Original Article - Evaluative Study

Reconstruction of Drug-induced Cleft Palate Using Bone Marrow Mesenchymal Stem Cell in Rodents

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Abstract

Background: Triamcinolone acetonide (TAC) (Kenacort*) is a commonly used synthetic glucocorticoid in today’s medical practice. The drug is also a potential agent in inducing cleft palates in rats. This drug has been used to induce cleft palate in the fetus of the pregnant rats to bring out a suitable animal model for human cleft lip and palate. The drug was given intraperitoneally to induce congenital cleft palate in pregnant mother rats. Aim: The aim of this study is to induce congenital cleft palate in pregnant Wister albino rats and reconstruct the defect with bone marrow mesenchymal stem cells (BMSCs) isolated from the same species along with PLGA (poly lactic co glycolic acid) scaffold. Methods: Twenty female animals were divided into two groups. Each group contains 10 animals. The animals were allowed to mate with male rat during the estrus period and the day in which vaginal plug was noticed was taken to be day 0. The pregnant rats were given triamcinolone acetonide (Kenacort* 10 mg/1 ml intramuscularly/intravenous [IM/IV] injections) injection intraperitoneally at two different dosages as the existing literature. The injection was given on the 10, 12, and 14th day of gestation. The clinical changes observed were recorded, and the change in the body weight was noted carefully. Group 1 which received 0.5 mg/kg body weight of TAC had many drug toxic effects. Group 2 which received 0.05 mg/kg body weight produced cleft palate in rat pups. The pups were divided into three groups. Group A control group without cell transplant, the cleft was allowed to close by itself. Group B containing palate reconstructed with plain PLGA scaffold (Bioscaffold, Singapore) without BMSC, Group C containing BMSC and PLGA scaffold (Bioscaffold, Singapore), Group C operated for the cleft palate reconstruction using BMSCs and PLGA scaffold. There was faster and efficient reconstruction of bone in the cleft defect in Group C while there was no defect closure in Group A and B. Results: There was complete reconstruction of the cleft palate in the group of rat pups which received BMSCs along with PLGA scaffold. Bone growth in the cleft defect was faster; complete fusion of the defect was achieved. Conclusion: The dosage of drug used for inducing cleft palate was standardized in rodents for a definitive congenital cleft palate model. The cleft palate induced was reconstructed using BMSCs and PLGA scaffold. This was compared with a control group and the other group with plain PLGA used for reconstruction of the palate. This study will invite future research in the effect of the drug on human beings, especially on pregnant mothers.

Keywords: Bone marrow mesenchymal stem cells, cleft palate, PLGA (poly lactic co glycolic acid)

Introduction

Rationale

Bone marrow mesenchymal stem cells (BMSCs) are mesenchymal in origin. They are from the marrow stroma and have special characteristics such as pluripotency, plasticity, and stemness. Mesenchymal progenitors differentiate into various lineages such as muscle, liver, nerve cell, endothelium, and bone. BMSC was cultured and differentiated as osteocytes and transplanted along with PLGA (poly lactic co glycolic acid) scaffold. Cleft palate is a congenital disorder arising from failure in the multi step process of palate development. The epidemiology of the disorder is 1/1000 live births in human beings (Vanderas 1987; Wyszynski et al. 1996; Mossey and Little 2002). Although cleft palate has been known to be associated with 300 syndromes. Nearly 50% of the cleft palate in human beings is nonsyndromic.

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The reason for the cleft palate may be due to lack of transcription factors such as Mnx1 or gene mutations or abnormalities associated with growth factors. Treatment for this disorder involves multiple surgeries at various stages of life. [6] To develop a suitable rodent model for the cleft palate reconstruction using BMSCs, this study was done. The current treatment methods are to use bone graft to reconstruct the defective site or using grafts, recombinant human bone morphogenetic protein-2 (rhBMP-2) to reconstruct the defective region. If the cleft lip and/or palate is associated with maxillary hypoplasia, then distraction is used for the maxillomandibular correction. The treatment modality involves donor site morbidity when graft is used; synthetic recombinant BMP-2 protein is very expensive and not affordable for lower and middle socioeconomic status. Allogenic autologous graft can become the safest method of reconstruction. Distraction is a painful procedure and requires a long-treatment time. To bring in an affordable, autologous, effective solution for cleft palate reconstruction, tissue engineering was employed. An animal model for nonsyndromic cleft palate was prepared. To induce cleft palate without gene mutations, triamcinolone acetonide (TAC) was used to induce cleft palate. [7]

**Rat an animal model for human disorders**

There is often a striking resemblance between naturally occurring cleft palate and those producing in the rat by teratogenic experiments. [8-9] The genomic patterns of the rats are more similar to the human pattern; hence, they are preferred animals models.

