

RESEARCH ARTICLE

Establishing a new alveolar cleft model in rats to investigate the influence of jaw reconstructions on orthodontic tooth movement



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ABSTRACT

Background: The aim of the present investigation was to develop a new cleft model in rats that allows alveolar cleft repair and subsequent tooth movement.

Methods: A complete continuity-interrupting alveolar cleft was performed on the left-side maxillae of 33 rats through ultrasonic surgery. The clefts were filled with bone wax, and microCT scans were done to analyze the cleft size. After four weeks, the cleft repair was completed using autologous, xenogeneic (human), or synthetic bone substitute. After an additional four weeks, the orthodontic tooth movement was initiated.

Results: Fourteen rats died during the research, and the study design was constantly adapted accordingly. The main reasons for death included breathing problems during or immediately after the experimental activities (eight animals), followed by two deaths due to circulatory failures. In the remaining 19 animals, the average cleft size was about $2.70 \pm 0.46 \times 2.01 \pm 0.25 \times 1.18 \pm 0.20$ mm, and the mean velocity of orthodontic tooth movement after seven days was between 0.21 ± 0.08 mm in the autologous group and 0.50 ± 0.54 mm in the xenogeneic group. After 56 days, the mean values ranged between 0.67 ± 0.27 mm in the autologous group and 0.82 ± 0.72 mm in the synthetic group.

Conclusions: Surgical interventions in the oral cavity of rats requires a stronger anesthesia and lead to increased risk of coolant and coagulated blood aspiration. The new alveolar cleft model in rats allows for subsequent orthodontic tooth movement after cleft repair, but only in the mesial root of the first molar.

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1. Introduction

Upper jaw reconstruction in the context of cleft repair can be done by different types of bone grafts, like autografts (e.g., iliac crest, cranium, tibia, rib, and mandibular symphysis), allografts or xenografts, and synthetic bone substitutes (e.g., bioceramics, polymers or biocomposites) (Aalami et al., 2004; Bajaj et al., 2003; Kamal et al., 2018; Sharif et al., 2016). However, bone grafts from the iliac crest have proven to be particularly suitable for the reconstruction of the upper jaw due to the crest's osteogenic, osteoinductive, and osteoconductive properties, and they are considered the gold standard for cleft repair (Canady et al., 1993). Nevertheless, efforts continue to improve surgical techniques and bone substitutes to

enhance the clinical outcome. However, the effects of different bone substitutes and materials, their long-term effects, and their influence on subsequent orthodontic tooth movement into the reconstructed jaw are uncertain.

In corresponding animal research, rodents are often preferred as a model that is accurate, stable, reproducible, and cost-effective (Nguyen et al., 2009). Rats belong to the smallest category of laboratory animals that are still large enough to tolerate the trauma of surgery, while being easier to handle and house. Additionally, compared to larger animals, they are less expensive with regard to purchase, husbandry, and operative costs (Aalami et al., 2004; Nguyen et al., 2009; Sun et al., 2015). Different rat models in experiential cleft research have been described as focusing the bone responses at recipient sites (Cheng et al., 2017; Jahanbin et al., 2016; Mehrara et al., 2000; Mostafa et al., 2014; Nguyen et al., 2009; Ru et al., 2016a,b; Sun et al., 2015, 2017). In general, a distinction can be made between mid-palate cleft (MPC) models in the anterior part of the maxilla (Cheng et al., 2017; Mehrara et al., 2000; Mostafa

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et al., 2014) and alveolar cleft (AC) models as a central (Jahanbin et al., 2016; Mostafa et al., 2014; Nguyen et al., 2009) or posterior (Ru et al., 2016a,b; Sun et al., 2015, 2017) cleft defect. Those in the posterior part are usually accompanied by the extraction of the first molars.

Mehrara et al. first introduced critical-sized MPC models in rats (Mehrara et al., 2000). They developed the MPC model with a size of about $9 \times 5 \times 3$ mm into both halves of the premaxilla and demonstrated complete bone healing by 12 weeks. In addition, Mostafa et al. and Chen et al. described alveolar cleft defects in the anterior part of the rodent maxilla behind the incisors (Cheng et al., 2017; Mostafa et al., 2014). In contrast, Nguyen et al. presented the first critical-sized AC in rats by creating a complete central alveolar defect from the maxillary incisors to the zygomatic arch in eight-week-old Sprague-Dawley rats (Nguyen et al., 2009). They reported healing rates of approximately $43 \pm 5.6\%$ at four weeks and $53 \pm 8.3\%$ at eight weeks. Furthermore, the regeneration of new bone was observable. A similar critical defect in size and location was reported by Jahanbin et al. (2016).

In addition, the AC defects are often accompanied by injuries to the incisor roots (Mostafa et al., 2014). Therefore, Sun et al. modified the AC models by the extraction of the first molar in combination with the use of bone wax (Sun et al., 2015, 2017). Hereby, it was possible to position the defect more dorsally, and it enabled subsequent tooth movement into the reconstructed part of the maxilla. They reported about an initial defect size of approximately $4 \times 4 \times 3$ mm and a significantly faster healing rate if no bone wax was used. They concluded that filling the artificial cleft with bone wax would affect the regeneration of new bone and could be used to create an alveolar cleft model.

However, the MPC and anterior AC are unsuitable for subsequent tooth movement through the reconstructed jaw, and the posterior AC does not represent a complete interruption of continuity of the alveolar ridge due to the remaining palatal bone and—owing to the defect location—both types of cleft models.

The present investigation introduces a new cleft model in rats that enables the creation of a complete alveolar ridge interruption. This cleft-like defect allows to study the use of bone substitutes for reconstruction that will only be supplied from two sides in context of a simplified way of orthodontic tooth movement. Maybe these special anatomical conditions will influence the healing and remodeling process in a different way compared to other cleft models. However, both the general anesthesia protocol and the operative procedure during the process of establishing this new cleft model were difficult.

Therefore, the aim of the present study was on the one hand to present a new alveolar cleft model in rats and on the other hand to demonstrate its implementation with focus on particular problematic incidents. These mainly concerned the general anesthesia protocol and the operative cleft creation, which will be described in detail to inform other researchers and improve animal welfare.

2. Material and methods

The original experiment called for the use of 24 animals, but the number had to be increased due to the high number of losses at the beginning as part of the establishment process. 14 animals died during the surgery or were forced to be excluded during the experiment due to a worsened state of health.

