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Mouse distraction osteogenesis model

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TITLE:

A Mouse Distraction Osteogenesis Model

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distraction osteogenesis, bone, angiogenesis, endothelial progenitor cells, bone marrow stromal cells, callus

SHORT ABSTRACT:

We present a mouse tibial distraction osteogenesis model developed using a custom-made distractor. The use of a mouse as an analysis target is advantageous for advancing research.

LONG ABSTRACT:

Distraction osteogenesis (DO) is a surgical procedure that involves skeletal tissue regeneration without cell transplantation. A DO model consists of the following three phases: the latency phase after osteotomy and placement of the external distractor; the distraction phase, wherein the separated bone ends are gradually and continuously distracted; and the consolidation phase. This custom-made distractor used for DO is comprised of two incomplete acrylic resin rings and an expansion screw. The process was initiated by making a mold with silicone impression material and then creating the custom-made distractor. Dental resin was poured into the formwork made of silicone impression material, and it was allowed to polymerize to create the incomplete resin rings required for the custom-made distractor. These rings were fixed with an expansion screw using transparent resin. The custom-made distractor created via this approach was attached to the tibia of mice. The tibia was fixed to the device using one pair of 25-gauge needles proximally, one pair of 27-gauge needles distally, and acrylic resin. After a latency period of 5 days, distraction was initiated at a rate of 0.2 mm/12 h. The lengthening was continued for 8 days, resulting in a total gap of 3.2 mm. The mice were sacrificed 4 weeks after distraction. Bone formation in the distraction gap was confirmed using both radiography and histology.

INTRODUCTION:

Distraction osteogenesis (DO) is an established treatment method for a variety of skeletal disorders, such as limb length discrepancies, bone defects, and limb deformities¹. This unique treatment strategy is based on the “tension–stress principle” proposed by Ilizarov. The method requires several days for latency, several weeks for active distraction, and several months for consolidation until the mature bone is formed².

The local hypoxic conditions due to blockage of blood flow^{3,4} and mechanical stimulation^{5,6} are particularly important in the healing process of DO. Hypoxia-induced angiogenesis carries oxygen, nutrients, soluble factors and cells necessary for tissue repair locally through the blood flow. Mechanical stimulation by extension operation causes biological reactions such as differentiation of mesenchymal stem cells, bone formation, calcification and remodeling. Serial DO treatment allows the formation of not only hard tissues but also soft tissues, including nerves, muscles, blood vessels, and skin tissues, without the need for stem cell transplantation. Therefore, a DO model is considered to be an excellent model for analyzing the regeneration of various tissues.

Rabbits and dogs are the most widely used animals in basic research for DO; however, there are few analysis tools available for these animals. The use of a mouse DO model facilitates a more detailed analysis. It is particularly suitable for experiments using knockout mice. However, when using a mouse as an experimental animal, an extension device should be created. Here, we present a mouse tibial DO model developed using a custom-made distractor created using a dental laboratory tool and technique, which has been used in a previous study.

PROTOCOL:

All experiments were carried out in accordance with protocols approved by the Animal Care and Use Committee of our institution.

1. Preparation of a Mold for Creating the Custom-Made Distractor

1.1. Make two incomplete rings (outer diameter, 20 mm; inner diameter, 10 mm), which are a part of the distractor, with one sheet of paraffin wax (145 mm x 74 mm) using an Evans wax carver.

1.1.1. Make 4 of the same pieces. Use a wax spatula heated with a gas burner. Stack the four rings for a thickness of 5 mm. Provide space for the expansion screw and needle, 8 mm x 2 mm and 5 mm x 2 mm respectively with the Evans wax carver (**Figures 1A, 1B**).

1.2. Embed wax patterns in the silicone impression material and create a mold for the resin rings (**Figure 1C**).

1.3. After curing the silicone impression material (approximately 5 min at room temperature), remove the wax patterns.

2. Production of the Custom-Made Distractor

2.1. Apply petroleum jelly thinly and evenly to the silicone mold. Mix 3 g of polymerization dental resin and immediately pour it into the silicone mold. Set for 5 min at room temperature (RT).

2.2. Remove the polymerized resin ring from the mold and polish it with a carbide bar and a dental micromotor to remove the burr (about 1 min). Two resin rings are required for each custom-made distractor.

2.2.1. Repeat the procedure to create the required number of resin rings (**Figure 1D**).

2.3. Leave a gap of about 2 mm to fix the resin rings with utility wax and secure the outside of the expansion screw completely with transparent resin. Set the resin for 5 min at RT. When polymerization is complete, remove the expansion screw knob (**Figure 1E**).