**Palate development in Wistar albino rats**

The occurrence of cleft lip and palate in rats is as common in human beings. The epidemiology of the cleft palate in rats is 1/7000 births which are 0.014%. [10] Cleft palate can be induced by drug teratogenesis, hypervitaminosis (excess Vitamin A). Hydrocortisone, irradiations, and using TAC (a synthetic glucocorticoid) are preferred method to induce isolated cleft palate [Table 1a]. [11-14]

**Normal palate development in human**

The development of pharyngeal arches takes place from the somitomeres S8–S11 which produces cranial base, sphenoid, and muscles of tongue, maxillary bone. Horizontal plate of maxillae develops from R1 and R2 somitomeres. Deficiency or faulty fusion of R1 and R2 derivatives [Table 1] can cause cleft palate. The palatal shelf cells have been regenerated using tissue engineering already, and it has been compared with the normal palatal cells. They can be compared by immunohistochemistry [Table 1b]. [6]

**MATERIALS AND METHODS**

Twenty-two animals were used for the study. Twenty-one were female, and 1 was a male. The study was carried out after getting permission from the Institutional Ethical Committee and Animal Ethical Committee (IEC/Research Board, Saveetha University). All female rats were 20–22 weeks old weighing 150 g weight and one male rat weighing 180 g. All the animals were fed uniformly with rat pellets (TANUVAS)*. They were maintained in a 12 h day and 12 h night cycle. The body weight was recorded and tabulated on day-to–day basis [Figure 1a].

Twenty-one female Wistar albino rats were allowed to mate with 1 male rat depending on the esterase cycle. The day of the vaginal plug was considered as day 0 [Figure 1b]. An 8-week-old female rat was sacrificed to isolate bone marrow. The marrow isolated was used for culture and osteogenic differentiation.

Twenty rats were divided into two groups containing 10 each. The normal gestation period for Wister albino rats is 20 days. Group 1 received TAC (triamcinolone acetoniod) at a concentration of 0.5 mg/kg body weight. In addition, Group 2 animals received TAC at a concentration of 0.05 mg/kg body weight. The drug was diluted in normal saline.

The diluted drug was given to the pregnant female rats intraperitoneally on the 10th, 12th, and 14th days of gestation. The effect of the drug on both groups were day wise during the entire gestation period. The observations include the reduction of appetite, significant weight loss in animals belonging to Group 1. Group 1 suffered many toxic symptoms. The weight of the rats was noted on the day of injections and after the injections. The toxic effects of the drug were studied in both the groups [Table 1].

The second group of 10 animals was allowed to mate in the esterase period, and they were given the same drug at a dosage of 0.05 mg/kg body weight. This was 0.0075 mg for a 150 g weighing rat.

The body weights of the animals were tabulated. The animals suffered loss weight; they lost about 40 g weight on an average. All the 10 animals delivered pups; there was no cervical bleeding observed. The pups were not disturbed till the completion of the weaning period. Artificial feeding was done to the pups with difficulty in sucking milk. The palates of the pups were examined after the weaning period. There were totally 12 pups delivered. The pups were weighing 6 g in weight on an average.

**Bone marrow mesenchymal stem cell isolation and culture**

One female rat 6-week-old rat weighing 150 g was anesthetized by ketamine 1 ml diluted in normal saline at 10 mg/kg body weight ketamine injected through the tail vein. [12] Long bone of the upper and lower limbs was used. The surgical area was sterilized using betadine solution. An incision placed in both the limbs, lower ends cut, and marrow was flushed into the test tube using phosphate-buffered solution into the culture medium Dulbecco’s modified Eagle’s medium. The marrow collected in the 15 ml Eppendorf’s tube. The flushed medium is centrifuged at a speed of 1250 rpm. The supernatant discarded out into T 75 and T 25 flask. The cells were retrieved and passaged. The first passage of cells done 21 days after culture is shown in Figures 1c-g.
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**Figure 1:** (a) Wistar Albino rat. (b) Vaginal plug noticed after copulation. Day of plug was taken day 0 (gestational age). (c) Isolation of bone marrow from the rat long bones. (d) Passaging of the cells under laminar flow. (e) Bone marrow mesenchymal stem cell showing CD44 positivity in FACS. (f) Polymorphous low-grade adenocarcinoma scaffold. (g) Centrifuged bone marrow mesenchymal stem cells.

