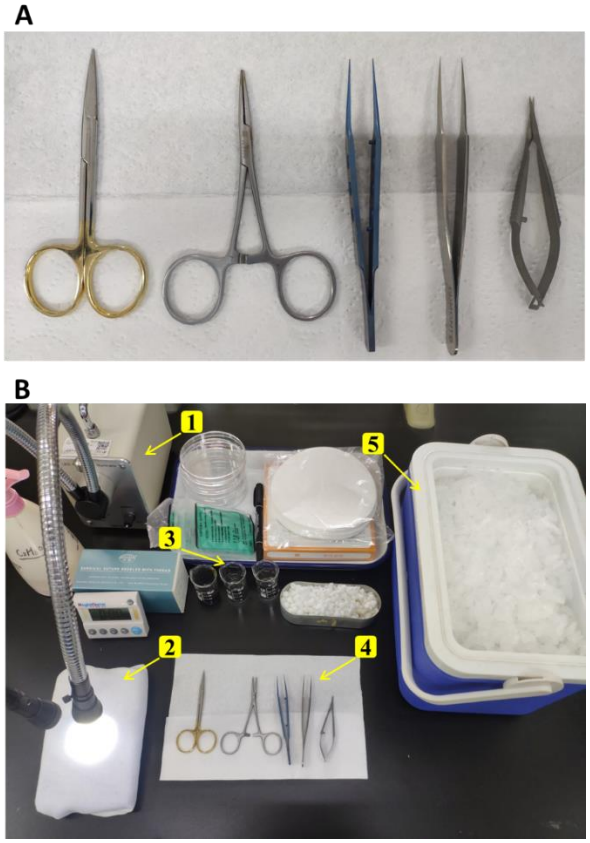

Figure 1. The main experimental device for establishing a visualized model monitoring cardiac injury and regeneration of the *Xenopus laevis albino*.

(A) Surgical instruments required for the operation (see Table 1 for details). (B) The operating table for establishing the visualized model: (1) light source; (2) low temperature operating table; (3) disinfectants such as iodophor and 75% alcohol; (4) surgical instruments, (5) ice box used to hibernate *Xenopus laevis albino* during surgery.

2.4 Establishment of a visualization model of cardiac heart injury and repair in Xenopus laevis albino

(1) The surgical instruments were autoclaved (104.0-137.3 kPa, 121-126℃, 30 minutes) before the surgery. The surgical area was covered with sterile gauze and all instruments were placed as shown in Figure 1.

(2) The *Xenopus laevis albino* was treated with 1% Tricaine solution for 2-4 minutes at room temperature, and then put into ice particles for about 60 seconds to hibernate. Loss of toe-pinch reflex indicates hibernation. Cautions: Check the Xenopus while on ice until there is no toe-pinch reflex. Prolonged exposure to Tricaine solution and hypothermia can lead to increased mortality.

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(3) The *Xenopus laevis albino* was placed on the frozen table with its abdomen upward and its four limbs were fixed with surgical tape. The skin of the chest and upper abdomen was disinfected with iodine tincture and 75% alcohol cotton ball.

(4) According to our previously reported method, about 10% of the ventricular tissue of *Xenopus laevis albino* was resected to establish a heart injury model [4]. A transparent film was placed in front of the *Xenopus laevis albino* chest cavity and sutured through the skin, film and lower muscle layer with a 6-0 suture needle. The edges of the sutures were coated with tissue glue to establish a visualization model that can monitor the cardiac injury repair process in *Xenopus*.

2.5 Observation of the injured area in the Xenopus laevis albino heart using stereo microscope

The observation was under a Leica fully motorized fluorescence stereo microscope. Setting parameters are as following. Exposure: 32.00 ms; gain: 3.8; photograph magnification: 9.45X.