3. Surgical Protocol

3.1. Use 8-week-old male mice.

3.2. Induce anesthesia with an intraperitoneal injection of medetomidine hydrochloride at 0.3 mg/g, midazolam at 4 mg/kg, and butorphanol tartrate at 5 mg/kg of body weight.

3.2.1. Carefully shave and disinfect the surgical area with 10% iodine solution, and then, administer 0.5% lidocaine hydrochloride at the right lower limb.

3.3. Make a longitudinal skin incision (approximately 15 mm in length) at the right lower leg with a No. 15 scalpel. Bluntly separate the underlying muscles, taking care not to remove all of the periosteum. Approach from the outside to easily reach the fibula. Cut the fibula with scissors.

3.4. Grasp the ankle with narrow thin forceps, and use a 27-gauge needle to make a hole in the bone about 5 mm from the heel. The needle should penetrate the skin, bone, and skin in that order. When the needle penetrates, cut the tip and root with a nipper so that the needle is about 15 mm.

3.4.1. Make another hole in the same manner, about 2-3 mm proximal. Hold around the ankle and pass two 25-gauge needles under the knee in the same manner. After the needles penetrate, cut the tips and roots with a nipper (**Figure 1F**).

3.5. Place the custom-made distractor such that it is parallel to the extension direction. Fix the needles and device with enough polymerization dental resin to fill the grooves of the device. Wait for the polymerization to complete (approximately 5 min) (**Figure 1G**).

3.6. Be careful not to damage the surrounding tissues. Cut the middle of the diaphysis of the tibia using a very thin cutting disc while applying a saline solution (1-2 mL).

3.7. Close the wound with a 4-0 nylon suture.

3.8. Give subcutaneous injections of buprenorphine (0.1 mg/kg) for analgesia immediately after operation. Continue buprenorphine every 12 hours through postoperative day 7 and as needed thereafter.

4. Distraction Protocol

Note: There are various reports on the latency period and distraction rate, but here, representative protocols are shown.

4.1. After a latency period of 5 days, start distraction at a rate of 0.2 mm/12 h. For extension, use the pin attached to the rapid expansion screw. Move the pin in the direction of the yellow arrow attached to the extension screw (**Figure 1E**).

4.2. Anesthesia is not necessary during extension. Hold the tail with the little finger and palm, and fix the extension device with the forefinger and thumb.

4.3. Perform extension (0.2 mm for 1/4 turn). Continue lengthening for 8 days, which will result in a total gap of 3.2 mm.

5. Analysis

5.1. Radiography analysis: After completion of extension, evaluate bone regeneration with computed tomography under general anesthesia by using 2.0% isoflurane.

Note: If care has been taken with regard to the position of the extension device, it is possible to evaluate it with simple radiography even with the device attached.

5.2. Histological analysis: Remove the apparatus carefully so as not to cause a fracture distraction site during sampling. Fix the sample in 10% neutral buffered formalin for 24 hours at room temperature. Decalcify with Morse's solution (10% sodium citrate, 20% formic acid) overnight at 4 °C.

REPRESENTATIVE RESULTS:

Figures 1A and **1B** present incomplete rings (outer diameter, 20 mm; inner diameter, 10 mm; thickness, 5 mm) with paraffin wax. Two wax patterns were embedded in silicone impression material, and a mold for the resin rings (**Figure 1C**) was formed. Polymerized resin was immediately poured into this mold, and resin rings were obtained (**Figure 1D**). A custom-made distractor was created by combining two resin rings and an expansion screw (**Figure 1E**). **Figure 2A** presents typical radiography findings at 4 weeks after distraction in the model. Newly formed bone was observed at the distraction site. **Figure 2B** presents the hematoxylin and eosin staining results. A newly formed bone bridge was observed, and the newly formed bone could be easily distinguished from the native bone. There was no intraoperative fracture or postoperative infection in the 5 mice used in this experiment.

FIGURE AND TABLE LEGENDS:

Figure 1. Custom-made distractor. (A, B) The approximate dimensions of the ring are as follows: outer diameter, 20 mm; inner diameter, 10 mm; thickness, 5 mm. The spaces for the expansion screw (white arrowhead) and needle (black arrowhead) are secured. (C) Wax pattern embedded in the silicone impression material. (D) Completed resin rings. (E) The two resin rings and the expansion screw are secured with resin. (F) Four needles are inserted into the proximal and distal metaphysis of the tibia. (G) Distraction device placed in the mouse limb.

