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Experimental Oral Biology

Alterations in enzyme histochemical characteristics of the masseter muscle caused by long-term soft diet in growing rabbits

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OBJECTIVE: Recently young people have an increasing tendency to intake an easily chewable diet and spend less time on mastication. The aim of the present study was to investigate the histochemical effects of long-term soft diet on the masseter muscle in growing rabbits.
MATERIALS AND METHODS: Twelve young male Japanese white rabbits were divided into two groups (*n* = 6 each) at weaning (1 month after birth) and fed a solid diet (control group) or a powder diet (soft-diet group). The duration of the experimental period was 6 months. Masseter fibers from the superficial and the deep portions were histochemically defined as type 1, 2A, 2B, or 2C fibers.
RESULTS: As compared with that of the control, the deep masseter of the soft-diet group showed a significantly lower ratio of type 1 fiber cross-sectional area to total area (6.3 and 10.1% for the soft-diet and control group, respectively), significantly more type 2A fibers (74.0% *vs* 50.3%) and significantly fewer type 2B fibers (4.3% *vs* 12.5%). However, fiber size did not differ between the two groups. NADH-tetrazolium-reductase (NADH-TR) of the masseter was less reactive in the soft-diet group, reflecting a lower oxidative capacity.
CONCLUSIONS: These findings indicate that the alteration of the functional activities contributed to selective disuse influences on the type 1 and type 2B fibers, and a resultant increase in type 2A fibers. This study suggests that long-term alteration of jaw function induced by a soft diet can lead to adaptations of the masseter muscle.
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Introduction

Recent studies have shown an increasing tendency for children and adolescents to intake an easily chewable diet and spend less time on mastication (Varrela, 1990, 1992; Teuteberg, 1991). This is of concern because soft diet has an unfavorable influence on masticatory muscles and craniofacial growth. A reduction in the recruitment of muscle fibers due to a decreased masticatory load may alter the morphological and histochemical characteristics of the masticatory muscles. Craniofacial skeletal changes caused by different food consistencies (Ito *et al*, 1988; Luca *et al*, 2003) and altered proportions of muscle fibers in the masticatory muscles (Engstrom *et al*, 1986; Maeda *et al*, 1987; Kiliaridis *et al*, 1988; Miyata *et al*, 1993; Sfondrini *et al*, 1996; Miehe *et al*, 1999) have previously been described, although the results of these studies are not necessarily consistent. Kiliaridis *et al* (1988) revealed a reduced frequency and smaller size of type 2A fibers in the masseter of rats administered a soft diet than in those receiving a normal diet, but found no changes in the digastric muscle. Maeda *et al* (1987) demonstrated that the fiber sizes of the masseter muscle and muscle spindles were significantly decreased in mice kept on a soft diet. Further, extraction of lateral teeth in combination with a soft diet has been reported to result in a shift in the muscle fiber composition towards the fastest fiber-type in rats (Miehe *et al*, 1999). However, Sfondrini *et al* (1996) did not find any change in the masseter muscles but did find changes in the temporalis and digastric muscles in rats. However, these studies used rats or mice as experimental subjects, and all fiber-type transitions observed in these studies occurred within the subpopulation of fast (type 2) fibers, rather than between slow (type 1) and fast (type 2) fibers, because in the jaw elevator muscles of rats and mice, the vast majority of the fibers are of type 2 (2A and 2B), and only a few type 1 fibers are recognized, whereas, humans have a considerable proportion of type 1 fibers in the masseter muscles (Eriksson and Thornell, 1983).

There have been a few histochemical studies of the adaptive responses of the masseter muscle to soft diet using rabbits (Negoro *et al*, 2001; Langenbach *et al*, 2003), which are similar to humans with respect to fiber-type composition and masticatory movement. The present study focuses on the effects of soft diet on the histochemical characteristics of fiber-type distribution in the masseter muscle in growing male rabbits. It can be expected that the group fed on a soft diet will show an increase in type 2B fibers as shown in inactivated muscles.