2.1. Sample size calculation

The original *priori* sample size calculation was performed using a one-way ANOVA as part of the main research project with regard to root resorption during orthodontic tooth movement in different

cleft repairs. The original study design (split-mouth, two clefts for dependent observations per animal) envisaged the use of eight animals per group (type of cleft repair), including one rat for dropout. Due to adjudgments in study design (one cleft for an independent observation per animal) 7 animals per group were necessary including an assumed drop-out rate of 25%. The final sample size calculation was based on the mean apical root resorption, estimated from Ru et al. with regard to their findings, in animals treated with xenogeneic and synthetic bone substitutes for cleft repair (Ru et al., 2016a). Sample size estimation relying on the large observed effect (0.0605 vs. 0.089) and the corresponding difference between xenogeneic and autologous bone was assumed to be half of this difference between the xenogeneic and synthetic bone substitute. The common standard deviation was considered 0.01, which corresponds to 10% of the highest value for mean root resorption reported by Ru et al. (2016a). The significance level was set to 0.0125 to reflect the measured root resorptions and an effect size of 1.3538 was characterized to reach a power of at least 80% in a one-way ANOVA model with three groups. No control group existed because the present study aimed to establish a new alveolar cleft model in rats to investigate the influence of jaw reconstructions on orthodontic tooth movements.

2.2. Animal welfare

All experiments were conducted in accordance with the German animal welfare law (Tierschutzgesetz, TSchG) and the EU Directive (2010/63/EU). The study protocol was approved by the Governmental Animal Care and Use Committee (Reference No.: 81-02.04.2018.A342; Landesamt für Natur, Umwelt und Verbraucherschutz Recklinghausen, Nordrhein-Westfalen, Germany; dated: 11.01.2019. The study protocol also complied with the ARRIVE Guidelines (Kilkenny et al., 2010) and the Guide for the Care and Use of Laboratory Animals. All animals were group-housed in filter-top cages (Type 2000, Tecniplast, Buguggiate, Italy), low-dust wood granulate was used as bedding (Rettenmeier Holding AG, Wilburgstetten, Germany) and for cage enrichment nesting material (Nestlet, 14010, Plexx B.V., Elst, The Netherlands).

General human endpoints were defined as body weight decrease $\geq 20\%$, body condition score (BCS) ≥ 2 , signs of severe disturbed circulation, and self-mutilation or uncoordinated behavior. Furthermore, trial-specific symptoms like loss of the bone substitute, acute infections with pus formation, massive bleeding, as well as locomotion disorders were set as specific human endpoints. Finally, 19 animals went through all the interventions of the experimental setup as planned and could be included in the analysis. These rats demonstrated clinically normal body conditions (BCS ≥ 3.0) (Ullman-Cullere and Foltz, 1999).

2.3. Surgical procedures

The final research process is shown in Fig. 1. Two operations were conducted. First, experimental alveolar clefts were created in the left side of the upper jaws of eight-week-old male Wistar rats with an average weight of 465 ± 34 g ($N = 19$). Four weeks later, this was followed by cleft repair in the then 12-week-old animals, which had an average weight of 504 ± 36 g ($N = 19$). All animals were randomized before participating in this investigation into three groups based on the kind of bone substitute used for cleft repair. Finally, with regard to the kind of cleft repair, the following distribution was shown to those animals that remained in the experiment over the entire period: autologous bone from the hip ($N = 6$), xenogeneic bone (human bone substitute material) (maxgraft, botiss biomaterials, Krems Austria) ($N = 6$), or synthetic material (biphasic calcium phosphate) (maxresorb, botiss biomaterials, Krems Austria) ($N = 7$).

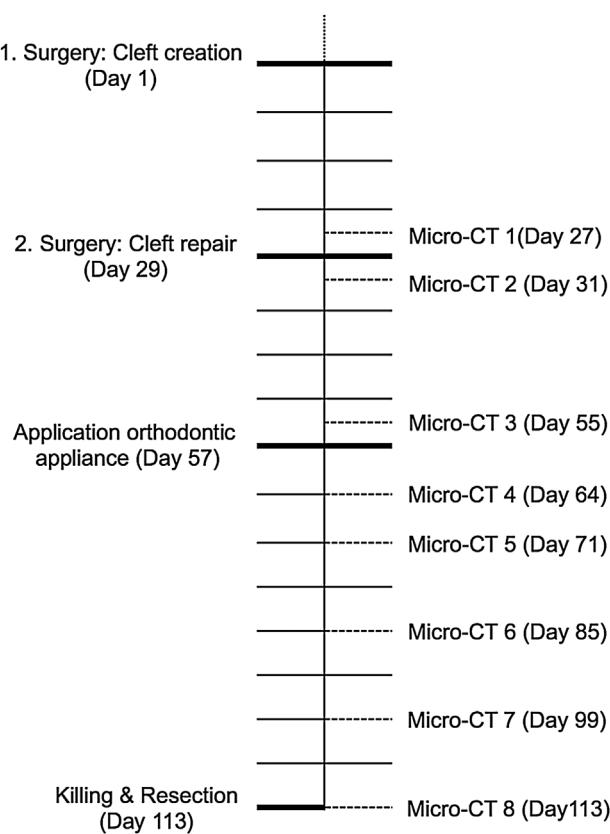


Fig. 1. Overview/timeline of the final animal research protocol. Thick, solid lines of experimental measures under intraperitoneal injection: Cleft creation, cleft repair, installation of orthodontic appliances; Dotted lines: radiological measures in micro-CT for follow-up monitoring under isoflurane anesthesia.

Before both surgeries, the rats were anesthetized with an intraperitoneal (IP) injection of a combination of ketamine (80–100 mg/kg, Ketavet, Pfizer, Berlin, Germany) and medetomidine hydrochloride (0.15–0.25 mg/kg, Domitor, Orion Pharma, Espoo, Finland). After adequate anesthesia was given, endotracheal intubation using a 15-gauge intravenous catheter was performed to substitute oxygen. Buprenorphine (0.03–0.05 mg/kg, Temgesic, Indivior Limited, Berkshire, UK) was applied subcutaneously as an analgesic. Cefuroxime (15 mg/kg s.c., Fresenius, Bad Homburg, Germany) for antibiotic treatment was started at 24-h intervals for seven days. Immediately after surgery, atipamezole hydrochloride (0.75 mg/kg, Antisedan, Orion Pharma, Espoo, Finland) was given as a reversing agent. Further analgesia was carried out if necessary, with buprenorphine (0.03–0.05 mg/kg) for a maximum period of five days.

