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ORIGINAL ARTICLE

Histomorphometric evaluation of delayed changes in masseter muscle after lengthening the rabbit mandible by distraction osteogenesis

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OBJECTIVE: The aim of the study was to evaluate the delayed responses and changes of gradually lengthened masseter muscles of rabbit mandibles.

STUDY DESIGN: Unilateral lengthening of rabbit mandibles was performed in 18 New Zealand rabbits for 7 days at the rate of 1 mm day^{-1}. Mandibles of animals were removed at months 3, 4, and 6 after distraction. Biopsy samples of distracted and contralateral side masseter muscles were histopathologically evaluated and mean area of muscle fibers (MAF) was evaluated with histomorphometric methods. Results were statistically analyzed.

RESULTS: Mild to moderate atrophy of the fibers, and necrosis and myophagocytosis in some areas were the leading features at month 3 and which were decreased at month 4 in distracted side-muscle specimens, with no statistically significant differences when compared with non-distracted side muscles at the same periods. Almost completely, normal fibers were detected in distracted muscle specimens at month 6. Evidence of myopathic changes was found to disappear at month 6 and no significant difference was found in the MAF of distracted side muscles.

CONCLUSION: This study showed that the masseter muscle could adapt to gradual lengthening of the mandible within 6 months. Regenerative features and some degree of atrophic changes that could be observed at months 3 and 4 disappeared at month 6, with adaptation of the fibers.
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Keywords: distraction; osteogenesis; mandible; masseter; muscle

Introduction

Lengthening the human facial bones has become a popular method of treatment (Kocaoğlu *et al*, 2003) years after the clinical introduction of the method by McCarthy *et al* (1992). Most of the studies focused on the technical improvement of the devices, histological features of the new bone formation, and surgical techniques, while the response of the soft tissues after distraction of the facial bones has gained less interest (Block *et al*, 1996; Grayson *et al*, 1997; Stucki-McCormick *et al*, 1999b; McCarthy *et al*, 2001; Kişnişci *et al*, 2002; Troulis *et al*, 2003; Gomez *et al*, 2005).

A number of muscles or muscle groups are affected during mandibular lengthening, whether the distraction vector is parallel or perpendicular to the long axis of the muscles. Earlier studies showed that the muscles were elongated and favorably adapt to gradual skeletal changes as the mandible is lengthened by distraction osteogenesis (DO) (McCarthy *et al*, 1992). Masseter, which is known to be one of the most load-bearing masticatory muscles, was shown to respond to horizontal distraction of the mandible with atrophy only after 20 mm of lengthening, whereas the digastrics muscle regenerated after 48 days of fixation (Fisher *et al*, 1997). Other experimental studies also reported that digastric muscle fibers regenerate starting in the distraction period and thus could adapt to the new position of the masticatory complex (Sato *et al*, 2007). On the other hand, the rate of distraction is considered to be one of the important factors that can affect the soft-tissue response of the distraction site. A 3 mm day^{-1} rate of distraction of sheep mandibles was shown to produce maladaptive responses in the muscle fibers of anterior digastrics muscles, whereas a 1 mm day^{-1} rate was found to be tolerated by the muscle fibers (Van der Meulen *et al*, 2005).

Studies of the extremities have shown that the associated muscles respond to progressive lengthening with hypertrophy and hyperplasia of the muscles, and that muscle fibers lengthen by the addition of new serial

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sarcomeres under favorable tension (Ilizarov, 1989). Experimental studies of the mandible have also shown that distraction of the mandible induces myocyte proliferation of the masseter muscle and this proliferative response is considered to improve the long-term stability of the distracted mandible (Castano *et al*, 2001). Studies of the lower extremities reveal that the procedure may lead to muscle contracture and loss of range of ankle movement (Williams *et al*, 1998, 1999).

The response of surrounding soft tissue and muscle changes during and after DO of the mandible may influence the procedure during activation period of the distractor and affect the overall anatomical outcome. Furthermore, delayed response of these muscles should also be evaluated for its negative effects on the stability of the new position of the bone.

The preliminary results of this experimental animal study reported the short-term changes in the masseter muscles of rabbit mandibles lengthened by DO. The structure of the masseter muscle was found to be influenced during and shortly after mandibular DO. Atrophic changes of the ipsilateral masseter muscles were the main characteristics and were attributed to the regenerative response that occurs during and shortly after distraction (Tuz *et al*, 2003).