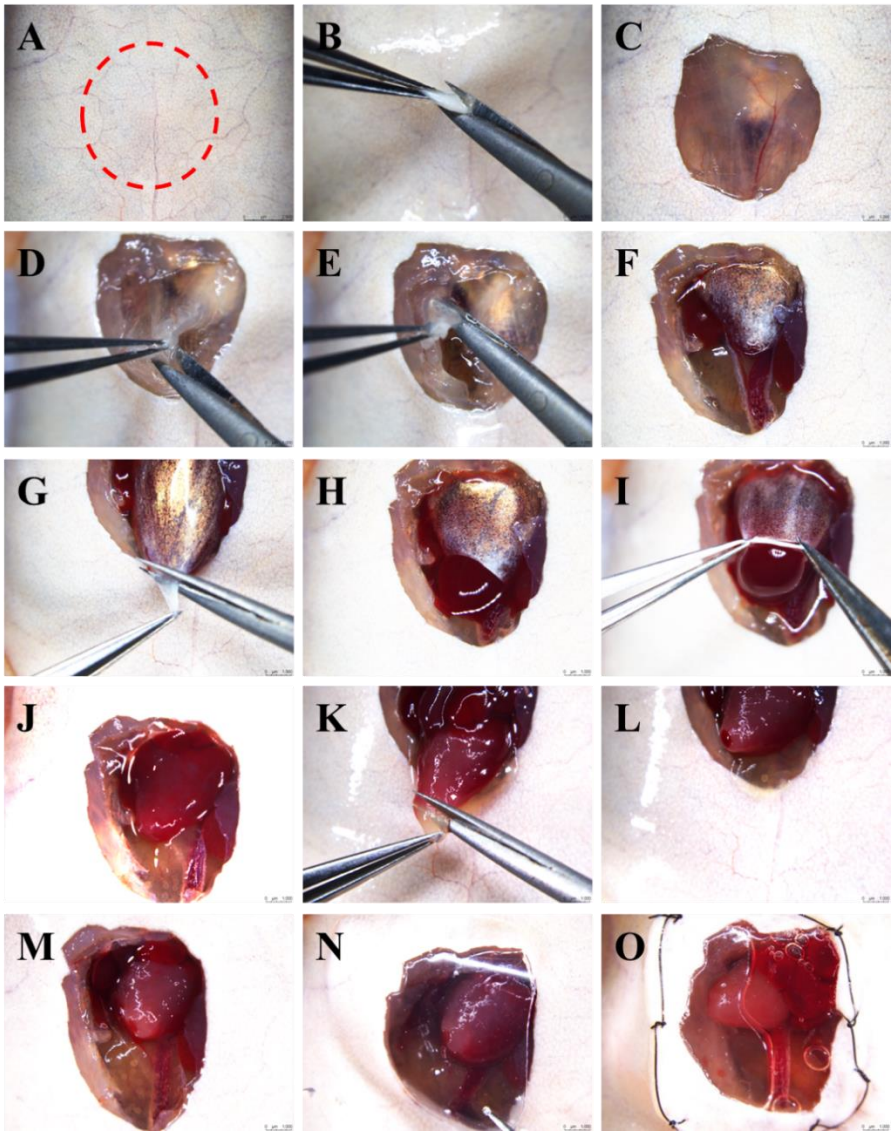

Figure 2. The main steps of a visualization heart model establishment in *Xenopus laevis albino*. (A) The red line shows where the skin was opened. (B-C): The skin was opened at the indicated position. (D-J): The thoracic cavity and pericardium were opened and the heart was exposed. (K): Approximately 10% of the ventricle was removed at the apex. (L): The bleeding was stopped in the damaged heart after 10% ventricle apical resection. (M): The injured heart was put back into the pericardial cavity and a transparent film was placed between the skin and muscle layer. (O): The skin, membrane and muscle layers were sutured together. Scale bar = 1 mm.