The animals were placed in the prone position and the mouth was disinfected (Cutasept, BODE Chemie GmbH, Hamburg, Germany). Afterwards, endotracheal intubation using a 16-gauge intravenous catheter was performed and the operation commenced (Fig. 2). First, an incision approximately one cm long was made using a scalpel (Number 13 blade) in the attached gingiva, beginning palatally on the first molar of the left side of the maxilla to the anterior part up to the first palate fold behind the incisors (Fig. 3A). The incision was carried down to the bone, followed by the tissue reflection using a caries excavator as a raspatory (Figs. 3B and C). A micrometric osteotomy between the roots of the incisor and the first molar was performed using an ultrasonic device with a titanium nitride coated, diamond grain osteotomy insert (\varnothing 1.7 mm, insert OT5, Mectron s.p.a., Carasco, Italy) (Fig. 3D) under irrigation with a sterile physiologic solution to prevent overheating. Afterwards, bone wax (Bonewax, Ethicon - Johnson &

Johnson Medical GmbH, Norderstedt, Germany) was applied to maintain the bone defect and the wound was closed with continuous resorbable sutures (7/0 Vicryl, Ethicon, Johnson & Johnson Medical, Somerville, NJ, US) (Fig. 3E). The animals were reinstated in their cages under intensive monitoring and observed until full recovery.

After four weeks, the second operation took place in the 12-week-old animals. The anesthetic protocol, the preoperative procedure, and the incision and soft tissue resection technique were the same as in the first operation. After the bone wax was exposed (Fig. 3F), it was removed and the surrounding bone of the cleft was refreshed. Afterwards, the jaw was reconstructed using autologous bone from the hip (Group 1), xenogeneic bone (human bone substitute material) (Group 2), or synthetic bone substitute (biphasic calcium phosphate) (Group 3), respectively (Figs. 4A–C). In the autologous group, the bone graft was first harvested from the tuberosity of the ischium as previously described (Möhlhenrich et al., 2020). Finally, the wound closure was again completed with continuous resorbable sutures (7/0 Vicryl, Ethicon, Johnson & Johnson Medical, Somerville, NJ, US), and the animals were replaced in their cages and observed until full recovery.

After all surgical interventions, the animals were given special soft food (DietGel Boost, Clear H2O, Portland, US) for seven days as refinement, followed by a standard diet (rat/mouse maintenance #V1534-300, 10 mm; ssniff Spezialdiäten GmbH, Soest, Germany) and water *ad libitum*.

2.4. Orthodontic treatment

After four weeks of bone consolidation, the orthodontic appliance was applied in all 16-week-old animals (with an average weight of 542 ± 32 g) ($N = 19$) via general anesthesia using the previously applied anesthetic protocol (ketamine/medetomidine) on the left side of the maxilla (Fig. 5). Next, a 0.14 N nickel-titanium closed coil tension spring (33-54495, PSM Medical Solutions GmbH, Gunningen, Germany) was fixed between the incisors and the upper-left first molar according to Kirschneck et al. (Kirschneck et al., 2017a,b; Kirschneck et al., 2013, 2015). To accomplish this, a notch was first milled into the base of both upper incisors and the upper left first molar. Afterwards, the spring was fixed into these notches by wire ligature (\varnothing 0.01") and dental composite (Venus flow, Kulzer GmbH, Hanau, Germany) using an acid-etching technique. A continuous force of about 0.14 N was able to be provided throughout the experiments, confirmed by an orthodontic calibrated spring balance (Correx, small model, Haag-Streit AG, Köniz, Switzerland). In addition, to prevent damage to the spring, the lower incisors were ground down in two-week intervals during radiological examinations.

2.5. Microfocus computed tomography (micro-CT)

Imaging of the rats was performed two days before and after cleft repair as well as two days before and seven days after orthodontic appliance installation using an *in vivo* microCT system (U-CT OI, MILabs, Utrecht, the Netherlands) under general anesthesia using isoflurane [induction: 5 vol% isoflurane+5 L O₂/min; maintenance: 2 vol% isoflurane+2 L O₂/min] (Abbott GmbH & Co. KG, Wiesbaden, Germany) (Fig. 1). In addition, follow-up imaging was carried out every two weeks as part of the orthodontic treatment for radiographic analysis. The animals were scanned with an ultra-focus magnification through 360° of rotation at an increment of 0.75° with 0.3 s/degree. The microCT data was reconstructed at an isotropic voxel size of 40 μ m (Fig. 6). For analysis, the data were down-sampled, by binning, to a voxel size of 80 μ m, thus improving the visual appearance of the scans. Images were eval-

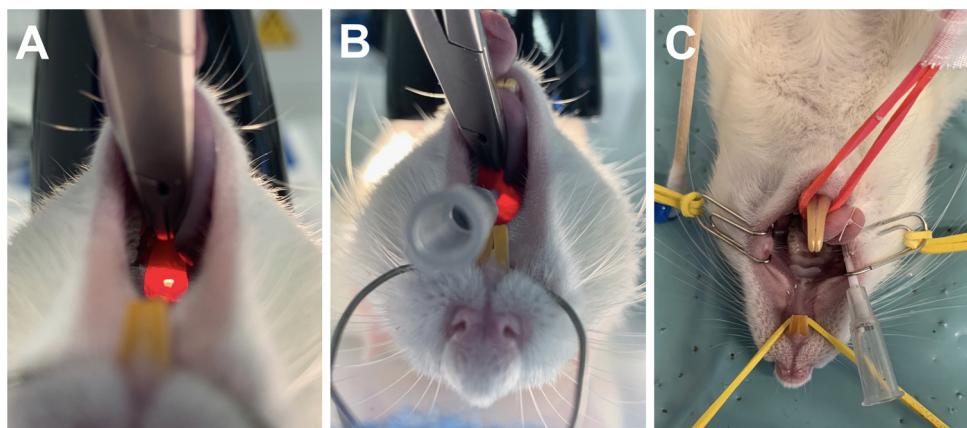


Fig. 2. Endotracheal Intubation: A) Animal in supine position on operation table. Tongue pulled and pushed to the floor of the mouth. Operation light transilluminated the trachea front wall and the glottis, B) A 15-gauge intravenous catheter with a maximum length of five cm (shorter for rats < 250 g) serves as an endotracheal intubation tube, C) Rat in supine operation position with a suture-fixed intubation tube.