However, long-term observations are still needed for a comprehensive understanding of the mechanism as well as the delayed structural and morphological changes of the masticatory system. In this second part of the study, the delayed response of the masseter muscles of distracted rabbit mandibles is evaluated and the results discussed, taking into account the previous part of this study and the literature.

Materials and methods

The protocol and guidelines for this study were approved by the Institutional Animal Care and Investigation Ethics Committee of Selcuk University, Faculty of Medicine, Konya, Turkey. Eighteen 20-week-old, male, New Zealand White rabbits, each weighing between 2.1 and 2.6 kg (mean weight 2.3 ± 0.17 kg) were used in this study.

Custom-made external distraction devices were used for unilateral mandibular lengthening, which was carried out on the left side of all animals, whereas no surgery was performed on the right side. All animals were operated under general anesthesia. Following a skin incision of 2.5 cm, the subcutaneous tissues were carefully dissected down to the periosteum, and the bone was exposed with a periosteal incision. A unicortical vertical osteotomy line was outlined with a fine fissure bur, starting between the premolars down to the inferior mandibular border.

Two self-tapping pins with a diameter of 1.5 mm were inserted bicortically on each side of the vertical corticotomy line. Bone cut was accomplished using a thin osteotome through the vertical corticotomy line and the bone fragments mobilized. A custom-made extraoral distraction device was mounted and fixed in place with screws. The flaps were repositioned and the surgical

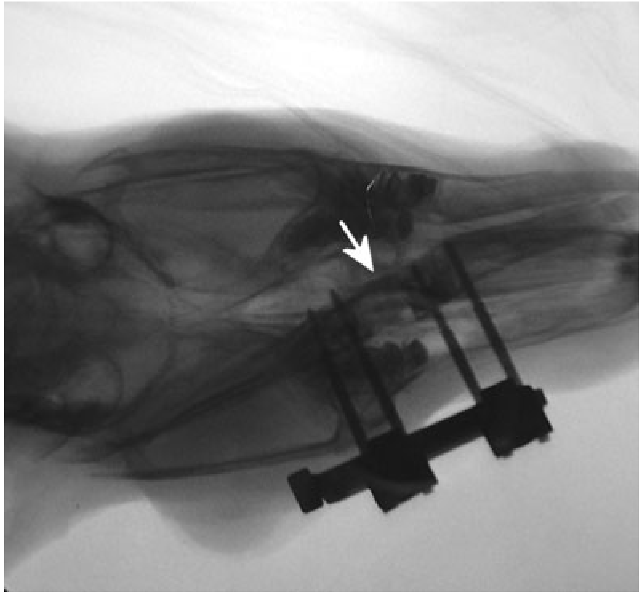

Figure 1 Radiograph of the animal at the end of distraction protocol (white arrow showing distraction gap)

closure of the wound completed with resorbable sutures. All animals were administered intramuscular penicillin G postoperatively twice a day for 5 days, given soft diet and water *ad libitum*, and kept single in cages during the study.

Distraction was started on postoperative day 5 at a rate of 2×0.5 mm day^{-1} and continued for 7 days. The device was held in place for another 30 days for skeletal fixation. The animals were divided into three groups with regard to the time intervals of specimen maintenance: seven animals at postoperative month 3; four at month 4; and seven at month 6. Radiographs of all animals were taken before distraction, after distraction was completed, and before the final period (i.e. months 3, 4 and 6) (Figure 1). The animals were killed for evaluation of the masseter muscles at postoperative months 3, 4 and 6. Experimental and contralateral non-operated sides of the mandibles and overlying masseter muscles of all animals were removed, and specimens were fixed in 10% formaldehyde solution.

Specimens were then taken from both masseter muscles of on both sides parallel and 3–5 mm superior to the inferior mandibular border. Sections were prepared and stained with hematoxylin–eosin. All the analyses and evaluation of the specimens were performed in the same manner by the same authors of the previous preliminary study, to achieve maximum standards. Biopsy samples were analyzed with histomorphometric methods on the images taken from a three-chip color video camera (Sony, Tokyo, Japan), mounted on a light microscope (Zeiss Axioscope; Zeiss, Oberkochen, Germany). Measurements were made with Zeiss Vision KS400 (version 3.0) image analysis program, running on Windows NT 4.0 (Microsoft, Redmond, WA, USA) with an IBM-compatible computer (IBM, Armonk, NY, USA). Thirty different regions were randomly selected from each specimen with 20× magnification lenses. The first muscle fiber sheath that includes the whole body of the point located in certain intervals on the monitor was outlined in each selected region. The mean area of muscle fiber sheath (MAF) and standard deviation (s.d.) were measured and recorded with the software used in the method previously reported (McCarthy *et al*, 2001). Results were statistically analyzed with a software (SPSS version 11.5.0; SPSS Inc, Chicago, IL, USA) working on Microsoft Windows XP.