**Figure 2:** (a) Trypan blue staining to show the vitality. (b) Neubauer chamber used to count the number of cells per ml. (c) PLGA (poly lactic co glycolic acid) scaffold diluted with normal saline and used along with the bone marrow mesenchymal stem cells on the defect area. (d) Flow cytometry of Bone marrow mesenchymal stem cells FACS analysis. (e) 6th passage cells with 90% confluent.

**Figure 3:** (a) Rat pups showing maxillofacial deformities. (b) Complete closure of the palate.

**Passing of the cells**

The medium in the flask was pipette out, and the flask was washed with Dulbecco’s phosphate-buffered solution to remove the remnants of the medium in the flask. Trypsin ethylenediaminetetraacetic acid 0.25% 500 µl was added to the flask for the detachment of the mesenchymal stem cells in the flask. The flask is incubated for 1 min at 37°C. After trypsinization, the morphology of the cells turns round and motile. When viewed under the microscope. Then, the cells split into 1:5 ratios. The cells are allowed to grow in the flask. Second passage was done at an interval of 25 days and 3rd passage at 11 days interval. After 85% confluence is reached. 4th passage was done at 6 days interval. Basic fibroblast growth factor (FGF) was added to enhance the growth of the cells. 6th passage cells were 90% confluent and were analyzed using FACS analysis. Trypan blue staining was done to check the vitality. The cell count was done using Neubauer's chamber. There were 18 million cells in 1 ml of the centrifuged content [Figure 2a-e] (90% confluent cells).

**Flow cytometry analysis**

Flow cytometry was done with Alexa Fluor product code (Invitrogen A 14798 × 1-MHCD10520 Alexa Fluor).
Table 1: Comparison of group drug effects in group I and group II animals

|                        | Group 1                                | Group 2                                |
|------------------------|----------------------------------------|----------------------------------------|
| Number of animals      | 10                                     | 10                                     |
| Drug dosage            | 0.5 mg/kg body weight                  | 0.05 mg/kg body weight                 |
| Mode of injection      | Intraperitoneally                       | Intraperitoneally                       |
| Average loss of weight | 60-70 g loss on an average             | 40 g loss of weight                    |
| Subcutaneous bleeding  | Subcutaneous bleeding seen on the pinnae and the lower limb | No subcutaneous bleeding seen |
| Severe gastritis/ulcerations in GIT | Gaseous bubbles seen in the inflamed intestines | Not seen |
| Cervical bleeding      | Spontaneous cervical bleeding was evident | Cervical bleeding not seen |
| Dural venous sinus engorgement | Dural venous sinus engorgement seen | No venous engorgement evident |
| Infant pups            | Did not deliver the infant pups        | Delivered infant pups, but they had maxillary hypoplasia, protruded zygomatic arches, growth rate was retarded |

Gastrointestinal tract

Table 1a: Somitomeres and their derivatives

| Somitomeres                  | Derivatives                        |
|-----------------------------|------------------------------------|
| Rhombencephalon (R1): Neural crest of R1 | Ethmoid<br>Presphenoid<br>Premaxilla<br>Vomer |
| Rhombencephalon (R2): Neural crest of R2 | Inferior turbinate<br>Palatine bone maxilla<br>Greater wing of sphenoid<br>Zygoma |

Table 1b: Biomechanic of normal palate development in rats and action of triamcinolone acetonide on inducing cleft palate

- Passage 1: Mesenchymal cell were like fibroblast-like in appearance. The cells were round shape and mixed population. The ratio between the mesenchymal cells and the mixed population was small. There were eccentric nuclei and process like extensions of cytoplasm.

- Passage 2: The second passage was done after 20 days after the first passage, they were changed from T25 to T75 flask. The growth rate was faster split in 1:5 ratio.