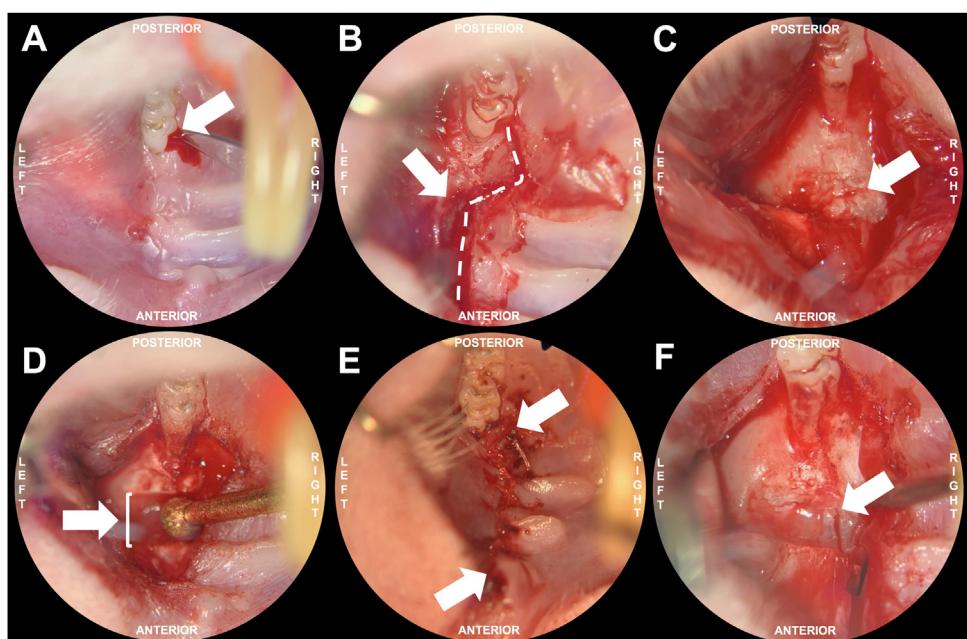


Fig. 3. View of the surgical site on the left maxilla and palate: tongue base above, mouth tip below. Operation procedure: A) Starting an incision at the palatal side of the first molar (arrow) using a knife with a No. 13 blade, B) Completed incision (dashed line) with vestibular course into the mobile mucosa (arrow), C) Subperiosteal elevation of the soft tissue in the operating field and exposition of the anterior part of the maxilla (arrow), D) Osteotomy of the anterior maxilla by ultrasonic surgery insert with an diameter of 1.7 mm to create a corresponding continuity-interrupting alveolar cleft (square bracket), E) Continuous wound closure (between the arrows), F) Re-entry and exploration of the artificial created cleft filled with bone wax (arrow).

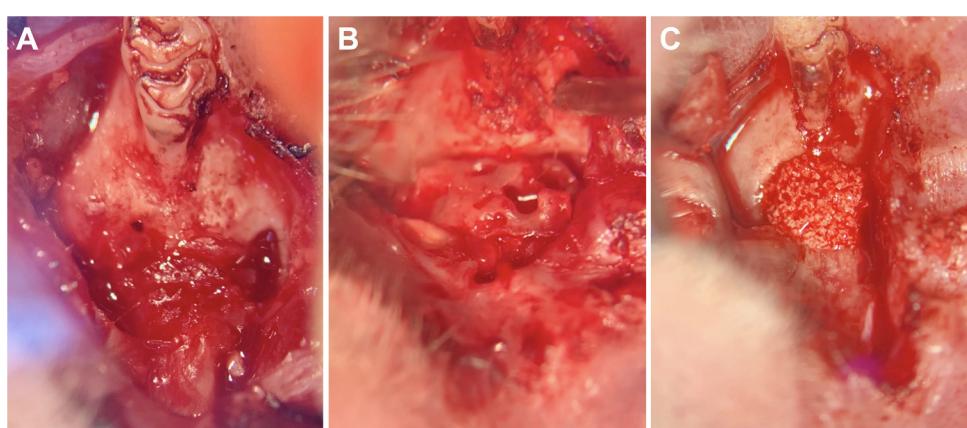


Fig. 4. Immediate reconstructed jaw using: a) autologous bone from the ischial tuberosity, B) Xenogeneic (human) bone block, C) alloplastic synthetically produced (calcium phosphate ceramics) block.