Results

Animals had no complications or infections during the distraction and consolidation periods. The distraction gap and new bone formation were controlled radiologically before distraction, at the end of distraction and before the animals were killed at months 3, 4, and 6 postoperatively.

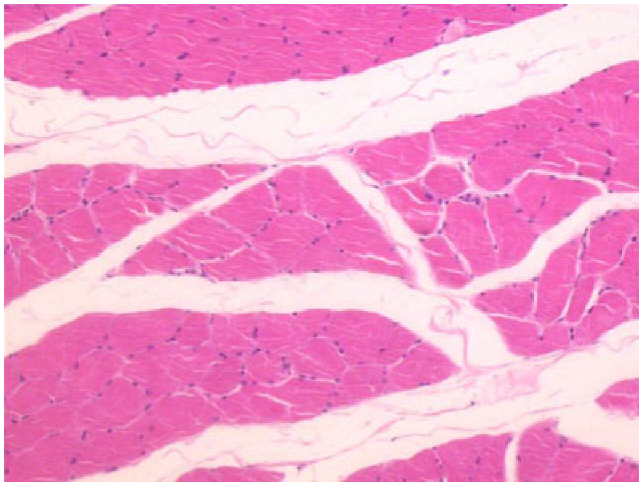

Figure 4 Normal muscle fibers could be observed at month 6 (hematoxylin–eosin stain; original magnification 100×)

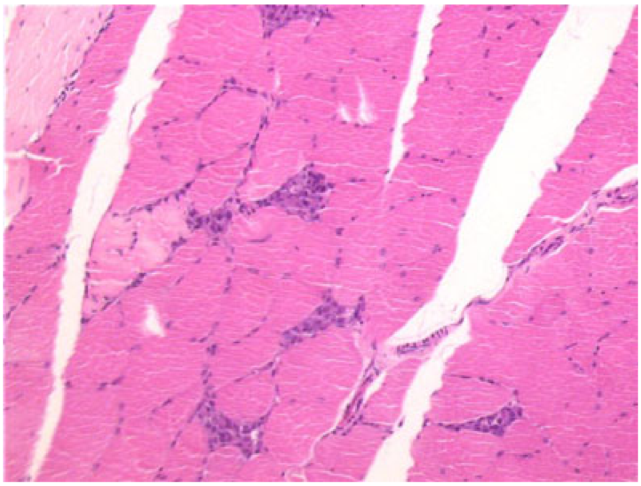

Figure 2 Myophagocytosis areas could be seen at month 3 (hematoxylin–eosin stain; original magnification 100×)

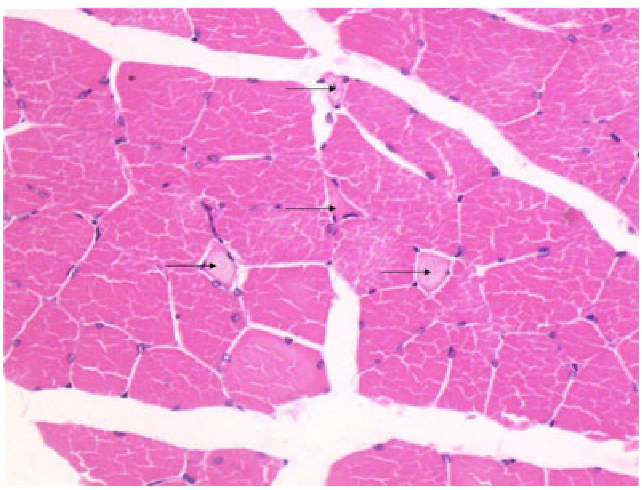

Figure 3 Arrows show atrophic fibers at month 4 (hematoxylin–eosin stain; original magnification 200×)