Materials and methods

The sample for this study consisted of 12 juvenile male Japanese white rabbits (Saitama Experimental Animals Supply, Co. Ltd, Sugito-Machi, Japan). All procedures of the study were approved by the Institutional Animal Care and Use Committee, University of Fukui. One month after birth, the animals were weaned with a mean body weight of 300 g and were randomly separated into two groups: an experimental group and a control group ($n = 6$ each). The control rabbits were fed their ordinary food, a diet of hard pellets (3.2 mm in diameter, normal diet; RC-4, Oriental Yeast, Co. Ltd, Tokyo, Japan), and the experimental rabbits were fed a fine-grained diet (soft diet; RC-4 powder) containing the same ingredients as the hard pellets. Each rabbit was housed, in an individual cage in a climate-controlled room kept at 20°C, and given water *ad libitum* until reaching 7 months of age. The experimental period was 6 months. Each animal was weighed every 7 days.

Histochemical analysis

After the experimental period of 6 months, the animals in both groups were sacrificed, following sedation with diethyl ether, by intravenous injection of an overdose of sodium pentobarbital (Nembutal; Abott Laboratory, Chicago, IL, USA). Masseters of both sides were excised from the bellies of the superficial mid-portion and the deep mid-portion of the masseter (Bredman *et al*, 1990), both perpendicular to the main direction of the muscle fibers. Each specimen was mounted on a cork board with tragacanth gum mounting medium, snap frozen in 2-methylbutane cooled by liquid nitrogen, and kept at −90°C until use. After allowing the temperature of the sample to rise in a cryostat chamber at −20°C, the muscles were cut into 8-μm serial transverse sections.

Serial sections were stained for myofibrillar ATPase reactivity after preincubation in specific pH buffers, NADH-tetrazolium-reductase (NADH-TR), and hematoxylin and eosin. Following alkaline preincubation (sodium barbital and $CaCl_2$; pH 10.3–10.7) and acid preincubation (sodium acetate and sodium barbital; pH 4.30–4.55), the muscle fibers were classified as type 1 (ATPase type 1 = immuno type I), 2A (ATPase type 2A = immuno type II A), 2B (ATPase type 2B = immuno type II X or II B), or 2C by means of an ATPase reaction (Dubowitz, 1985). Type 2C fibers not only showed a high reactivity after alkaline preincubation but also had a residual positive reaction at pH 4.30.

A preliminary study confirmed that both the diameter and the fiber-type distribution (the ratio of each fiber type) were similar between the right and left masseter muscles. In the present study, the right masseter muscle was used for histochemical evaluation in each subject. Four areas in the specimen were selected to measure the ratio of type 1 fiber cross-sectional area to total area, the fiber-type distribution, and the mean diameters of the muscle fiber type using an image analyzer (Mac Scope; Mitani Co., Ltd., Fukui, Japan) (Kitagawa *et al*, 2000). In each specimen, at least 500 muscle fibers were classified and measured. The ratio of type 1 fiber cross-sectional area (%) was calculated as the formula: (cross-sectional area of type 1 fibers/total area) × 100. The fiber size was measured across the minimum diameter of each type. The data presented in this paper are expressed as the mean ± s.d. The statistical significance of differences between the two groups was evaluated with Mann–Whitney U-test.

Results

Animals in both groups were adjusted to their food within days after weaning. The control rabbits did not show any visible change in their masticatory pattern. The rabbits in the soft-diet group were observed to use mainly licking movements to feed. The body weight of all the rabbits regularly increased with development. After the experimental period, however, there was a significant difference ($P < 0.05$) in body weight between the control (3505 ± 263 g) and the soft-diet group (3018 ± 151 g).

Histochemical features in the muscle fibers

The masseter muscle of both groups consisted of mainly type 1, 2A, and 2B fibers (Figures 1 and 2). Type 2C fibers were fewer in number. In the deep masseter, the percentage of the type 1 fiber cross-sectional area to total area was significantly lower ($P < 0.05$) in the soft-diet group (6.3 ± 1.9%, $n = 6$), than in the control group (10.1 ± 3.1%, $n = 6$) (Figures 1 and 3). In the superficial region of the masseter, there was a similar tendency (5.2 ± 1.2 and 7.8 ± 3.5% for the soft-diet and the control group, respectively), but no statistical difference was found ($P = 0.12$).