- Passage 3: The cells were typical of the adherent spindle and fibroblast-like cells however under differentiation, these spindle-like cells growing as adherent cultures can be induced to grow in an attachment independent fashion, forming spherical type with confluence. The morphology of the cell clusters appeared like islets.

- Passage 4: After being induced for 28 days, 40% confluence was seen. The cells were polygonal or round shape. Fibroblast-like spindle-shaped.

- Passage 5: The cells grew faster and appeared homogeneous when compared to the earlier passages. The duration between the 5th and 6th passage was only 3 days and 90% confluent cells showed 100% positivity for CD44 monoclonal antibody when analyzed by flow cytometry. The 6th passage cells were 90% confluent and CD44, CD29, CD90 positive CD45 negative cells. The cells were transplanted in the defects in the palates [Figure 3a].
Observation of the rat pups delivered to the triamcinolone acetonide injected mother rats
The 12 pups were divided into three groups each containing four in each group.
- **Group A**: control group without cell transplant the cleft was allowed to close by itself
- **Group B**: containing palate reconstructed with plain PLGA scaffold (Bioscaffold, Singapore) without BMSC
- **Group C**: containing BMSC and PLGA scaffold (Bioscaffold, Singapore).

The pups had difference in sound production. Maxillofacial growth retardation was observed. Zygomatic protrusion was seen with maxillary hypoplasia. No polydactyl and syndactyl were seen. The palate was examined after the weaning period. Their involvement of the floor of the nasal cavity was evident. The extent of the cleft was measured by placing an iconic contrast dipped in cotton and placed in the defect, and radiograph was taken.

The depth was around 0.61 cm × 0.42 cm size on an average. The depth involved the nasal floor and premaxilla.
- **Group A**: Animals the defect was retained without closure. The defect existed even after 3 months after birth
- **Group B**: The group of animals which received plain PLGA scaffold in reconstruction of the defect in the palate had the defect in the palate, there was no closure of the palate
- **Group C**: The pups which had received the PLGA and BMSC reconstruction of the palatal defect showed bone development within 21 days postoperatively. The defect was completely closed after 2 months, and the postoperative-dissected palate showed complete closure of the defect.

**RESULTS**

The results were tabulated and studied with difference in TAC dosage the pregnant mother rats which received TAC at a dosage of 0.05 mg/kg body weight [0.075 mg for 150 g weighing rat] showed cleft in the premaxilla region involving the nasal floor. The diluted drug was given to the pregnant female rats’ intraperitoneal on the 10th, 12th, and 14th days of gestation showed evident clefing of palate.

The palatal closure in Wistar albino rats takes place during the 10th–14th day of gestation. Calculated dosage of TAC injected interfered with closure the palatal shelves.

**Culture of bone marrow mesenchymal stem cells**

The 6th passage cells with 100% vitality checked with trypan blue test was used for transplantation and closure of the palatal defect. The cell count was around 8 million cells in 1 ml of the transplanted. The cell count was done in Neubauer’s chamber.

**Palatal defect closure**

Pups belonging to Group C which received BMSC and PLGA scaffold showed faster closure of the palatal defect when compared to Group A and Group B. There was a complete closure of the palatal defect after 21 days post-BMSC transplant surgery [Figure 3b].

**DISCUSSION**

Cleft palate is a well-known, most commonly occurring congenital anomaly affecting the human race. Treatment to this defect has been done for more than a century. Hilliard et al. in 2005 states that secondary palate develops on the embryonic day 11.5 in rats.〔15〕Palatogenesis culminates when the palatine shelves make contact and adhere to each other along the midline forming the epithelial seam that is later replaced to form adjunctive palate. Definitive palate fuses with the primitive palate and nasal septum in the anterior and midline regions, and this separates the oropharynx from the nasopharynx. This complex multiple step process involving multiple steps is caused due to failure of fusion of derivatives of the first rhombomere and second rhombomere derivatives.〔9,10〕In rat, the frequency of its occurrence is 1 in 7000 live births which are very rare. The affected pup usually cannot survive. To prepare a nonsyndromic cleft palate animal model, Wistar albino rats were selected, and drug teratogenicity was used to induce cleft palate.