Histologically, atrophic fibers were seen at month 3 distracted side-muscle specimens. Internalization of the nucleus was detected in a few of the fibers. Muscle fibers in the process of necrosis, regeneration, and myophagocytosis could be seen in some areas. Split fiber and connective tissue increase was also detected in some areas (Figure 2). Similar findings were found, but they were reduced in the month 4 distracted side-muscle specimens (Figure 3). However, month 6 distracted muscle specimens showed almost completely normal appearance (Figure 4). Slight myopathic changes (minimal alteration of fiber dimension, nucleus internalization) in some focal areas were seen in non-distracted side muscle specimens at months 3, 4 and 6. Evidence of slight to moderate myopathic changes at months 3 and 4 distracted side-muscle specimens were found to disappear at month 6 and almost completely normal muscle tissue was observed.

Morphometric analysis

The MAF of the masseter muscles of the distracted and non-distracted side specimens were recorded and statistically compared by using 'paired-samples *t*-test' and no statistically significant difference was observed at month 3. Although a mild increase in the MAF of distracted side muscles at both months 3 and 4 was seen, no statistical difference was seen in 4-month control specimens as well. Six-month control muscle specimens showed a slight decrease in MAF; however, this difference was also not found to be statistically significant (Tables 1 and 2).

Discussion

Distraction osteogenesis is the method commonly used to lengthen bones, where new bone formation is needed in most parts of the body skeleton. Traumatic, pathologic, post-surgical defects, and congenital and

Table 1 Distribution of MAF and s.d. of experimental and control side masseter muscle fiber biopsies at: (a) month 3; (b) month 4; (c) month 6

Animal	*Distracted sides*		*Non-distracted sides*	
No.	*MAF (μm²)*	*s.d.*	*MAF (μm²)*	*s.d.*
(a)				
1	1495.543	330.614	1608.144	362.784
2	2232.321	879.452	1690.811	668.094
3	1971.002	700.302	1093.829	559.112
4	1384.875	311.113	1788.097	1022.194
5	2416.139	787.024	1787.825	438.655
6	1736.062	798.502	1965.952	549.988
7	1353.948	399.181	1628.542	501.056
(b)				
1	1406.692	487.162	1045.502	339.935
2	1277.023	421.327	1252.882	258.813
3	1356.38	619.901	1439.308	747.697
4	1273.373	397.936	1009.75	292.435
(c)				
1	1083.025	392.774	1332.375	620.694
2	1471.674	446.469	1617.531	506.508
3	1482.737	453.836	1237.373	373.586
4	1074.216	519.602	1096.153	474.05
5	1241.216	347.121	1012.023	367.187
6	912.986	214.99	1267.047	560.075
7	997.026	368.105	1574.928	601.795

Table 2 Statistical results of MAF and s.d. of experimental and control side biopsies at months 3 and 6

	MAF (μm²)			
	Experimental side	*Control side*	*Difference*	*Statistic*
Month 3	1798.55	1651.88	146.67	$P = 0.883 > 0.05$
Month 6	1180.41	1305.35	−124.94	$P = 0.819 > 0.05$

developmental deformities are the cases in which DO is used to reconstitute missing bone tissues, in addition to remodeling deficient or abnormal growth of parts of the skeleton (Tong *et al*, 1998; Takahashi *et al*, 2002; Kocaoğlu *et al*, 2003; Birch and Samchukov, 2004).

In maxillofacial surgery, DO has been most prominently used in the mandible and the maxilla, and less frequently in other facial bones. Widening and lengthening the mandible, lengthening both the mandible and the maxilla, augmenting the alveolar bone height, and even carrying a dental segment horizontally with the alveolar bone, extending a missing part of the bone and approximating defects like a resected condyle are some of the applications of DO in the jaws (Polley *et al*, 1996; Epker, 1999; Stucki-McCormick *et al*, 1999a; Rachmiel *et al*, 2001; Kişnişci *et al*, 2002; Uckan *et al*, 2002; Alkan *et al*, 2005).

Among other facial bones, the mandible appears to have a different role as it is the only mobile bone, which is continuously active during most vital functions like chewing, swallowing, talking, mimicking, and even breathing. Thus, the muscular structure is extremely important not only for its functions but for the remodeling of the mandible and other facial bones via the attachment forces that they have to bear as well.

Changes in the vector or alterations in the structure and volume of any bone and particularly the mandible are likely to influence the anatomy, morphology, and the function of the muscle or muscle groups that bond to it. This new state of bone, muscle, and other soft-tissue complex may influence the lengthening procedure, consolidation process, long-term stability of the elongated bone, and the functional and morphological characteristics of the area.