Compared with the control group, significantly more type 2A fibers [74.0 ± 10.3% for the soft-diet group ($n = 6$) *vs* 50.3 ± 19.6% for the control group ($n = 6$)] and significantly fewer type 2B fibers (4.3 ± 2.3% *vs* 12.5 ± 10.0%) were observed in the deep masseter of the soft-diet group (Figures 2 and 4). Similarly, in the superficial masseter of the soft-diet group, significantly more type 2A fibers (66.1 ± 6.1% *vs* 42.5 ± 16.7%)

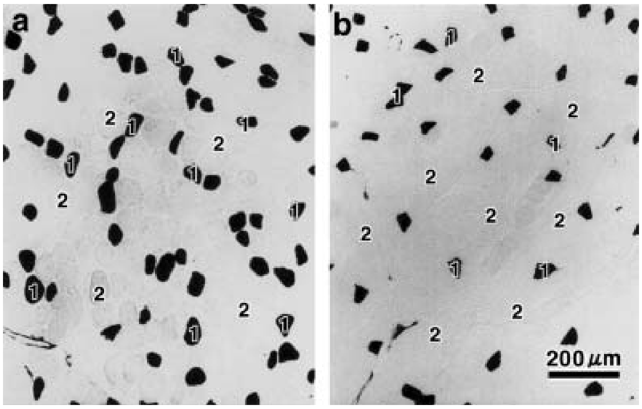

Figure 1 Photomicrographs of cross-sections of the deep masseter in the control (a) and the soft-diet group (b) at 6 months after weaning. Type 1 fibers are shown in black and type 2 fibers in white (ATPase stain, pH 4.30). Compared with the control, fewer type 1 fibers were located in the soft-diet group

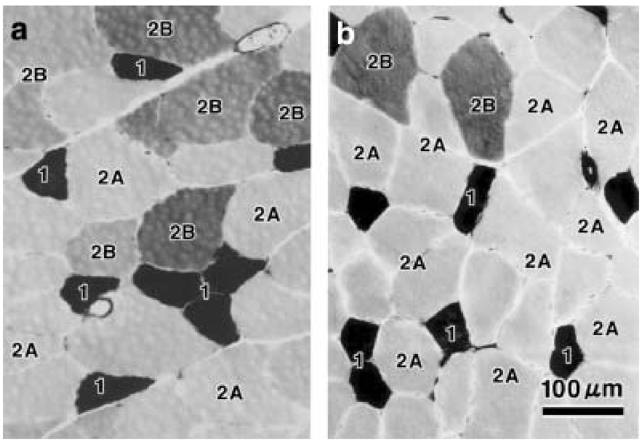

Figure 2 Photomicrographs of cross-sections of the deep masseter in the control (a) and the soft-diet group (b) at 6 months after weaning (ATPase stain, pH 4.55). Compared with the control, a higher proportion of type 2A fibers and a lower proportion of type 2B fibers was observed in the soft-diet group

