Are Mesenchymal Stem Cells Implanted in Lip Defect Capable of Returning Orbicularis Oris Muscle Function?

Baratali Ramazanzadeh, MD,* Arezoo Jahanbin, MD,* Neda Eslami, MD,† Mojgan Kazemian, MD,† Nasser Mahdavishahri, MD,§ and Samaneh Habibi, MD¶

Introduction: This study aimed to evaluate the amount of orbicularis oris muscle formation and function after using scaffold-free cells and mesenchymal stem cells scaffold in repaired cleft lip of rats.

Methods: Forty male Wistar rats were used. A triangular cut (7 × 7 × 4 mm) was made on the upper lip of 30 rats and then divided into 3 equal groups. In the first group (control), the defected cuts were closed by routine surgical procedures. The second group consisted of cell scaffold and the third scaffold-free cells (5 × 5 × 3) in the area of the incision. Two months after restoration, muscle electromyography activity was examined alongside 10 normal rats and tissue samples were histologically examined. Data were analyzed statistically by SPSS software and ANOVA and the Kruskal–Wallis test. The significance level was considered at P < 0.05.

Results: Electromyography activity in second and third groups was not significantly different from each other and with normal muscle. The control group had, however, higher activity than normal muscle (P < 0.001). The amount of newly formed muscle tissue in the scaffold group had a clear statistical difference with the cell scaffold (P = 0.018) and control group (P < 0.001). Furthermore, the inflammatory reaction showed a significant statistical difference in the control scaffold group (P = 0.001) and the scaffold and cell-scaffold groups (P = 0.007). There was no significant difference in the number of formed fibroblast cells and collagen tissue in the area between the 3 groups.

Conclusions: The results of this study show that extracellular matrix scaffold can reduce tension in the repaired cleft lip area by increasing muscle formation and decreasing scar tissue contraction, confirming the benefits of this type of scaffold.

Key Words: Cleft lip, electromyography, extracellular decellularized matrix, orbicularis oris muscle, stem cells

Cleft lip and palate is one of the most common congenital malformations and its prevalence varies from 1/500 to 1/2000 live births worldwide. Chromosomal abnormalities, genetic factors, stress, folic acid deficiency, and some drugs have been proposed as etiological factors. Routine methods of surgical closure of cleft lip usually accompany common side effects including decreased forward growth of maxilla and lingual tipping of incisors. Contraction of scar tissue is suggested as a contributory factor for these side effects. Strategies for closure of the bone defect include autologous bone grafts and soft tissue repair with local flaps.

Tissue engineering has recently introduced new strategies for reconstruction of cleft lip and palate defects. The principle of tissue engineering is based on selection and preparation of cells, designing scaffold matrix, and applying biologic signaling molecules. Behnia et al investigated secondary repair of alveolar cleft in 2 patients. The patients were treated with the composite scaffold of demineralized bone mineral and calcium sulphate loaded with mesenchymal stem cells. Computed tomograms showed only 34.5% formation of regenerated bone after 4 months in 1 case and 25.6% in the other. This implied that conventional bone substitute is not a suitable scaffold for mesenchymal stem cells induced bone regeneration.

Buono et al utilized stem cells derived from orbicularis oris muscle to enhance bone regeneration at the alveolar cleft area. These multipotent cells were able to differentiate into chondrogenic, adipogenic, osteogenic, and skeletal muscle cells, and showed the same phenotype and behavioral characteristics as other stem cells. They speculated that these cells are a potential source of stem cells for reconstruction of alveolar clefts, especially in young patients with cleft lip and palate.

Although there is some literature on the reconstruction of bone defects in cleft palate patients using stem cells, there are limited data available on the reconstruction of orbicularis oris muscle utilizing stem cells. The aim of the present study was to evaluate the orbicularis oris muscle function and structure after implantation of mesenchymal stem cells to repair the surgically induced lip defect in rats.

METHODS

Forty male Wistar rats, weighing 320 to 420 g, were studied. All animals were kept individually in 22°C temperature, and in a light and humidity-controlled environment.