Phylogenetically in birds and in some reptiles cleft palate is an ancestral trait.〔15〕The reason for selecting rat as an animal model for nonsyndromic cleft palate has been supported by Schüpbach 1983.〔16〕He stated that human and rodents share greater similarity in palatogenesis. Poswillo in 1968 also supported the striking resemblance between naturally occurring cleft palate in human and those produced by rat in teratogenic experiments.〔17,18〕

The animals that received high dosage of triamcinolone acetonide 0.5 mg/kg body weight have suffered drug toxicity suffered with severe weight loss, loss of appetite, subcutaneous bleeding in the limb, and pinnae. Similar toxicity by TAC has been reported by Tripathy pharmacology 5th edition.〔1〕He also further reported cleft palate, cleft lip, cardiovascular and musculoskeletal deformity in TAC-induced cleft palate. In this study also, similar findings were observed in the animals. Gu et al., 2008 have stated the critical period in the palatogenesis is embryonic day 11.5. The palatine shelves elevate during the E14.5–15.5 days of rats.〔5〕During this interval, there occurs a series a molecular steps occurs in the differentiation of the palate one or more of which is blocked by TAC thus causing cleft palate. In Group 1 animals, 0.5 mg/kg body weight triamcinolone acetonide was injected intramuscularly (IM). The drug dosage was given according to the study performed earlier by Furukawa et al. in 2004.〔11〕The drug was given IM to pregnant Wistar albino rats during the E11–E14. The same dosage was given to the Indian strain Wistar albino rats during the same E11–E14. After the injection, all the 9 animals suffered severe weight loss. This would have been due to appetite loss and body protein catabolism. Breakdown of body protein reserves results in muscular weakness, especially in the shoulder, arms, pelvis, and thigh. The psychological behavior of the animals was inactive. They were passive when
compared with their behavior when compared with their earlier behavior before the injections. This would have been due to the myopathy, and psychological disturbances may be due to stress maintenance cycle and altered hypothalamo pituitary adrenal (HPA) axis. TAC injections given on the consecutive days, the long and moderate acting steroid injections on the alternate days. Subcutaneous bleeding spots were evident on the extremitis and in the pinnae which is due to the effect of the drug on the skin making it fragile and easy bruising, purple bleeding spots indicate abnormal platelet counts in the blood. There was unexplained hair loss seen on the mid-dorsal body wall of the animals. The body hair became sparse, and alopecia was observed. The actions of the TAC on the hair loss are still unknown which requires extensive studies. In a normal rat, the proliferation of the cranial neural crest cells within the maxillary process results in primordial palatal outgrowth which takes place on the 11.5 days. Fibroblastic growth factor receptor 1 (Fgfr1) and Fgfr2 expression have been reported by Richman and Crosby et al. in 1990[19] and Richman et al. in 1997,[20] Liu et al. in 2008,[21] these primordial palatal shelves in the palatogenesis grow vertically downward beside the tongue in the E12.5–E13.5 days are localized in the epithelium. Sylvia et al. 2005 state that elevation and reorientation of the palatal shelves bring them in horizontal position. Adhesion and fusion of the shelves take place by the transformation of medial edge epithelial (MEE) cells and apoptosis of MEE. The fusion of the shelves is initiated in the third palatal Rugae region and extends anteriorly, and posteriorly growth factor β3 is specifically required for the adhesion and fusion of the palatal shelves. The phenotype analysis shows that delayed closure of the secondary palate at the anterior end leading to a failed fusion of primary and secondary palates. According to Gu et al. 2008,[5] there is a considerable role of SHOX2 belonging to Homeobox gene family. There have been reported animals experiments, in which SHOX2 inactivation causes significantly reduced bone formation in the hard palate probably due to downregulation of Run × 2 and Osterix. According to Nawshad and Hay in 2003,[22] there is evident cross-talk between Wnt and canonical TGF-β Signaling pathway during epithelial-mesenchymal transition of the palatal epithelial seam. Cui et al. in 1998[24] state that TGF-β3 receptor was restricted more to the palatal shelves. The 17-day old fetus was dissected from the uterus showed cleft palate. The fusion was normally complex on the 14th day of gestation. There was inhibition in the fusion of the palatal shelves. TGF-β3-mediated phosphorylation of Smad activation domain 2 inhibits the MEE cell proliferation a prerequisite for palatal fusion. The TGF-β3-mediated phosphorylation is brought by the glucocorticoid and its receptor complex present in the palatal shelves of the rat. The glucocorticoid dephosphorylates the receptor protein on the cell membrane of the palatal cells activates the receptor and helps it make a complex with glucocorticoid. The activated complex binds to the translocated nucleus binds to the chromatin acceptor sites and alters specific transcription factors such as Msx1, Lhx8, Pax9, Pitx1, or Pitx2. Alteration of these transcription factors causes cleft palate according to Satokata and Maas in 1994,[25] Peters et al. in 1998,[26] Liu et al. 1999,[21] Szeto et al., 1999,[26] Zhao et al., 1999. Although cleft palate was seen in the dead fetus. The animals could not be thrived alive. Group 1 animals has severe drug toxicity. Group 2 animals received 0.05 mg TAC injections on the 10, 12, and 14th day of gestations. The injections were given on the alternate days of gestations. The injections were given on the alternate days because 48 h interval was required for moderate and long-acting steroids. To prevent the suppression of the HPA axis, the injection was given as a single dose once a day to prevent immunological suppression. The size of the delivered infants was small for their age. Glucocorticoid is known to cross the rodent placenta without getting metabolized. The placental barrier is crossed, and it affects the fetus. Hence, the delivered pups also showed slow body weight gain. They were looking small for their age. Growth retardation was significant. According to Zimmerman et al.in 1970,[13] glucocorticoid is being used as anti-inflammatory that is by suppression of the mucopolysaccharide synthesis. Growth inhibitory action is profoundly due to the inhibition of m RNA synthesis mat be the reason for blocking of translation of protein causing cleft palate. Altered protein synthesis may be responsible for abnormal facial changes. There was maxillary hypoplasia and zygomatic protrusion evident. Molecular genetically studies performed by Gu et al. in 2008[5] have been shown that there are specific genes responsible for the fusion of anterior and posterior palates. Factors such as Msx1, Shh (Sonic Hege Hog signaling), BMP-2, BMP-4, Fibroblastic growth factors (Fgf10 and SHOX2 are expressed in the anterior palatal region[5] (Hilliard et al. 2005[15]). In contrast, the genes responsible for the fusion of the posterior palate are the TBX22 and MEOX-2. The anterior and posterior palate are heterogenetic in their epithelial-mesenchymal interaction and in the cellular responses to growth factors in rats a (Zhang et al. 2002,[27] Hilliard et al. 2005).[15] The cleft palate formation starts in the posterior region of the palate and progress anteriorly.