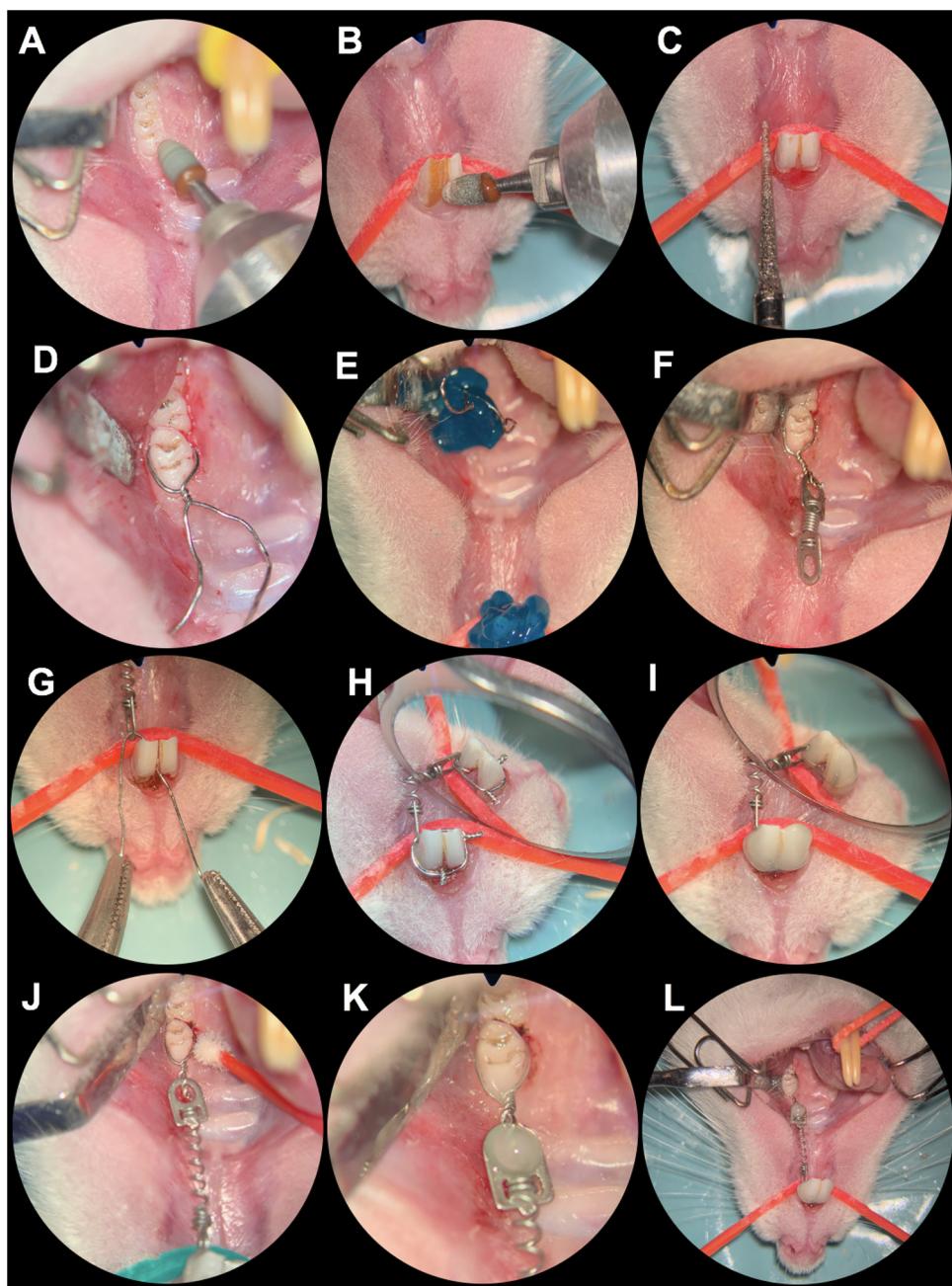


Fig. 5. Application of the orthodontic appliance four weeks after cleft repair: A) Roughening the first molar of the left side using a milling cutter, B) Roughening both incisors, C) Creating a groove at the transition to the gingiva for later wire retention, D) Attachment of the retention wire in the groove of the first molar, E) Conditioning of the teeth in an acid-etching technique using 39% phosphoric acid, F) Application of a 0.14 N nickel-titanium closed coil tension spring on the first molar retention wire, G) Fixation of the tension spring at the left incisor, H) Additional securing of the wire retention connecting the left and right incisor, I) Final fixation using a bonding agent and dental composite, J) Application of the bonding agent at the first molar, K) Application of the dental composite at the first molar and the connecting point between the wire and the coil tension spring, L) Overview of the activated orthodontic appliance.

uated using cross-sectional slices and rendered three-dimensional iso-surfaces. The alveolar cleft size was measured in the postoperative microCT, and the missing bone volume was segmented using all the anatomical planes. Images were evaluated using cross-sectional slices and rendered three-dimensional iso-surfaces. The alveolar cleft size was measured in the postoperative μ CT, and the missing bone volume was segmented using all the anatomical planes. The tooth movement distance was measured in the sagittal projections of μ CT. The visible landmarks, i.e., the edges of the left first and second molar, were chosen to quantify the tooth movement. The distance was measured 3 times between thereof and averaged at each time point to obtain reliable results (Ru et al., 2016b). During

the general anesthesia and after the last imaging, the animals were killed by cervical dislocation.

2.6. Statistical analysis of tooth movement

Statistical evaluation of tooth movement within or between the groups was carried out using an ordinary two-way ANOVA with Bonferroni post hoc tests. The level of significance was set at p -value ≤ 0.05 . All results are expressed as mean \pm standard deviation (SD). Statistical analysis was performed with Prism (version 9, GraphPad Software Inc., La Jolla, CA, USA).

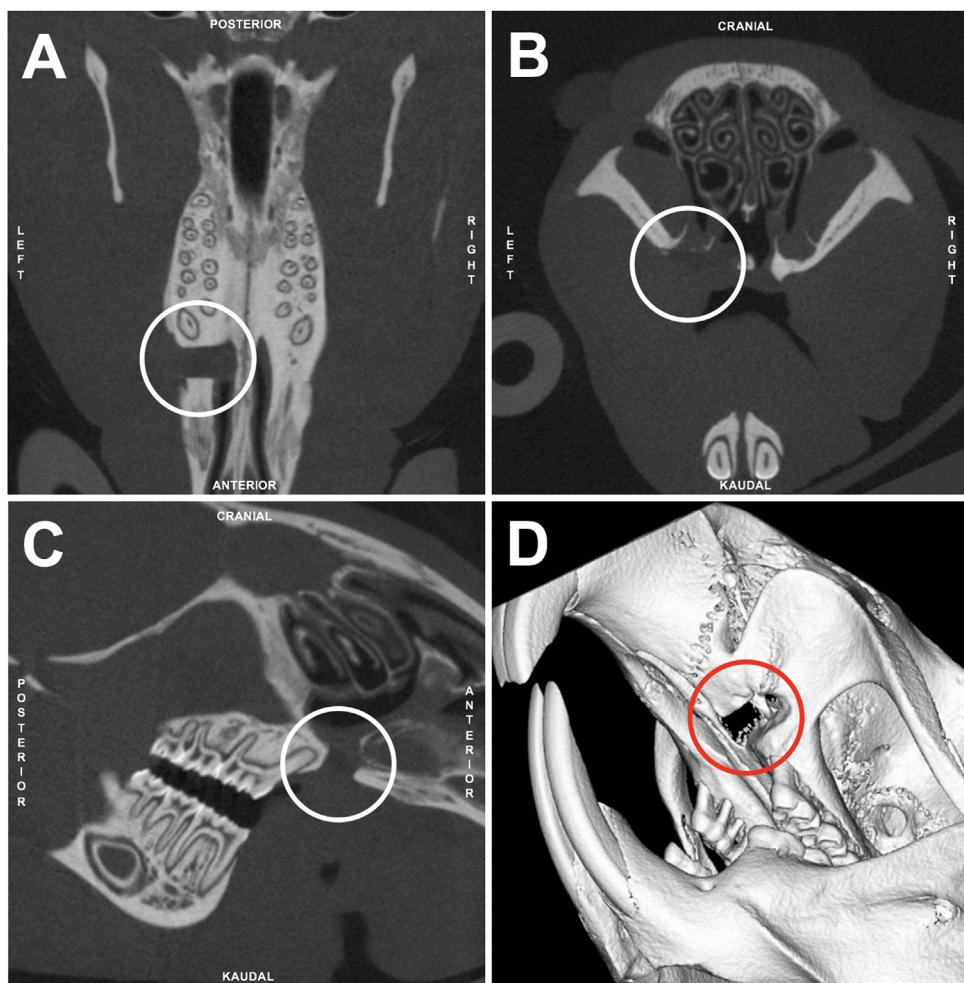


Fig. 6. Radiological imaging of the alveolar cleft defect (white/red circle) in left side of the maxilla two days before jaw reconstruction (Micro-CT 1) in the A) transverse plane, B) coronal plane, C) sagittal plane, and D) the corresponding three-dimensional microCT volume rendering. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3. Results