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3. Results

3.1 Establishment of a visualization model of cardiac heart injury and repair in Xenopus laevis albino

The surgery location in *Xenopus laevis albino* was determined by the position of heartbeat approximately at 0.8 cm below the middle line of the two forelimb (Figure 2A). The skin was lifted above the sternum with tweezers, and was cut out of an area about 1cm^2 with fine surgical scissors (Figure 2B, C). The muscle layer was cut to expose the heart (Figure 2D-F). The pericardium was carefully removed by blunt separation, so that the whole heart was completely exposed to the field of vision (Figure 2 G-J). The apical position was fixed, and the required size of heart tissue (about 10% of the whole ventricle) was removed with Vannas scissors along the horizontal direction (Figure 2 K). The excised area was pressed with absorbent cotton for about 10-30 seconds to stop bleeding (Figure 2 L). The injured heart was put back into the thoracic cavity, and a piece of polyethylene transparent film with a slightly larger area than the cut area of the skin was placed between the skin and the muscle layer of the *Xenopus* (Figure 2M-N). The skin, membrane and lower muscle layer was firmly adhered with 6-0 sutures (Figure 2O). The edges of the sutures were coated with tissue glue (3M Vetbond). After the glue was solidified, the *Xenopus* was transferred to clean water for a few minutes. The *Xenopus* was then transferred to the conventional breeding system after regaining consciousness.

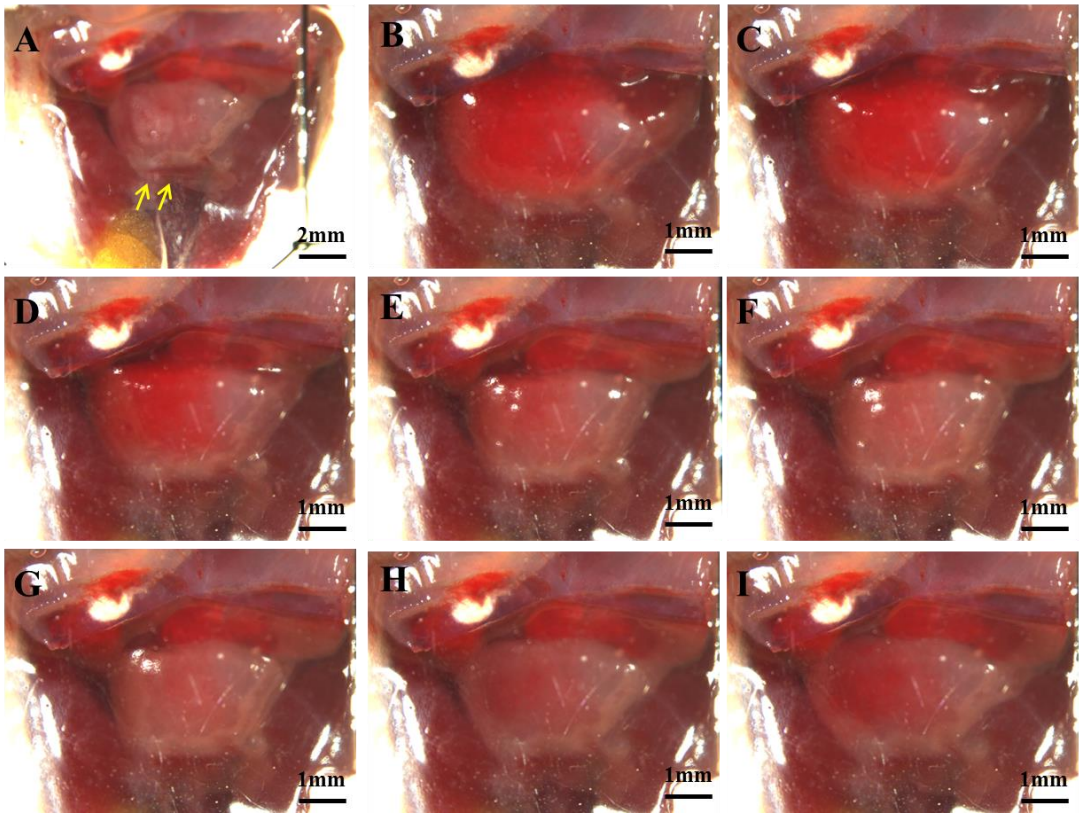

Figure 3. The site of heart injury and heart beating of *Xenopus laevis albino* were observed by stereo microscope. A: the overall appearance after surgery. The yellow arrow indicates the heart apical injury area. Scale bar = 2 mm. B-I: *Xenopus* (1 day after surgery) completed a cardiac contraction and pulsation. Scale bar = 1 mm.