Figure 2. Radiological and histological findings. (A) Radiologically, newly formed bone is observed 4 weeks after distraction. (B) Histologically, the gap is filled with newly formed bone (bar = 500 μ m).

DISCUSSION:

When a large animal is used as an experimental model, a ready-made extension device can be used, and it is easy to obtain good fixation and assess the extension operation itself and the extension amount. However, when a mouse is used as an experimental model, it is necessary to develop some or all of the equipment. Isefuku *et al.* and Tay *et al.* made the device and created a mouse model^{7,8}. Carvahjo *et al.* adopted a method involving fixation of a ready-made expansion device (track distractor: KLS Martin) with a ligature wire⁹. We could also create a mouse DO model using existing products for extension devices and devising a fixation method. This approach would make it easy to manufacture the extension device, perform extension, and assess the extension amount. Moreover, it is expected that a tibial segmental defect model might be created using the same device. Additionally, the length of the defect can be adjusted in steps of 0.2 mm, and thus, the device is considered to be versatile.

In such an experiment, it is technically difficult to thread the needle through the tibia. In particular, the diameter of the distal part of the tibia is small, and some training is required to pass two needles. If sufficient bone is not noted, it is necessary to check whether good fixation is obtained. In order to obtain good fixation, it might be better to pass the needles at an angle and with a gap as wide as possible between the needles^{7,8}. Additionally, as the rate of healing and the histology during healing would change according to the method of osteotomy, it is necessary to perform the procedure in a certain manner.

The size of the experimental animal can be considered as a limiting factor of this experiment. There are no problems when the mouse is large; however, if it is too small, surgery itself may not be possible. Previously, we used 4-week-old mice and were able to perform experiments without any problems¹⁰. With regard to the evaluation period, extension devices could be installed without problems until 42 days after cutting the bone. However, as further observations were not made, additional research is necessary in the future. The occurrence of complications might be an issue when advancing experiments. Fracture during surgery might occur in approximately 1 out of 30 mice. Similarly, postoperative wound infection might occur in approximately 1 out of 30 mice.

In conclusion, we presented a mouse tibial DO model developed using a custom-made distractor.

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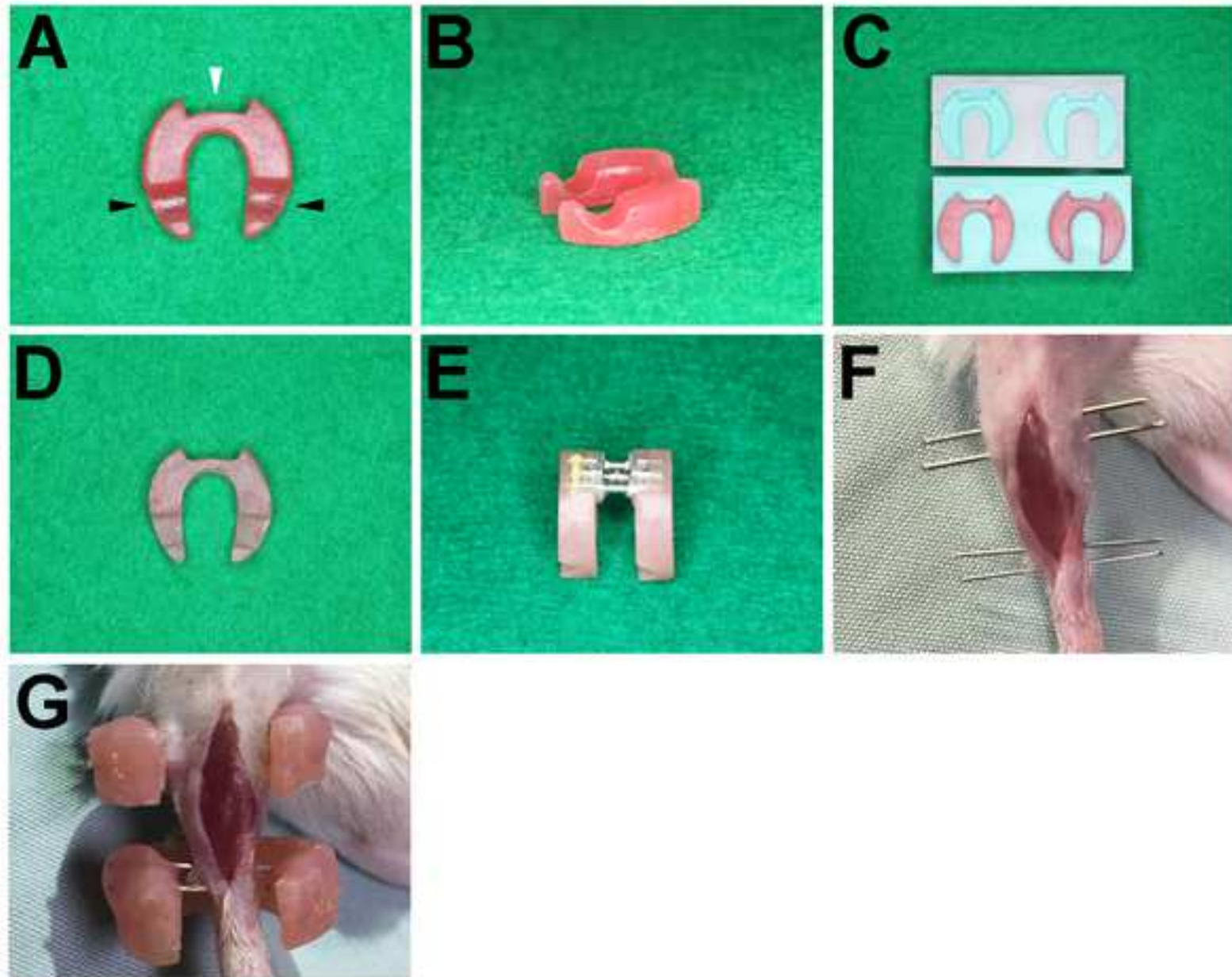
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The authors have nothing to disclose.