The original study design was intended for a split-mouth study with 24 total animals; eight animals per group and one of each group designated as a potential drop out. Due to the high number of animal losses during or immediately after the first operations ($N = 11$), the study design was changed in accordance with the Governmental Animal Care and Use Committee according to the procedure described above in Section '2'. Finally, 33 rats were included in the establishment process, but only 19 animals reached the application of the orthodontic appliance phase and participated in the entire animal experiment. The process of implementation is shown in Fig. 7.

3.1. General anesthesia protocol

The originally planned anesthetic protocol was based on a combination of ketamine (80–100 mg/kg, Ketavet, Pfizer, Berlin, Germany) and xylazine (2.5–5 mg/kg, WDT, Garbsen, Germany) with an IP injection. However, the depth of anesthesia was insufficient. Although the first three rats showed no reactions to the pain stimuli at the tail tip or paws, they even reacted to the surgical interventions in the mouth. This required repeated injections until the maximum dose was reached. In one case, one of these animals had to be taken out of the experiment early due to early awakening from anesthesia despite maximum dose of narcotics due animal

welfare. Afterwards, the anesthetic protocol was changed to a combination of medetomidine (150 µg/kg), midazolam (2 mg/kg), and fentanyl (5 µg/kg) in the next four animals. Here, the depth of anesthesia was also insufficient. Whereas, the combination of ketamine (80–100 mg/kg) and medetomidine (0.15–0.25 mg/kg) combined with buprenorphine (0.03–0.05 mg/kg) in oral intubation led to anesthesia, which also allowed for the planned surgery in the oral cavity.

3.2. Surgical procedure

The initially planned surgical procedure was based on an osteotomy of the left and right part of the maxilla to create an alveolar cleft for a split-mouth study design on each side. However, this had to be avoided due to an increased risk of bleeding of the well perfused palate. The first two animals died intraoperatively due to sudden bleeding and the associated aspiration of blood, respectively. In both cases, the death occurred quickly and unpredictably under general anesthesia. To reduce the risk for further animals, the setup was changed and the surgery was continued only on the left side of the maxilla and the additional application of vasoconstrictive nasal drops (Otriven, GlaxoSmithKline Consumer Healthcare, Brentford, United Kingdom).

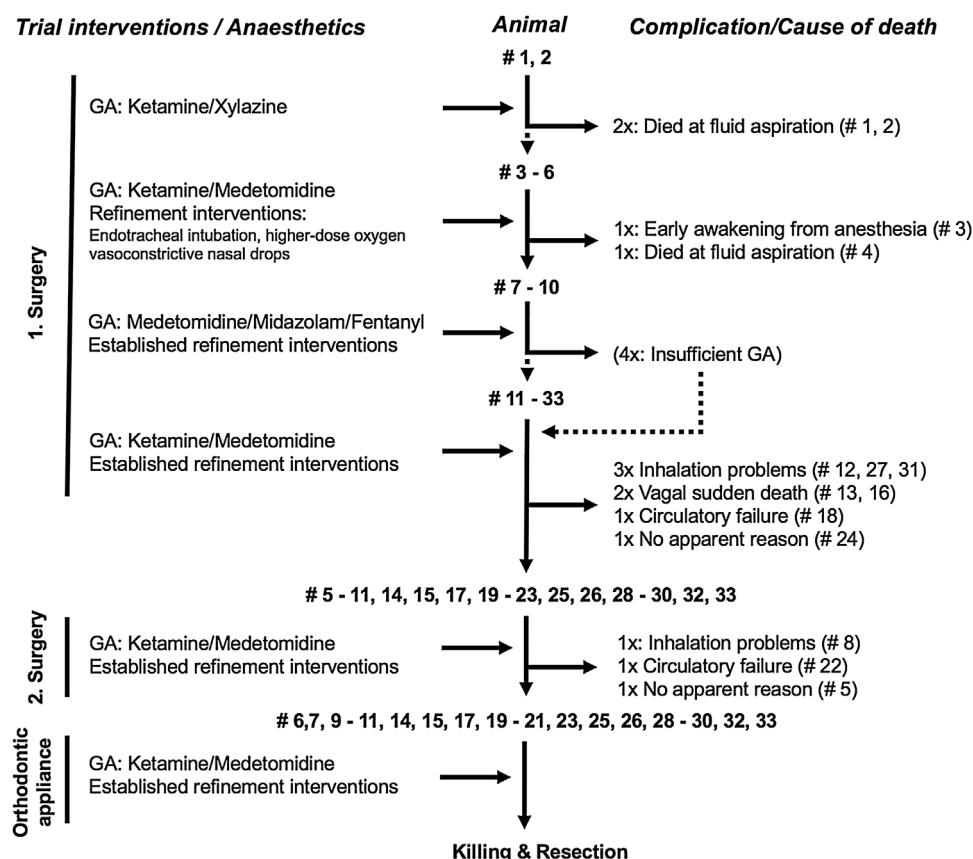


Fig. 7. Flowchart of the establishment process of the artificial cleft creation.

GA: general anesthesia, #: individual number of the research animal.

3.3. Breathing

Inhalation problems caused by aspiration of coolant or blood during or after the surgery were the main causes of death. In total, eight animals died in the context of insufficient breathing. The first two rats died due to unpredictable blood aspiration, and a third died from inhaled irrigation fluid. Therefore, light supported endotracheal intubation was combined with an insertion of a pharyngeal tamponade. This allowed the extraction of aspirated liquid during the operation, pre-oxygenation and the inhalation of higher-dose oxygen. However, even though this method of intubation worked very well, two animals died from vagal sudden death. In these cases, an intravenous catheter larger than 16 gauge was used. Furthermore, two other animals had to be euthanized due to their poor general condition associated with postoperative breathing problems on the second or third postoperative day.

3.4. Unexplained deaths

Two rodents died of sudden circulatory failure during the first or second surgeries, and three more were found dead in the cage for no apparent reason. One died immediately after the first surgery, while the second passed two days after the first surgery and the third 36 days after the second surgery.