It was previously reported that the masseter muscle showed evidence of regeneration with significant atrophy and mild hypertrophy at the end of month 1 (Tuz *et al*, 2003). Evaluating the long-term response of this particular muscle group, results of this study showed that regenerative changes in the muscle fibers continue at months 3 and 4 with the process of adaptation. It is also observed that the masseter muscle is capable of adapting to the distraction to the mandible in the horizontal vector at the end of 6 months.

Previous studies dealing with the histologic and objective determination of the response of the associated soft tissues of the mandible after DO reported clinically adaptive or even enhanced responses of these deficient tissues, which was attributed to the functional matrix (McCarthy *et al*, 1992). Muscle is capable of adapting to an increase or decrease in functional length by the addition or removal of serial sarcomeres (Williams and Goldspink, 1985). Structural changes in the size of the fibers may occur as a result of physiologic or pathologic stimulation and alterations in the tension of the muscle that may initiate the atrophic or hypertrophic changes in the fibers (Dubowitz, 1985). Clinical studies have also shown that mandibular lengthening by means of gradual distraction leads to hypertrophy of the medial pterygoid muscle up to 75% of its pre-distraction volume in pediatric patients (Mackool *et al*, 2003).

The first part of this study emphasized the atrophic changes of the muscle fibers of the horizontally distracted side of the mandible in the early phase after distraction. Thirty days post-distraction, results exhibited accentuated interstitial edema and connective tissue increase in deep muscle fibers of all distracted side-muscle specimens. Atrophic and hypertrophic changes in muscle fibers and basophilic cytoplasm, and enlargement of the nuclei noted in the cells of the distracted side-muscle fibers were attributed to regenerative changes (Tuz *et al*, 2003). Other studies showed that the masseter muscle shows evidence of atrophy if the distraction process exceeds 20 mm. However, the vector of the distraction force is considered to have an important role in the physiologic and functional adaptation of a muscle and muscles in the same vector of distraction reported to adapt to elongation with compensatory regeneration and hypertrophy, whereas muscles lying in a different vector show prolonged evidence of atrophy during later periods of distraction (Fisher *et al*, 1997). Data on the delayed response of these fibers to the same kind of mandibular lengthening with DO was lacking and needed to be evaluated for understanding the overall treatment outcome.

The results of this study showed that, although atrophic fibers were still visible at month 3 in distracted sides, a mild increase in the MAF of distracted side muscles was observed but no statistical difference was found when compared with non-distracted sides. A mild decrease in MAF was observed in the 4th month period specimens. Muscle fibers in the process of necrosis, regeneration, and myophagocytosis could be seen in some areas. Split fiber and connective tissue increase was also detected in some areas.

Almost completely normal appearance could be observed at postoperative month 6 in distracted muscles. Morphometric analysis of the month 6 distracted side muscles also did not show any statistical difference when compared with the muscles of the non-distracted sides. Slight myopathic changes in some focal areas of non-distracted side muscle specimens at months 3 and 4 were attributed to morphological changes in the structure of the mandibles and nutritional differences of the animals. Evidence of slight to moderate myopathic changes in month 3 and 4 distracted-side muscle specimens was found to disappear at month 6.

Month 6 muscle specimens appeared normal. This finding was supported by histomorphometric analysis, a more objective method, and no significant difference was observed between non-distracted side muscles. When considered with the short-term results, the overall effects of DO on the masseter muscle in rabbit mandibles may be described as significant atrophic alterations with regenerative changes at month 1 after distraction; non-significant differences but regenerative features at months 3 and 4 with reduction of atrophic changes; and finally almost normal appearance of the masseter muscle at month 6 with adaptation of the fibers. This study evaluated the adaptation of the rabbit masseter muscle that lies perpendicular to the vector of distraction of the mandible but not the other muscles in different vectors. Considering the complex structure of the masticatory system, further studies of other muscle groups and anatomic structures may contribute more information to the topic.

Author contributions

Dr. Hakan H. Tüz designed the study, did the surgery and wrote the paper. Dr. Doğan Dolanmaz designed the study and did the surgery. Dr. A. Alper Pampu did the surgery. Dr. Reha Ş. Kişnişci designed and wrote the paper. Dr. Ömer Günhan did the histological evaluations.

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