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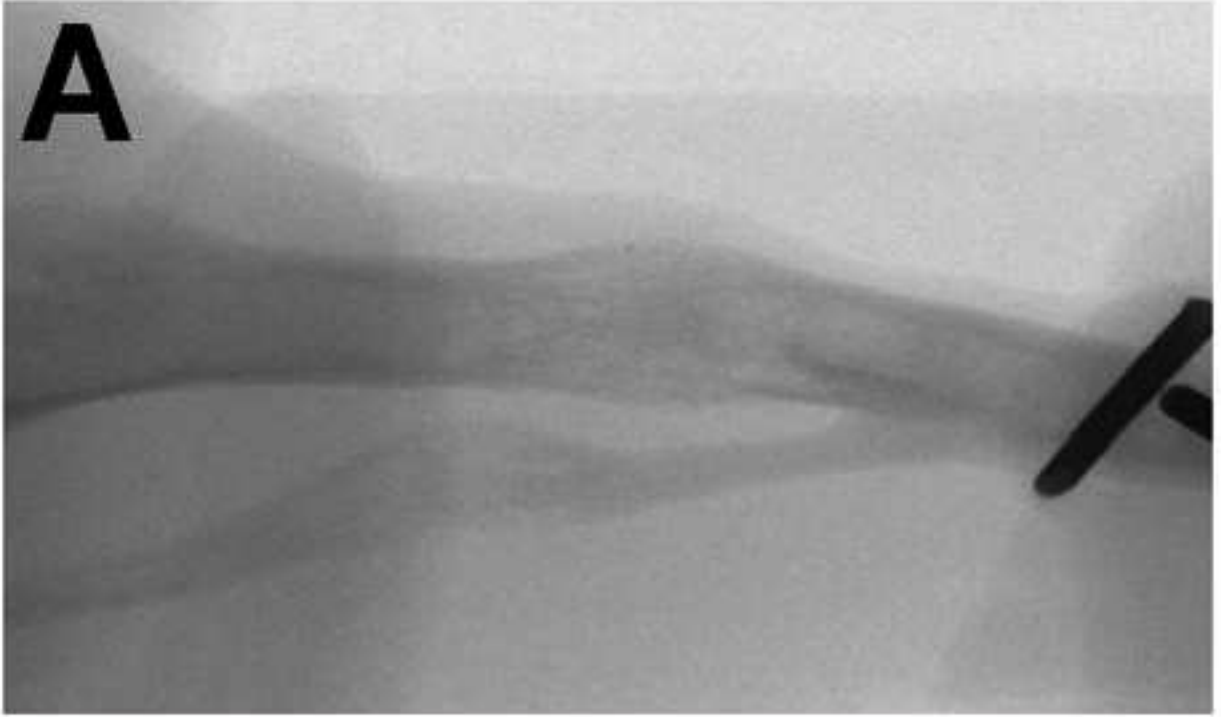
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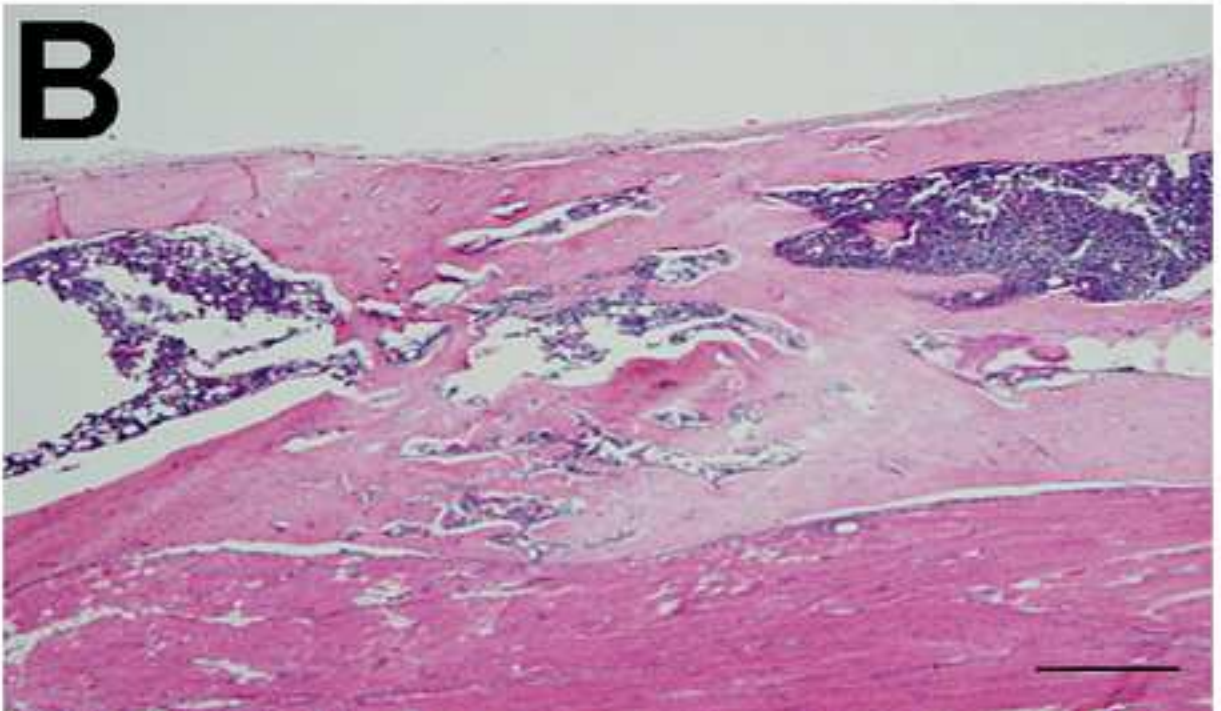