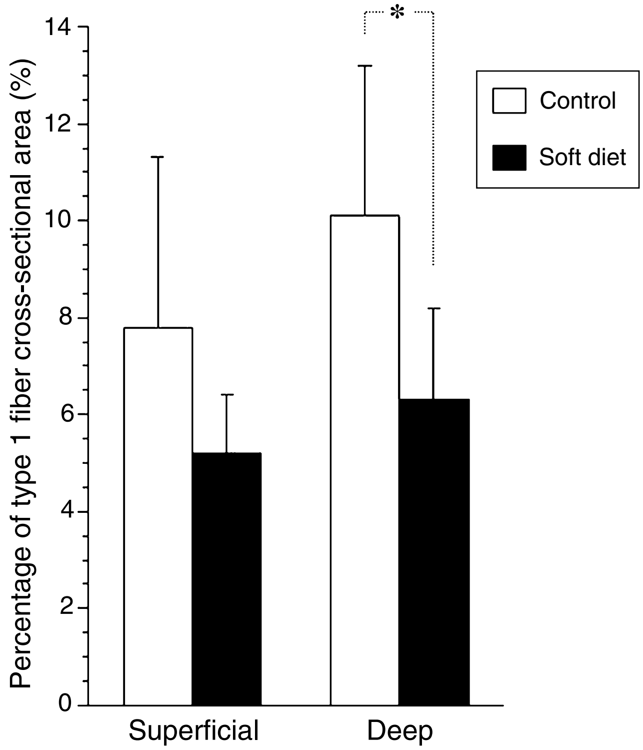

Figure 3 Mean percentage of type 1 fiber cross-sectional area in the superficial and the deep masseter. The soft-diet group showed a significantly lower ratio of type 1 fiber cross-sectional area than the control group in the deep masseter (Mann–Whitney U-test, $P < 0.05$, $n = 6$ in each group)

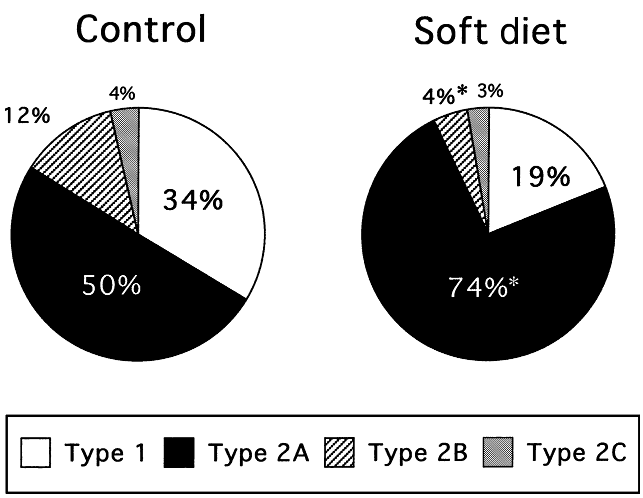

Figure 4 Mean relative percentages of the different types of fibers in the deep masseter in the control and soft-diet groups. The soft-diet group showed significantly more type 2A fibers and fewer type 2B fibers compared with the control group (Mann–Whitney U-test, $P < 0.05$, $n = 6$ in each group)

and significantly fewer type 2B fibers (6.4 ± 3.2% *vs* 21.8 ± 4.5%) were observed (Figure 4).

Regarding the fiber size, there were no differences in the size of any of the fiber types between the two experimental groups (Figure 5).

NADH-TR staining showed lower reactivity at each muscle site of the soft-diet group, reflecting lower oxidative activity, in comparison with that of the control group.

Discussion

Muscle fibers have been characterized by their physiologic properties unique to each type. These properties have been shown to be closely related to muscle function. As to the masticatory muscles of mammals, the composition of these different fiber types varies with the structure and function in each species. According to the enzyme histochemical studies of the masseter muscles in different mammals (Suzuki, 1977; Tuxen and Kirkeby, 1990), the masseter muscle fibers of rats and guinea pigs consist almost entirely of type 2 fibers, indicating that the masseter muscle of rodents is well adapted to a powerful or sudden activity for biting and

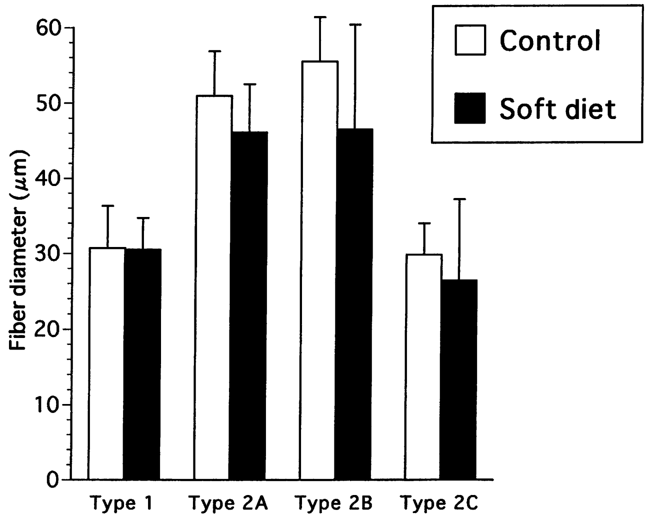

Figure 5 Mean diameter of the different types of fibers in the deep masseter. Comparing control and soft-diet groups, no significant differences in fiber size were observed