3.2 The site of heart injury and heart beating were observed by stereo microscope

After the visualization model of *Xenopus* was set up as described above, we could use low dose of tetracaine to anesthetize the *Xenopus* and put it in ice water bath for 30-60 seconds. Then we can

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directly observe the repair process and heart beating of the injured part in the *Xenopus* heart by stereo microscope under the condition of maintaining heart beating and body immobility of *Xenopus*. Through observation from the transparent film, we found that the cardiac wound of *Xenopus* had stopped bleeding after surgery (Figure 3A), and the whole beating cycle of the heart could be clearly observed. The *Xenopus* carried out normal contraction and pulsation after operation (Figure 3B-I). The *Xenopus* has achieved long-term survival after the cardiac wound healing and regeneration visualization *in vivo* model has been established.

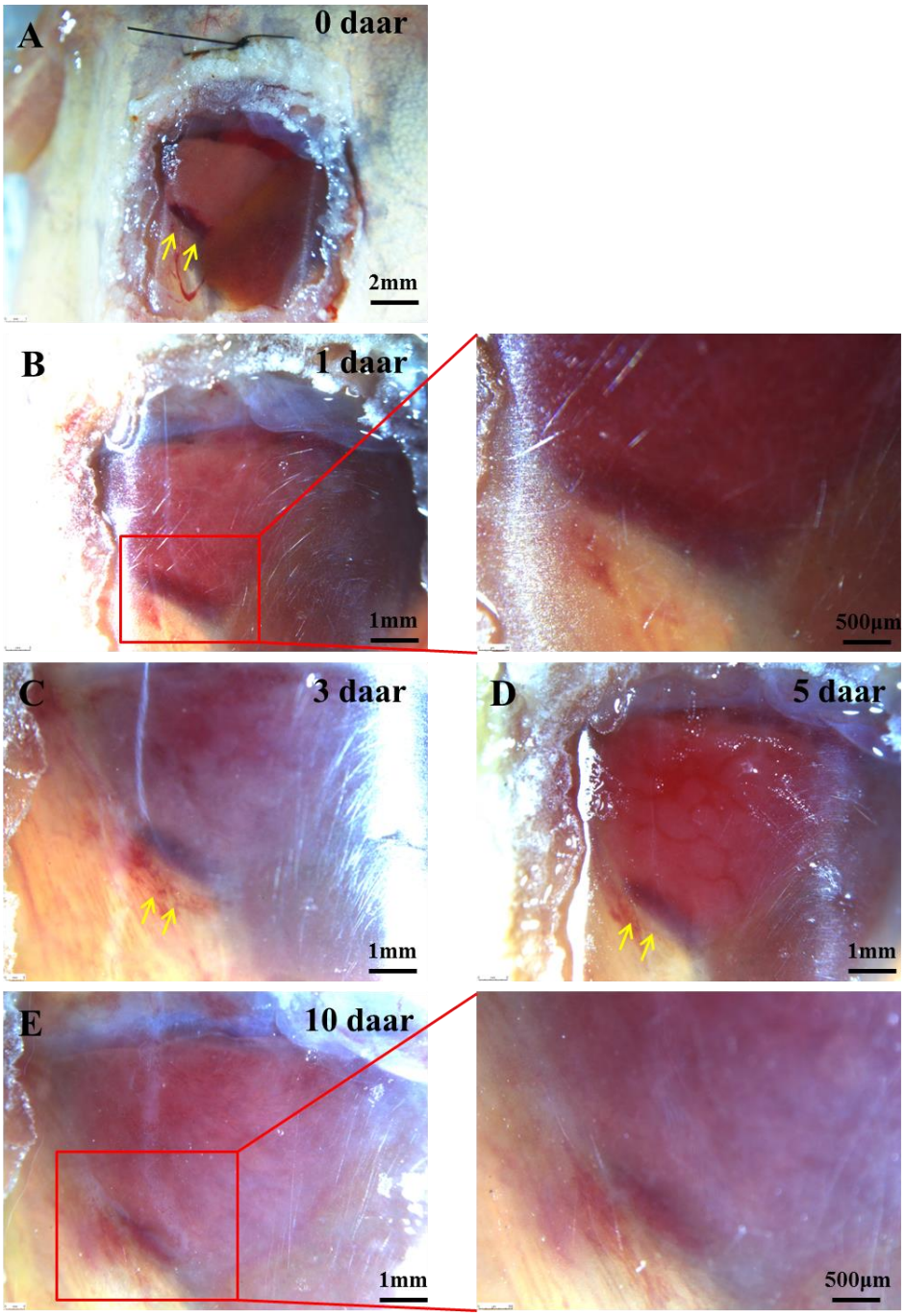

Figure 4. Regeneration and repair process of damaged heart in *Xenopus laevis albino* at different time was observed under stereo microscope.

A: Overall appearance after operation (scale bar = 2 mm). B-E: The images showed the repair process of amputated heart at 1daar, 3 daar, 5 daar, 10 daar (daar: day after operation; scale bar = 1 mm). The yellow arrow indicates the heart apical injury area.

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3.3 Regeneration and repair of damaged heart in Xenopus laevis albino at different time

The dynamic observation for more than 10 days of the injured heart in *Xenopus* from the very begining has been realized using our model under stereo microscope. We found that the damaged heart in *Xenopus laevis albino* carried out normal contraction, and the blood entered and exited the heart with the contraction. The blood scab was formed at the edge of the wound area of the injured heart after operation. With time, the color of the blood scab gradually became lighter, and the area of the blood scab was decreased. On the 1st day after the operation, the wound area of the injured heart had been filled with blood scab, and there was no bleeding in the injured heart with normal heart beating. On the 3rd day after operation, the dark red blood scab in the wound area began to lighten. On the 5th day after operation, the area of blood scab in the wound area decreased significantly. On the 10th day after operation, the area of blood scab in the wound area was further reduced (Figure 4).

3.4 Survival