Preparation of Scaffold

In this study, extracellular matrix derived from the rats’ orbicularis oris muscle was used for preparation of scaffold. Initially, 5 adult Wistar rats were sacrificed and muscles of their upper lip were dissected.

To make the samples free of cells physical and chemical methods including incubation of samples in 1% sodium dodecyl sulfate for 24 hours were applied. Then, they were placed in phosphate buffer saline for 2 hours, before being immersed in 75% ethanol. This procedure was repeated 8 times. After complete washing, the samples

From the *Department of Orthodontics, Dental Research Center, Faculty of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran; †Dental Research Center, Department of Orthodontics, Dental Research Center, Faculty of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran; ‡Department of Oral and Maxillofacial Surgery, Dental Research Center, Faculty of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran; §Department of Biology, Ferdowsi University of Mashhad, Mashhad, Iran; and ¶Department of Orthodontics, Birjand University of Medical Sciences, Birjand, Iran.
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Address correspondence and reprint requests to Arezoo Jahanbin, Department of Orthodontics, School of Dentistry, Park Square, Mashhad, Iran, 91735. E-mail: jahanbin@sums.ac.ir;
Dr_arizzoojahanbin@yahoo.com
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Behavior of mesenchymal stem cells in the prepared scaffolds at day 3

were placed in 70% ethanol for 30 minutes to sterilize the acellular scaffold. After washing with sterile water, they were immersed in phosphate buffer saline for 1 hour (53). The scaffolds were then placed in Dulbecco’s Modified Eagle’s Medium (DMEM) and incubated at 37°C and 5% CO₂. (Fig. 1)

Mesenchymal Stem-Cell Culture with Rat Muscle Scaffolds

Stem cells were provided by the Cell Bank of the Biotechnology Research Institute of Ferdowsi University of Medical Sciences, Mashhad, Iran. These undifferentiated mesenchymal stem cells were derived from human adipose tissue and were labeled with green fluorescent protein. To cultivate the cells on the prepared scaffolds, they were smeared with trypsin enzyme to be separated from the flask. Then, the cells were counted, centrifuged, and extracted from the culture medium. Finally, adding DMEM to cellular sediment, cellular suspension of 5 × 10⁶ cells in 50 μL was obtained.

Two types of scaffolds (cellular and acellular) were prepared (n = 12 each). After removal of scaffolds from the culture media, 5 × 10⁶ cells were cultured on each scaffold in the cellular group. In the acellular group, only 50 μL DMEM was added to culture media. After 1 hour, 1 mL of DMEM was added and the scaffolds were then incubated at 37°C and 5% CO₂. Culture medium was refreshed every 2 to 3 days.

Histologic Evaluation of Cellular Scaffolds Before Graft

Four prepared scaffolds were histologically studied at 7, 14, 21, and 28 days to evaluate the interaction of mesenchymal stem cells with the scaffolds. Paraformaldehyde was used for 24 hours to stabilize the samples. Then, they were dehydrated using 70% ethanol and paraffin blocks were prepared. Sections of 5 to 7 μm thickness were prepared and studied under a microscope. Samples were stained either with hematoxylin–eosin (H&E) or DAPI (4–6-Diamidino-2-Phenylindole).

Figure 2 shows the behavior of mesenchymal stem cells in prepared scaffolds at day 7 using a fluorescent microscope and DAPI staining.

Electromyographic Evaluation

Electromyographic signals of the orbicularis oris muscle at the defect area were evaluated using Powerlab 4.30 (AD Instruments, Bella Vista, Australia). Ten normal rats served as the control group. All 3 experimental groups and control rats were anesthetized similar to the previous stage. Electromyographic recording (EMG) was performed by placing 2 recording electrodes on the orbicularis oris muscle with a 2 mm distance from each other. Also, 2 stimulating electrodes were placed on the area of the buccal nerve, a ground electrode was attached to the leg (Fig. 5) and a 0.5 V voltage was used for nerve stimulation, which lasted for 20 ms per stimulation. The peak EMG activity was measured and the mean value based on the 5 stimulatory pulses was calculated.
Postoperative Histological Evaluation

The rats were sacrificed 2 months after the operation. After which 5 to 7 mm sections of the repaired area were removed. Tissue samples were fixed in paraformaldehyde and Bouin’s fluid for 24 hours, dehydrated, and paraffin embedded. They were stained with H&E, and picrofuchsin for observation of connective tissue.