3.5. Orthodontic appliance failures

Despite all the fixation measures, the orthodontic appliance has loosened. In the course of the radiological follow-up imaging, a total of 11 broken devices were found, in one animal even twice. The reattachment was then carried out again under IP general anesthesia with a continuous transition from isoflurane anesthesia.

Table 1

Mean velocity of orthodontic tooth movement at different times depending on the type of cleft repair.

	Day	Mean	SD
Autologous bone group	7	0.21	0.08
	14	0.26	0.26
	28	0.36	0.23
	42	0.63	0.20
	56	0.67	0.27
Xenogenic bone group (Human bone)	7	0.50	0.54
	14	0.50	0.70
	28	0.61	1.04
	42	0.70	0.73
	56	0.78	0.69
Synthetic bone group (Hydroxyapatite)	7	0.29	0.12
	14	0.10	0.14
	28	0.32	0.15
	42	0.60	0.46
	56	0.82	0.72

3.6. Cleft size and orthodontic tooth movement

The average size of the generated alveolar clefts before corresponding repairs was about 2.70 ± 0.46 mm in length, 2.01 ± 0.25 mm in width, and 1.18 ± 0.20 mm in height. The mean cleft volume was about 5.04 ± 1.22 mm³. The tooth movement differed between the three different groups (Table 1). After an initial period of seven days (T1), the mean rat tooth movement was 0.21 ± 0.08 mm in the autologous bone group (G1), 0.50 ± 0.54 mm in the human bone group (G2), and 0.29 ± 0.12 mm in the synthetic bone group (G3). After eight weeks (T5), the largest tooth movement distance was found in the synthetic bone group (G3) for about 0.82 ± 0.72 mm, followed by the human bone group (G2) for about 0.78 ± 0.69 mm, and the autologous group (G1) for about 0.67 ± 0.27 mm. Neither

between the groups on the first till last measurement day (T1, G1 vs. G2: $p = 0.58$; G1 vs. G3: $p = 0.96$; G2 vs. G3: $p = 0.74$; T5, G1 vs. G2: $p = 0.92$; G1 vs. G3: $p = 0.85$; G2 vs. G3: $p = 0.99$) nor between the times of measurement within the respective group (e.g.: G1, T1 vs. T5: $p = 0.52$; G2: T1 vs. T5: $p = 0.87$; G3: T1 vs. T5: $p = 0.29$) were any statistically significant differences found.

4. Discussion

Autologous bone grafts as well as tissue-engineered materials, like allografts or xenografts, and synthetic bone substitutes can be used for cleft repair (Aalami et al., 2004; Bajaj et al., 2003; Kamal et al., 2018; Sharif et al., 2016). Up to date, autologous bone from the hip is considered as gold standard due to its osteogenic, osteoinductive, and osteoconductive properties (Canady et al., 1993). However, to enhance the clinical outcome and to avoid complaints associated with iliac crest autologous bone grafts, alternative synthetic or xenogeneic bone substitute have been investigated increasingly. In this context, different rat models in experimental cleft research have been described as focusing the bone responses on recipient sites (Cheng et al., 2017; Jahanbin et al., 2016; Mehrara et al., 2000; Mostafa et al., 2014; Nguyen et al., 2009; Ru et al., 2016a,b; Sun et al., 2015, 2017). However, these models have weaknesses with regard to an additional orthodontic examination. Either the constructed cleft defect is too far away from a tooth or the defect is based on extraction of the first molar combined with additional bone removal, but without creating a complete continuity-interrupting alveolar cleft. Therefore, in a current research project, a new animal cleft model was developed. It allows the creation of a complete interrupting alveolar cleft, the opportunity for subsequent orthodontic tooth movement after previous cleft repair by using different bone substitutes.

However, in the initial phase of this investigation, there were disproportionate losses of animals, especially due to unpredictable surgical and anaesthesiological events, which almost led to the termination of the trial. This prompted us isolated from the actual results of this study to demonstrate possible sources of error in establishing the model and to describe the most promising approach in the present investigation. The intention of the present work is to focus on method development in order to spare other research groups our frustrating experiences and, above all, to prevent unnecessary suffering of laboratory animals.

In general, the implementation was difficult due to a high initial complication rate at the beginning. These complications mainly comprised difficulties in finding a suitable anaesthetic protocol that allows for surgical procedures in the oral cavity as well as breathing problems during and immediately after the surgical procedure.

Different general anesthesia protocols were introduced for cleft creation in rats. Cheng et al. anesthetized with isoflurane (4%–5%), followed by intraperitoneal injection of ketamine (40 mg/kg) and xylazine (10 mg/kg) (Cheng et al., 2017). While other groups reported regarding the use of an intraperitoneal injection of 10% chloral hydrate between 2–4 ml/kg (Ru et al., 2016a; Sun et al., 2015). Mostafa et al. reported about intra-peritoneal injections of Ketamine (75 mg/kg) and Domitor (0.5 mg/kg) as well as an additional local injection of 0.25 ml of 0.4% lidocaine (Mostafa et al., 2014). In the present study a similar protocol based on a combination ketamine (80–100 mg/kg) and medetomidine (0.15–0.25 mg/kg) was finally used to reach a sufficient depth of anesthesia.

Unfortunately, the framework and problems with the study design with regard to inhalation problems caused by aspiration of coolant or blood were not described in the previously published research, but represented the main problem during the establishment process in the present investigation. With exception of the research group of Sun et al. (2015), we received no personal feed-

back on our inquiries. It was recommended to us to use tampons because of their valid effect of absorbing fluid and blood. However, in our opinion this was not sufficient, since liquid inhalation would take place apparently via the nasal passages. Therefore, we decided to reduce the risk of fluid development by minimizing the surgical area as well as operating time by applying a split mouth design. In addition, we carried out intubation according to Rivard et al. (2006) and added a throat tamponade. This considerably reduced postoperative breathing problems and additionally incapacitated aspiration of fluid into the lungs. It can be recommended to use a 16-gauge intravenous catheter with a maximum length of 5 cm (shorter for rats <250 g). After these refinement activities the maintenance of animal welfare could largely be achieved.

