SURGICAL CREATION OF CLEFT-LIKE PALATAL DEFECTS IN NEW ZEALAND RABBITS (A PILOT STUDY)

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ABSTRACT

INTRODUCTION: Surgically Created cleft palate models in animals have solved most of the problems associated with congenital models. Recent studies have preferred the use of rats and rabbits as they are easy to handle and observe.
OBJECTIVE: The aim of this study was to optimize surgically created palatal defects in New Zealand white rabbits.
MATERIALS AND METHODS: A total of 7 rabbits were used in this pilot study; for defect optimization. Three different defect dimensions 7×2.5×1 mm³, 7×2.5×5 mm³ and 5×2.5×4 mm³ were created in the mid-palatal region. Maxillae were taken immediately after surgery and at 2 weeks interval for radiographic and histological analyses.
RESULTS: 7×2.5×1 mm³ defects although showed success of the created animal model, they were shallow such that spontaneous healing of defects occurred. 7×2.5×5 mm³ defects with nasal mucosa removal showed death of all animals immediately and after 2 weeks with severe injuries in teeth and vital structures. On the other hand, 5×2.5×4 mm³ defects with intact nasal mucosa showed preservation of vital structures without closure of the defect at 2 weeks.
CONCLUSIONS: 5×2.5×4 mm³ defects with intact nasal mucosa were shown to be the largest yet safest dimensions that could be used to create mid-palatal defects in rabbits in this region.
KEY WORDS: Palatal defects, Cleft palate, Bone engineering, Rabbit model, Cleft animal model.

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INTRODUCTION

Establishment of an animal cleft palate model to simulate the human condition and test efficacy of grafts and scaffolds has been addressed in studies that proposed both congenital and surgically created models. Congenital models have been carried out either transgenetically by genetic mutation such as the use of Twirler gene (Tw/Tw) in mice (1) or using teratogenic drugs as phenytoin and corticosteroids (2-4). Both models have helped studying the morphology and contributing factors to clefts; but have been shown to be less beneficial in testing new approaches for cleft treatment. This may be related to variability in the size, site of the defect, the association with other congenital disorders that led to death of animals. In addition to the need of skillful techniques, some studies reported that the congenitally defected newborns were neglected by their mothers (5).

Surgically created cleft models were first reported by Harvold in 1950, where he developed an alveolar and palatal cleft in a rhesus monkey (6). Following this; Chierici et al. in 1970 produced 3mm unilateral clefts of premaxilla and hard palate while leaving intact nasal mucosa in monkeys (7&8). Boyne in 1974(9) and El-Deeb et al. in 1985 (10) used monkeys for creation of bilateral alveolar clefts with involvement of nasal mucosa. Jonsson

and Stenstroin in 1978 were the first to create palatal defects in dogs by removing a 5mm wide segment of palatal bone and leaving nasal mucosa intact (11). Marx et al. in 1984 used dogs for creation of alveolar clefts (12). Freng in 1979 used domestic cats for removal of 5mm of palatal bone with the involvement of nasal mucosa (13). These models mostly used large animals as primates, dogs and pigs. Small animals such as rabbits and rats have been included in bone defect studies due to their availability, lower cost and ease of their handling and observation particularly as they can comply with the 3R principles of experimental research including replacement of large animals with smaller ones if applicable. Rabbits were first included in cleft palate research by Schultz in 1964(14); where he created a soft palate defect. Verwoerd et al. in 1976 was the first to report using rabbits in surgically created alveolar defects (15) However, recent studies that used rats (16-19) or rabbits (20-23) mainly discussed alveolar defects.

Some studies discussed the creation of palatal defects in rabbits; such as El Bokle et al. in 1993(24) and Liceras-Liceras et al. in 2017 (25), but those did not include the mid palatal suture. Mostafa etal. In 2014(26) reported a reproducible and reliable mid palatal defect including the mid palatal suture. However, this latter defect was created in rats while in the current study the same model (26) was used in rabbits for the first time. The rationale was to optimize the dimensions of surgically created mid-palatal defects in rabbits. Rabbits are easy to handle and observe. Being the largest of the small animal group, they can bear the surgical procedures. They also have similar bone mineral density as humans; in addition to high remodeling rate compared to other rodents (27). The aim of this study was to surgically create a palatal defect in white New Zealand in rabbits. These defects were assessed radiographically and histologically to select the most reliable, reproducible and least invasive dimensions for creating a cleft-like palatal defect suitable to be used in future research to assess the regenerative capacity of new materials for cleft palate management.