gnawing. In contrast, the masseter muscles of cattle and sheep, which ruminate for about 4–9 h per day, are uniformly composed of type 1 fibers. Thus, the masseter muscle of ruminants is considered to be well adapted to a slow, long-term, and fatigue-resistant function. The masseter muscle of humans, dogs, swine, monkeys (Maxwell *et al*, 1979) and rabbits has intermediate characteristics and comprises type 1 and type 2 fibers with different proportions. Furthermore, different types of myosin isoforms, e.g. alpha-cardiac and neonatal isoforms, have also been described in the masseter muscle (English *et al*, 1998; Yu *et al*, 2002; Sciote *et al*, 2003). These unusual histochemical features of the masticatory muscles may be closely related to characteristic and unique functions, including chewing, maintaining resting mandibular posture, and other movements of the mandible such as parafunctional recruitment (Kitagawa *et al*, 1999).

In order to investigate adaptation of the skeletal muscle to a reduction in activity, various experimental models have been provided: denervation (Bacou *et al*, 1996; Bobinac *et al*, 2000), tenotomy (Noirez *et al*, 2000), immobilization (Malathi and Batmanabane, 1988), and spaceflight (Jiang *et al*, 1992). With respect to orofacial muscles, functional changes induced by removing teeth (Maxwell *et al*, 1980), denervating the facial nerve (Kitagawa *et al*, 2002) or the trigeminal nerve (Mineta, 1990), or raising the bite height (Ohnuki *et al*, 1999), have been shown to affect the histochemical profiles of muscle fibers in the masticatory muscles. Although these models may have shown drastic changes in muscle, they do not represent natural conditions. The soft-diet feeding used in the present study is considered to keep the animals in a relatively natural condition. The authors confirmed by visual inspection that the rabbits in the soft-diet group performed licking movements to take their food and did not need to exert a large force by the masseter muscle during mastication. Such features probably resulted in a reduced activity level of the masticatory musculature. The masseter muscle of rabbits comprises type 1 fibers in relatively high frequency like the masseter muscle of humans, and rabbits perform a grinding movement with lateral excursion of the mandible during chewing (Weijs *et al*, 1989), which also resembles the masticatory movement of humans. Administration of a soft diet to young rabbits is therefore an excellent experimental model as it inaugurated new experimental protocols.

Several previous studies have used rabbits to study the effects of food consistency on fiber-type properties of the masseter muscle (Negoro *et al*, 2001; Langenbach *et al*, 2003). Langenbach *et al* (2003) recently demonstrated that the muscles of rabbits fed a soft diet were composed of fibers with a smaller cross-sectional area than the hard-diet group, but no changes were found in fiber composition. However, their experimental setting was different from our design: the age of the animals at the start of their experiment was 16 weeks, versus 4 weeks for our rabbits, and the incisors of the soft-diet group were cut back to minimize gnawing. We used very young male rabbits at the age of weaning (4 weeks), because it is known that large developmental changes in fiber-type composition occur after this age (Bredman *et al*, 1992; Eason *et al*, 2000). In addition, it is reported that great sexual differences develop after the age of 6 weeks (English *et al*, 1999; Eason *et al*, 2000). It may be easier for muscles to adapt to functional alterations before the establishment of any definitive functional pattern. Negoro *et al* (2001) showed that the diameter and ratio of type 1 fibers was most remarkably decreased, and that the ratios of type 2A and 2B fibers were increased in the fine-grained diet subjects. The present study reported that soft-diet treatment increases type 2A fibers at the expense of type 1 and 2B fibers. Negoro *et al* (2001) demonstrated similar findings to some extent, however, the duration of their experimental period was only 3 months (that of our experiment was 6 months) and their results were critically different from ours in that type 2B fibers were increased (our study showed decreased type 2B fibers in the soft-diet group).