With regard to the orthodontic appliance, we had minor problems. In 10 animals there was a loosening of the apparatus, whereby one appliance was affected twice. This happened despite the supply of special food for the days immediately after the experimental interventions as well as soft food during the orthodontic tooth movement according to reports of de Carlos et al. (2006) and Drevensek et al. (2009). It was noticeable that most of the time, the appliance detached itself from the first molar. In contrast, Kirschneck et al. reported a similar orthodontic setup that proved to be stable enough to withstand the masticatory forces. They reported that during an experimental period of four weeks only one apparatus was lost in 24 animals. Whereas, in the present investigation the experimental period was about 8 weeks, which could be one reason for the higher loss rate. Another possible reason could be the long intervals between consecutive reductions of lower incisors, as they were performed during radiological examinations, which extended to a period of up to 2 weeks. A modification of the orthodontic appliance according to Kirschneck et al. (2017a) and (2015), which provides anchorage on the first and second molar can potentially improve the orthodontic intervention.

The primary aim of the present investigation is to introduce a new way of creating an alveolar cleft for the rat model. It was found that ultrasonic surgery allows for good control in creating a continuity-interrupting alveolar cleft. The standardized inserts with a diameter of 1.7 mm allowed defects of a similar size without the use of additional aids like surgical guides.

In the previous experiential cleft models in rats, a distinction was made between mid-palate cleft (MPC) models in the anterior maxilla (Cheng et al., 2017; Mehrara et al., 2000; Mostafa et al., 2014) and central (Jahanbin et al., 2016; Nguyen et al., 2009) or posterior (Ru et al., 2016a,b; Sun et al., 2015, 2017) alveolar cleft (AC) models. The latter are based on a supporting extraction of the first molar. Regarding the potential cleft dimension, a mean defect size of between 7 × 2.5 × 1 mm (Mostafa et al., 2014) and 9 × 5 × 3 mm (Mehrara et al., 2000) for MPCs and about 7 × 4 × 3 mm for central ACs was reported. The size of the posterior AC was described as between 3 × 2 × 2 mm (Ru et al., 2016a,b) and 4 × 4 × 3-mm of the complete alveolar cleft (Sun et al., 2015) or 5 × 2.5 × 1 mm (Mostafa et al., 2014), respectively. In the present investigation, a mean cleft size of about 2.75 × 2 × 1.2 mm with a mean cleft volume of about approximately 5.04 mm³ was found. Therefore, the defect is significantly smaller than in the other presented models. However, the present model at least allows for the movement of the mesial tooth root into the reconstructed maxilla.

The mean velocity of orthodontic tooth movement after 14 days was between 0.26 ± 0.26 mm in the autologous group and 0.50 ± 0.70 in the human bone group, depending on the kind of bone substitute used for cleft repair. After 28 days, the mean values ranged between 0.32 ± 0.15 mm in the synthetic group and 0.61 ± 1.04 in the human bone group. In this context, Kischneck et al. reported for the first molar movement, in a surgically unaffected maxilla, a mesial tipping of about 0.8 ± 0.2 mm and a mesial root torque of about 0.4 ± 0.3 mm after 14 days. The corresponding values mea-

sured after 28 days were around 0.9 ± 0.2 mm and 0.4 ± 0.3 mm, respectively (Kirschneck et al., 2013). In a subsequent research, the authors modified the orthodontic appliance by its fixation on the first and second upper molar using a continuous wire ligature. With regard to the velocity of tooth movement they reported a value for the second molar of around 0.4 ± 0.1 mm and 0.7 ± 0.1 mm after 28 days (Kirschneck et al., 2017a). However, in the present study the speed was measured for the first molar. Therefore, the present values are not totally comparable with the measurements by Kirschneck et al., as the first molar's movement was based on a translational movement combined with a mesial tipping. Nevertheless, the present tooth movements seem somewhat low compared to the reported values in current literature. This is possibly due to increased bony resistance caused by the different bone substitutes. This issue will be investigated in subsequent studies.

Obviously, findings based on this study design show limitations in a direct transfer into clinical practice. Although critical size defects, like alveolar clefts, in rodent models have been very useful to understand the biology of cleft repair using bone substitutes, comparative age of the animals differs from that of humans. In literature, postnatal maturity for rats is described as peri-adolescent phase starting at day 49 and young adulthood period at day 70. In this investigation the artificial cleft was created around day 56 (8-week-old animals). Thus, the animals in the present study were in the pubertal age according to Sengupta (Sengupta, 2013). However, cleft repair took place around postnatal day 84 (12-week-old animals). At this time the animals were already in the adolescent phase and even in adult period during orthodontic treatment. Furthermore, it must be taken into account that this animal model is based on an artificially created cleft, a defect model, unlike an innate alveolar cleft that can be found in clinical practice.

5. Conclusions

The present research demonstrates a demanding model that seems to be suitable for processing relevant questions about the influence of cleft repair on tooth movement. With regard to animal welfare, the following refinements can be recommended:

- 1 General anesthesia based on the combination of ketamine (80–100 mg/kg) and medetomidine (0.15–0.25 mg/kg) is sufficient for cleft creation.
- 2 To avoid the risk of bleeding, surgery should only be performed on one side of the jaw.
- 3 Ultrasonic surgery allows for good control in creating a continuity-interrupting alveolar cleft.
- 4 Endotracheal intubation using a 16-gauge intravenous catheter combined with throat tamponade can prevent the ingress of fluids and allows for an adjuvant administration of oxygen.
- 5 If needed orthodontic appliance fixation at the upper first two molars can be favorable (M1, M2).

Ethics approval and consent to participate

The experimental animal study protocol was approved by the Governmental Animal Care and Use Committee (Reference No.: 81-02.04.2018.A342; Landesamt für Natur, Umwelt und Verbraucherschutz Recklinghausen, Nordrhein-Westfalen, Germany; dated: 11.01.2019). The study protocol conforms to the ARRIVE Guidelines and with the Guide for the Care and Use of Laboratory Animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Conflict of interest

All other authors declare that they have no conflict of interest.

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Authors' contributions

SCM contributed to conception and design, performed animal surgeries, micro-CT scans and data acquisition, drafted the manuscript, coordinated the research project, gave final approval. MH contributed to conception and design, supported animal surgeries, reviewed the manuscript and gave final approval. ZM performed micro-CT scans and data acquisition, drafted the manuscript and gave final approval. FG contributed to conception and design, drafted the manuscript and gave final approval. SC performed data acquisition, contributed interpretation of the data, reviewed the manuscript and gave final approval. GD contributed interpretation of the data, reviewed the manuscript and gave final approval. FH contributed to conception and design, reviewed the manuscript and gave final approval. AM contributed to conception and design, coordinated the research project, supported animal surgeries, reviewed the manuscript and gave final approval.

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