MATERIALS AND METHODS

The protocol of this study was reviewed and approved by the staff members of the Oral and Maxillofacial Surgery Department and the Research Ethics Committee at Faculty of Dentistry, Alexandria University, Egypt (IRB NO: 00010556 IORG0008839, 20/9/2018). It was in accordance with the ARRIVE guidelines (Checklist attached). This study was conducted at the Tissue Engineering Labs, Faculty of Dentistry, Alexandria University, Egypt.

2.1 Animals

This study used 7 male white New Zealand V-line rabbits (Faculty of Agriculture, Alexandria University). Rabbits were healthy and with good oral health weighing 3-3.5 Kg and 3.5-4 months of age. V-line New Zealand rabbits are a hybrid strain from the set that was brought from Department of Animal Science of the Universida de Politecnica de Valencia, Spain. Six rabbits were used; where different palatal defect dimensions were surgically created. One rabbit was used for studying normal anatomy and histology, and so did not receive any surgery.

Rabbits were kept under observation in the animal unit at the Tissue Engineering Labs, Faculty of Dentistry, Alexandria University. They were kept in separate cages; each cage was supplied with a feeder and water supply. Standard environmental and nutritional conditions were maintained with enough period of day
light. Monitoring of the rabbits' general health conditions, nutrition, and excretion were carried out three times daily. The animals were left for adaptation for a week before the beginning of the experiment.

2.2. Experimental procedures

2.2.1. Surgical procedures (25) (Fig. 1 a-d).

Rabbits were anesthetized using 3-5 mg/Kg Xylazine HCL 2% (Adwia, Egypt) followed by 20-50 mg/Kg Ketamine HCL (Sigma, Egypt) intramuscularly. Following Betadine disinfection of the site, injection of local anesthetic solution of Mepivacaine HCl 2% containing 1:20000 Levonordefrin vasoconstrictor (Alexandria Co. for Pharmaceuticals, Alexandria, Egypt) was administered. Vertical incisions were done posterior to the upper second incisors (peg) till the apex of the anterior palatine foramen was reached, with dissection of mucosa attached on the peg incisor teeth and retraction of the flap using periosteal elevators (Surgicrafts, Pakistan). Defects were created with a fissure bur using a low speed handpiece at 30000 rpm under continuous external cooling. Defects were posterior to the peg incisors; where the coronal side (top) of the defect was towards the palate and the apical side (bottom) was towards the nasal apparatus (Fig. 2).Defects created were as follows:

$7\times2.5\times1\ mm^3$ defects were the first used dimensions (n=2) (Fig. 1 g);guided by a model that was created in rats (26), that coincided with our observations on cadaveric rabbit's maxillary bone dimensions using plain radiographs (Fig.1 f).

$7\times2.5\times5\ mm^3$ defects with nasal mucosa removal were the second used dimensions (n=2) (Fig.1 h) guided by the measurements on cadaveric rabbit's maxilla that showed that the distance from the most palatal side to the beginning of nasal apparatus is ≈5mm (Fig.2 a).

$5\times2.5\times4\ mm^3$ defects leaving nasal mucosa intact were the third used dimensions (n=2) (Fig.1 i), to

overcome problems that occurred in the first two defect dimensions.

Mucosal flaps were sutured using 3-0 polyglactin absorbable sutures in interrupted manner(Huaiyin Medical Instruments Co, China) (Fig.1e).

Rabbits were transferred to the animal care unit and monitored till recovery before returning them to their cages. For 3 days postoperatively, rabbits were fed crushed moistened food and were given intramuscular injection of 1g Cephotaxime antibiotic (Egyptian.Int.Pharmaceutical (E.I.P.I.C.O), 10th of Ramadan, Egypt) in a dose of 150 mg/Kg/day and 60 mg/day Ketorolac analgesic (AmriyaPharm.IND, Alexandria, Egypt). They were monitored for any postoperative complications till sacrifice.