2

A



B



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Paraffin wax	YAMAHACHI DENTAL MFG. CO.	—	For preparation a mold for resin rings
Labocone putty	GC Corporation	—	For preparation a mold for resin rings
Utility wax	GC Corporation	—	For preparation a mold for resin rings
Expansion screw	Ortho Dentaurum	600-301-30	Component of custom-made distractor
Unifast III	GC Corporation	—	Immediate polymerization resin Component of custom-made distractor
Ortho Crystal	NISSIN	—	Transparent resin Component of custom-made distractor
25-gauge needle	TERUMO	NN-2516R	For custom-made distractor
27-gauge needle	TERUMO	NN-2719S	For custom-made distractor
ICR mouse	Chubu Kagaku Shizai Corporation	—	Experimental animal
Somnopentyl	Kyoritsu Seiyaku	—	Pentobarbital sodium salt
Isoflurane	FUJIFILM Wako Pure Chemical Corporation	099-06571	Isoflurane inhalation solution



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1. Please expand the Introduction to be 3-6 paragraphs with sufficient references.

- The authors expand the Introduction to be 3 paragraphs with references.

2. Additional details are needed in the manuscript. Please see the comments in the attached manuscript.

- The authors rewrote them basically according to the instructions of the editor. Also, I think that lack of information is sufficiently supplemented with video.

3. Veterinary Review:

The use of the needles is an unique method for providing external fixation for the fracture that was created (see attached picture). Thus, the use of the needles don't specifically concern me. However, the lack of analgesia is a major welfare concern and I am quite surprised that this was not brought up by the reviewers. Fracture models are considered to cause significant pain and only using lidocaine on the skin is unacceptable. The mice should have received pre-emptive analgesics prior to the surgery and been maintained on appropriate analgesics for the length of the study.

- We apologize for the incomplete description. I described the current anesthesia that improved the method that was done in the past, consideration for distress.