As described in this protocol, the entire procedure of modeling only took about 30 minutes using low-dose anesthetics and low-temperature. The visualization model can be completed by simple operation, and the survival rate was more than 80%. The consciousness of the *Xenopus laevis albino* can be recovered after about 10 minutes after operation, and its activity was basically normal. On the 11th day after operation, the observation was terminated due to the shedding of the sutured transparent film. The frequent molting of *Xenopus* after operation may result in the shedding of the film. Therefore, searching for a film with good biocompatibility and optimizing the operation technology may improve the survival rate and survival time of the *Xenopus* in our model. The key step is to reduce the food intake of *Xenopus* during 1-3 days after operation, so as to avoid tearing the wound due to excessive food intake.

4. Discussion

In this study, we successfully established a visualization model to observe the repair and regeneration process of the injured heart *in vivo*. A transparent polyethylene film was sutured in front of the thoracic cavity in *Xenopus laevis albino* to construct a closed chest window after apical resection of 10% of the ventricle. After proper suturing which ensured the chest was watertight, the *Xenopus* could survive for at least 10 days with normal activities.

On the basis of maintaining the survival of our *Xenopus* model, we can set different intervals, use low-dose anesthetics and low-temperature methods to keep the *Xenopus* motionless, with heart beating normally. We can directly observe the repair and regeneration of injured heart under the microscope.

Our study showed that this modeling is feasible, and the *in vivo* dynamic observation for more than 10 days of the repair and regeneration process of injured heart in *Xenopus* from the very begining can be successfully achieved. The details and process of heart injury and successful regeneration can be recorded. The survival rate of the visualization model is more than 80%. The establishment of this model does not need the use of tracheal intubation and ventilator, with the advantages of fast, low-cost, highly reproduction, easy operation, and modest labor requirement. In the future, this model can also be combined with fluorescence cell labeling and *in vivo* tracking technology, so that we can dynamically observe the detailed events and processes of heart repair and regeneration that cannot be observed by many common methods, and reveal the process of cardiac repair and regeneration from different aspects. Studies using this model will provide valuable information on mammalian heart regeneration.

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