2.2.2. Radiographic imaging

Following sacrifice, radiographic images for rabbit's maxillae were taken using digital periapical x-rays (Heliodent DS - Sirona – Germany).The exposure parameters were 60 KV, 7mA for 0.04 seconds. Data analysis was performed using Sidexis software (Heliodent DS - Sirona –Germany).

2.2.3. Histological and histomorphometric analysis

Processing of specimens was done following the hard tissue resin embedding technique (28). In Brief; 10% formalin was used for specimens' fixation and then specimens were dehydrated gradually in 40%, 70%, 85%, 90% &100% ethyl alcohol. Clearing was done using xylene and finally, embedding of the specimen was done in methacrylate (Sigma-Aldrich, USA) with benzoyl peroxide (Alpha Chemika, India). Sections were cut using a hard tissue microtome (EXAKT 300CP, Germany) of 180-220µm thickness. Staining was done using Stevenel's blue and Alizarin red (LobaChemie, India). Three examiners used Light Microscope (Optika, Italy) for examination and photographing of sections.

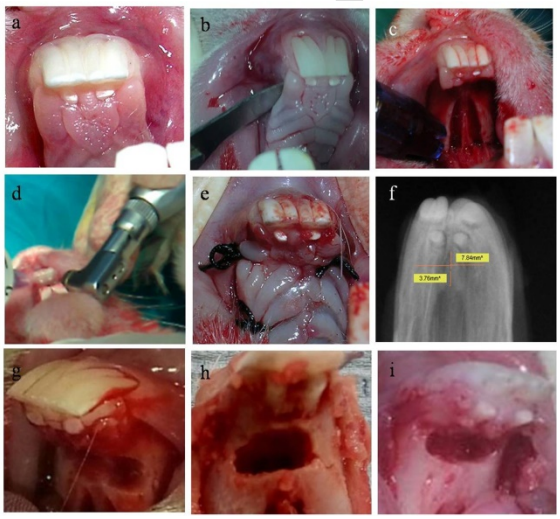
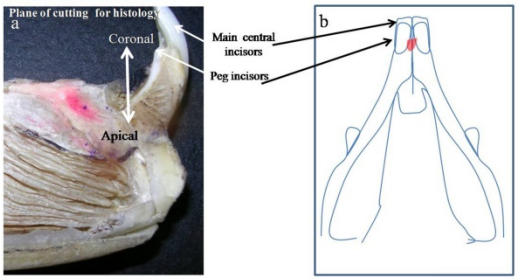


Figure1: Showing surgical procedure; (a) Rabbit's palate preoperatively; (b) Vertical releasing incisions on both sides of premaxilla were done posterior to the upper second incisors (peg incisors); (c) Flap retraction using periosteal elevator; (d) Bone removal was done using a fissure bur attached to a low speed handpiece at 30000 rpm under saline irrigation for cooling;; (e) Mucosal flaps were closed using 3 interrupted 3-0 polyglactin absorbable sutures; (f) Radiographic imaging for rabbit's maxilla. (g-i) Palatal defects of different dimensions; (g) 7×2.5×1 mm³; (h) 7×2.5×5 mm³ with nasal mucosa removed; (i) 5×2.5×4 mm³ with intact nasal mucosa.



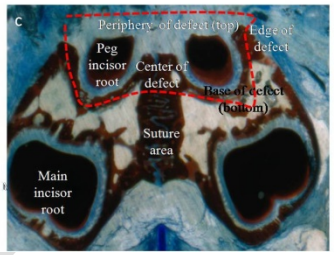


Figure 2: Showing illustration of defect boundaries and plane of cutting for histological assessment; where (a, b & c) are linical image, diagram and histological scanned section respectively, showing the defect area (red spot (b) and frame (a & c)).

RESULTS

Clinical criteria of success of animal model (Fig.3)

7×2.5×1mm³ defect dimensions were reproducible with no postoperative complications.