According to Gu et al. in 2008,[5] mildest form of the palatal cleft arises posteriorly. Bifid uvula is the mildest form of cleft palate. Posterior most structures are mostly affected severe cases have been anterior extension of clefting. Rarely, there can be an oval opening in the mid-palatine region without any abnormality in the soft palate. In human beings, fusion signaling progresses from medial to lateral directions. Cleft defect had involvement of nasal floor perforation depicting the actual nonsyndromic cleft palate model in human beings. The cleft defect did not involve the premaxillary region. The results clearly depict that interrupted epithelial-mesenchymal interaction prevents fusion of the palate. Control group which did not receive the transplant of BMSC and group which received plain PLGA reconstruction did not show closure of the palatal defect. Group of rats which received BMSC plain PLGA scaffold showed normal healing was able to close the intact closure of the defect. The scaffolds induce cell adhesion and migration. These scaffolds mimic extracellular matrix proteins. This allows cell-to-cell communications. There
are different types of scaffolds available. PLGA has been used in the present study because it is the most biologically acceptable when it is mixed with water it produces an acidic environment leading to postoperative inflammation. Hence, the PLGA was diluted in normal saline to neutralize the PH. The 90% confluent 6th passage was centrifuged at high speed, and cell count was done using trypan blue test. Each milliliter contained 18 million cells. The cell count above 6 million/ml produces faster regeneration. The photograph taken at 3 months postoperative shows the complete fusion of the defect.

CONCLUSION
Bone marrow mesenchymal stem cells induced faster closure of cleft palate in rodents. BMSC can used in faster and complete reconstruction of palatal defects.

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Conflicts of interest
There are no conflicts of interest.

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