We revealed that soft diet-induced changes in masticatory function in growing rabbits led to alterations in the enzyme histochemical profile of the masseter muscle. The masseter in the soft-diet group showed a significantly smaller percentage of type 1 fiber cross-sectional area and a tendency toward a decreased frequency of type 1 fibers compared with that of the control group fed a normal diet. According to the ‘size principle’ theory, smaller motor units are recruited during weaker contractions requiring less tension, and larger motor units are recruited increasingly as the contractile force increases. This theory has also been confirmed to be applicable to the jaw muscle functions (Scutter and Turker, 1998). Generally, type 1 fibers are optimally suited for sustained contractions requiring relatively low force and thus are recruited during biting or masticating (Maxwell *et al*, 1980). In our experiment, the rabbits fed a soft diet could be considered to be performing a lower-resistance exercise when chewing and grinding food than the control rabbits fed a normal hard diet. The smaller cross-sectional area and decreased number of type 1 fibers indicate a decrease in the load resisted by these fibers. The reduction of load on type 1 fibers may have been the result of an altered recruitment pattern of these fibers due to the decreased masticatory effort. The alteration of these functional activities in the soft-diet group most likely contributed to a selective disuse effect on the type 1 fibers, and might have thereby caused a conversion from type 1 to type 2A fibers. NADH-TR reaction showed lower reactivity, reflecting lower oxidative capacity and less fatigue resistance in the soft-diet group, which in turn indicate the disuse effect of type 1 fibers.

Similar findings were reported in a study of tooth extraction by Maxwell *et al* (1980), and in a study of facial nerve denervation by Kitagawa *et al* (2002). Maxwell *et al* (1980) studied the effects of complete tooth removal on the histochemistry of jaw muscles in rhesus monkeys. They demonstrated that the jaw elevator muscles of edentulous monkeys had fewer and smaller slow fibers than did the muscles of normal rhesus monkeys. Kitagawa *et al* (2002) investigated maxillofacial growth and the histochemical effects of long-term unilateral chewing caused by unilateral facial paralysis on the masseter muscle in growing rabbits. The rabbits with facial abnormality on the paralyzed right side were observed to perform unilateral chewing only on the surgically unmanipulated side. Type 1 fibers of the masseter muscle on the denervated side, i.e. the non-chewing side, were significantly fewer in number and smaller than those on the unmanipulated side. These two studies on experimental disuse of masticatory muscles support our findings.

In the present experiment, another important finding was that the soft-diet group also showed a greater proportion of type 2A fibers and lesser proportion of type 2B fibers compared with the control group. Type 2B fibers are optimally suited for sudden and powerful contractions requiring relatively large force. The control rabbits had to increase occlusal forces to crush the tougher pellets. Several studies have reported that tougher foods require higher levels of the activity of masticatory muscles (Weijs *et al*, 1989; Liu *et al*, 1998). In contrast, the rabbits fed a soft diet did not need to perform crushing movements and thus did not generate large occlusal forces. This diminished activity of the masticatory muscles probably resulted in the fiber-type shift in the present study. The reduced proportion of type 2B fibers in the soft-diet group was the result of a conversion from type 2B to type 2A fibers due to a sustained alteration in the recruitment pattern of these fibers in response to less adaptation of the musculature for powerful contractions. This is in agreement with the findings of Ringqvist (1973), who reported that maximum bite force correlates with type 2 fiber size, not with type 1 fiber size. In our study, it is speculated that the significantly increased proportion of type 2A fibers probably resulted from a simultaneous two-way conversion; i.e. a conversion from type 1 to type 2A fibers, and another conversion from type 2B to type 2A fibers. This two-way conversion may have been caused by the continuous adaptation to the reduction of load by the masseter muscle of the soft-diet group, concomitant with disuse effects on both type 1 and type 2B fibers.

The enzyme histochemical characteristics of the masticatory muscles provide a cumulative picture of neuromuscular alterations related to the masticatory functions. The results presented in this study suggest that the long-term alteration in jaw function induced by a soft diet can lead to adaptations of the masseter muscle. These adaptations of masticatory muscles could also occur in humans who regularly ingest a soft diet. Further research regarding the myosin heavy chain gene transcription or regarding other masticatory muscles such as the pterygoid muscles should be conducted in order to better understand the correlation between food consistency and masticatory muscles.

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