7×2.5×5mm³ defect dimensions showed death of the two animals; one on the second day of surgery and the other after 2 weeks. Animals' general activity and weight were affected by difficulty in feeding.

5×2.5×4mm³ defect dimensions; with one animal was sacrificed after 24 hours for comparison with other defects in the same interval of time and the other one acted normally until it was sacrificed.

Radiographic and histological assessment

7×2.5×1 mm³ defect dimensions showed preservation of incisors teeth roots (Fig.4 a & 5 a - b) . At 2 weeks, these defects showed almost full healing histolgically (Fig. 4 b & 5 c- d) and couldn't be distinguished from surrounding sound bone radiographically.

7×2.5×5 mm³ defect dimensions showed severe injuries in main and peg incisors teeth roots and nasal mucosa that affected the animals' health and led to their death (Fig.4 c -d &.5e-h).

5×2.5×4 mm³ defect dimensions showed that defects were confined within the peg incisors roots with preservation of all surrounding structures; except for very limited injuries in the peg incisors roots, yet at 2 weeks defect borders could still be distinguished from surrounding bone (Fig.4 e - f &5 i-l). So these defect dimensions proved to be the largest and most reliable to surgically create mid palatal defects in rabbits in that area.

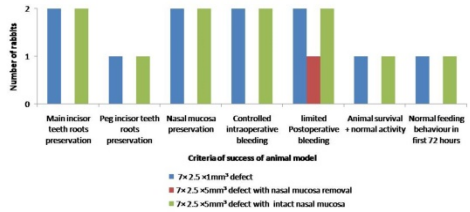


Figure 3: Showing graphical representation of clinical criteria for success of animal model.

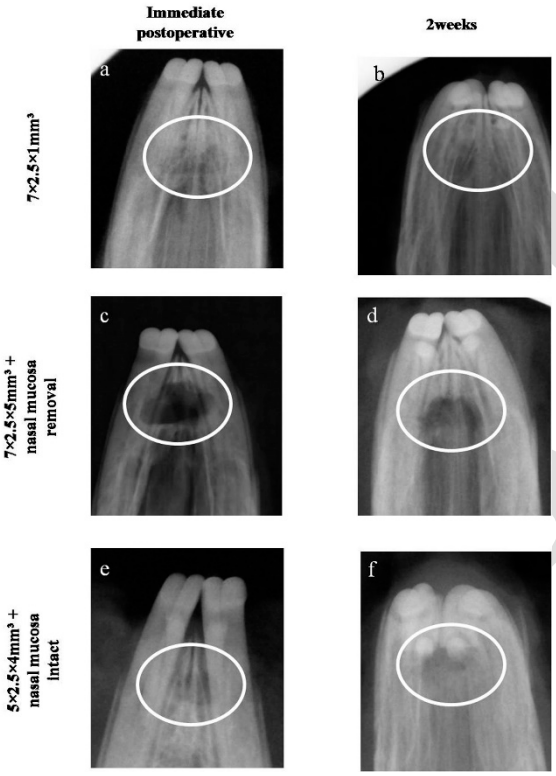


Figure 4: Showing representative radiographic images with frames representing defect areas of different defect dimensions used for optimization of the created model. (a, c & e) Images represent immediate surgically created defects; whereas (b, d & f) represent 2 weeks postoperative interval defects. (a & b) Images show defects of 7×2.5×1 mm³ dimensions with injury in the roots of main and peg incisors teeth root and almost complete closure of the defect restoring the normal palate contour at 2 weeks. (c & d) Images demonstrate defects of 7×2.5×5 mm³ dimensions showing extensive injuries in peg tooth roots and bone overlying main incisor. (e & f) Images show the optimized created defects of 5×2.5×4 mm³ dimensions with defect confined between the roots of the peg teeth with no injuries of main and peg teeth roots and without spontaneous healing of the defect.