For quantitative histologic evaluation (histomorphometric analysis), microscopic pictures with 40x magnification were taken, and areas where muscular fibrils could be observed were measured. For qualitative analysis, density of inflammatory cells was considered an indicator of inflammation. Also, formation of new capillaries, proliferation of fibroblasts, and fibrous tissue formation (collagen accumulation) were measured as the criteria of tissue repair (56).

Each criteria was assigned a number of 0 (normal), 1 (mild), 2 (moderate), or 3 (severe). Moreover, density of inflammatory cells, fibroblasts, and new capillaries were classified into 3 consecutive groups: mild, 1–10 moderate, 11–30 and severe ≥31.

Statistical analysis was performed using SPSS (Version 16.0, Chicago, IL). One-way ANOVA and Tukey tests were used for comparison of groups. To compare the quantitative data between groups, the Kruskal–Wallis and Mann–Whitney U test were used. $P < 0.05$ was set as significant.

RESULTS

Mean EMG activity of the orbicularis oris muscle of the normal group is shown in Table 1. Also, mean EMG recordings at the repaired area of the cleft lip in the control, scaffold, and cellular scaffold groups are shown in Table 1.

ANOVA analysis showed a significant difference between the 4 groups ($P < 0.001$). The Tukey test showed a significant difference between the control and normal group ($P < 0.001$). Moreover, there was a significant difference between the control and scaffold ($P = 0.04$) and cellular scaffold ($P = 0.022$) groups.

Table 2 shows the distribution of samples in regard to intensity of inflammatory cellular response, formation of new capillaries, number of fibroblastic cells, and formation of collagen fibers in the control, scaffold, and cellular scaffold groups. According to this table, there was a significant difference between the 3 groups regarding intensity of inflammation and new capillary formation. Mann–Whitney analysis revealed a significant difference between the control and scaffold groups ($P = 0.001$) and scaffold and cellular scaffold groups ($P = 0.007$).

Similarly, a significant difference in new capillary formation was observed between the control and scaffold ($P = 0.007$) and scaffold and cellular scaffold groups ($P = 0.04$). No significant difference in other variables was, however, found between the other groups.

Microscopic view of the repaired cleft area stained with picrofuchsin in the 3 groups is shown in Figure 3.

DISCUSSION

Cleft lip-palate is a very common congenital craniofacial anomaly. Previous studies have shown that routine surgical procedures of lip repair usually result in deficient maxillary growth. Contraction of postoperative scar tissue at the cleft area has been proposed as a contributory factor for maxillary deficiency.

Introducing tissue engineering methods and recent progress in deriving multipotent stem cells have suggested alternative regenerative techniques to repair the cleft areas.

The present study aimed to regenerate orbicularis oris muscle at the cleft lip using stem cells and scaffolds. Electromyographic activity of the repaired area was also recorded to confirm the histological findings.

In this study, natural heterogeneous extracellular matrix derived from the rat’s orbicularis oris muscle was used as scaffold. Recent studies have confirmed the nonantigenic potential of extracellular matrix. Therefore, it can be transplanted from an animal to another without being rejected. Also, undifferentiated human mesenchymal stem cells derived from adipose tissue were used in this study. Considering the increased frequency of liposuction surgeries, ease of access to these types of cells justifies their use. Ability of allogeneic transplantation of adipose-derived stem cells allows the surgeons to use them in emergency cases.