Figure 5: Showing representative scanned histological images (a, c, e, g, i& k) with frames representing selected areas in the microphotographs of the relevant sections (b, d, f, h, j & l) respectively of samples of different defect dimensions used for optimization of the created model. (a, b, e, f, i & j) Images represent immediate surgically created defects; whereas (c, d, g, h, k & l) represent 2 weeks postoperative interval defects. (a - d) Images show defects of 7×2.5×1 mm³ dimension with presence of fine bone rim covering the peg tooth root (a &b) then spontaneous healing of the defect at 2 weeks(c &d). (e - h) Images demonstrate defects of 7×2.5×5 mm³ dimensions showing extensive injuries in peg tooth roots bone overlying main incisors and nasal mucosa. (i - l) Images show the optimized created defects of 5×2.5×4 mm³ dimensions with preservation of main, peg teeth roots and nasal mucosa without closure of the defect (k &l).

DISCUSSION

Surgically created cleft animal models have solved many problems that have resulted from congenital models. Several animal models were surgically created to test the efficacy of new materials in treatment of cleft palate. Bardach and Kelly in 1988 discussed surgically created alveolar and palatal

defects in rabbits and dogs and reported similar growth of maxillary bones in both animals (29).

Most recent studies that used rats (16-19) or rabbits (20-23) mainly discussed alveolar defects. Palatal bones stabilize the skull, support masticatory loads (33) and include the mid-palatal suture with its critical function in maxillary expansion; thus palatal bones are of great interest to study the effect of new grafting materials and scaffolds on their regeneration. Some studies discussed the creation of palatal defects as Pilanci, O et al. in 2013 (31) used the model created by El Bokle et al. in 1993 (24); where hemipalatal defects were produced but did not include the mid-palatal suture. Liceras-Liceras et al. in 2017 created palatal defects at the 2nd molar region (25); but when this model was used by Naudot et al it lead to death of six animals during follow up period(32) and Sun et al in 2019 reported the self healing capacity of this model (33). This could be explained by the rich blood supply of the hard palate; making it less reliable to study the effect of any material targeting bone regeneration in this defect.

The established model in the current study adopted the model that was reported by Mostafa et al. in 2014 as a reliable and reproducible model (26). Similar to the present study, they solved the problems of other created models (16, 17&34) as they avoided exposing the incisor roots, which resulted in periodontal pocket formation that could delay bone regeneration or lead to material loss as what was reported by El Deeb et al., in 1982 (10). Mostafa et al's model (26) also avoided the injury of the palatine foramen; where the depth of the defects was limited to the palatal bone without injury of nasal mucosa. Removing the nasal mucosa would increase the surgery time and may affect the health of the animal due to difficulty in oral intake, the risk of aspiration and bleeding into the nasal cavity postoperatively.

Despite the fact that that model was created in rats; similarities between rats and rabbits in maxillary measurements encouraged the use of this model in rabbits in our study. Using the same defect dimensions as those used by Mostafa et al in 2014 (26) showed spontaneous healing of 7×2.5×1 mm^3 defects at 2 weeks; despite that that did not occur in the same defect in rats at 8 weeks. This could be related to the high remodeling rate in rabbits. 7×2.5×5 mm^3 defects with nasal mucosa removal showed severe injuries of vital structures and death of animals; so modifications in dimensions were done to decrease the length of the defect not to injure the peg teeth roots; as they are a unique feature not present in rats and decrease the depth to reach nasal mucosa while leaving it intact to avoid any aspiration complications; so the final optimized defect dimensions are 5×2.5×4 mm^3. However, the small sample size and lack of micro CT setting were limitations in the current study. Future studies should focus on creating a typical cleft model similar to the anatomical condition of human cleft that could enable accurate evaluation of new tissue engineering scaffolds regarding their regenerative properties and their effect on maxillary growth in this critical area.

CONCLUSIONS

To our knowledge, this is the first study to create a palatal defect in rabbits at this region of the palate including the mid palatal suture. Optimization of the defect dimensions was an important phase for further using this defect to test efficacy of bone engineering scaffolds. 5×2.5×4 mm^3 defects have been found to be the largest yet most preservative dimensions for creating palatal defects in that region in rabbits without allowing spontaneous healing till 2 weeks.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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