Stem cells derived from human orbicularis oris muscle in conjunction with collagenase membrane have been shown to regenerate cranial defects in rats. Costa et al showed new bone formation at the site of cranial defect using stem cells derived from the pulp of primary teeth.

We found statistically higher electromyographic activity of the upper lip in rats from the control group compared with normal, scaffold, and cellular scaffold groups. This implies the contraction of scar tissue in rats with routine surgical lip closure and thus increased electromyographic activity records, which is in line with similar studies. On the other hand, there was not a significant difference in the muscular activity of scaffold and cellular scaffold groups in comparison to the normal rats. This can be the result of decreased contraction of scar tissue due to the placement of the scaffold. Also, histologic findings showed less formation of muscle fibers.

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tissue at the repaired cleft area in the control group, whereas the scaffold group showed the most frequent number of muscle fibers. These findings confirm the advantages of implantation of scaffolds (cellular or acellular) in the cleft area. Similarly, Chaturvedi et al.\(^\text{18}\) observed regeneration of muscle tissue after placement of bioactive scaffolds.

It seems that in the present study, the scaffolds played a similar role as extracellular matrix and stimulated the migration, proliferation, and differentiation of peripheral cells to regenerate new functional tissue. In a similar study, Buneo et al.\(^\text{14}\) used orbicularis oris muscle as a new source of stem cells with potential for alveolar bone reconstruction in cleft lip/palate patients. Thus, it is probable that muscle formation in the acellular scaffold in this study is induced by stem cells of the peripheral tissues. On the contrary, some studies have reported the potential of activation, proliferation, and differentiation of satellite cells after minor damage to muscle tissue throughout life. In case of severe damage and formation of fibrous tissue, these cells are, however, not able to repair the muscle tissue. Therefore, they can be known as stem cells in the muscles.\(^\text{\text{30}}\) It is probable that these cells have played a role in the induction of muscle fibers before fibrous tissue formation in the acellular scaffold group in the present study.

In this study, we found less number of inflammatory cells in the acellular group compared with the other two groups (Table 2). Ophof et al.\(^\text{14}\) observed less formation of scar tissue and inflammatory reactions in skin-derived substrates implanted in palatal wounds compared with the control group, which is in agreement with our findings.

In the present study, more severe inflammation reaction was found in the control group, which could be the result of wound strain at the repaired area.\(^\text{\text{32}}\)

On the contrary, more severe inflammation was observed in the cellular scaffold group when compared with acellular scaffolds. Reaction of the host immunity system to the stem cells can be a contributory factor for this finding. It should be remembered that labeled stem cells could not be seen at the repaired cleft area after 2 months. This could be the result of either an immunity defense response and thus rejection of the labeled stem cells or the accumulation of inflammatory cells at the cleft area.

Studies on transplantation of myoblasts have shown different results. Rapid rejections of transplanted myoblasts have been observed in rats, which can be the result of activation of the cellular and humeral immunity system.\(^\text{\text{33}}\) Most of the successful myoblast transplantsations have been reported in immunodeficient rats.\(^\text{\text{34}}\)

In the present study, antigenic difference between human stem cells and host tissue might be an etiologic factor for rejection of stem cells and severe inflammatory response.

Better results were found in terms of muscle tissue formation and functional activity of the muscle in the cellular scaffold group compared with control rats. Although labeled stem cells were not observed in the cellular scaffold group in the peripheral muscle, migration of other stem cells in the peripheral muscle might induce muscle regeneration at the repaired area.

It seems that more a severe inflammatory reaction in the cellular scaffold group is the main reason for decreased formation of muscular fibers in the cellular scaffold group in comparison with the scaffold group (Table 2).

In this present study, significantly less number of capillaries was found at the repaired cleft area in the scaffold group in comparison with the other 2 groups (Table 2). It should be remembered that formation of new capillaries increases during the fibroblastic phase of wound repair, whereas it usually decreases during the reconstructive phase. Therefore, rapid maturation of the wound healing phases might be the reason for less observation of new capillaries in the scaffold group, which could be due to the mild inflammation observed in this group compared with the other groups.

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