Reduced masticatory function is related to lower satellite cell numbers in masseter muscle

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SUMMARY The physiology of masseter muscles is known to change in response to functional demands, but the effect on the satellite cell (SC) population is not known. In this study, the hypothesis is tested that a decreased functional demand of the masseter muscle causes a reduction of SCs. To this end, twelve 5-week-old male Sprague–Dawley rats were put on a soft diet (SD, $n = 6$) or a hard diet (HD, $n = 6$) and sacrificed after 14 days. Paraffin sections of the superficial masseter and the m. digastricus (control muscle) were stained with haematoxylin and eosin for tissue survey and with anti-myosin heavy chain (MHC) for slow and fast fibres. Frozen sections of both muscles were double-stained for collagen type IV and Pax7. Slow MHC fibres were equally distributed in the m. digastricus but only localized in a small area of the m. masseter. No differences between HD or SD for the m. digastricus were found. The m. masseter had more SCs per fibre in HD than in SD (0.093 ± 0.007 and 0.081 ± 0.008, respectively; $P = 0.027$). The m. masseter had more fibres per surface area than the m. digastricus in rats with an SD group (758.1 ± 101.6 and 568.4 ± 85.6, $P = 0.047$) and a HD group (737.7 ± 32.6 and 592.2 ± 82.2; $P = 0.007$). The m. digastricus had more SCs per fibre than the m. masseter in the SD group (0.094 ± 0.01 and 0.081 ± 0.008; $P = 0.039$). These results suggest that reduced masseter muscle function is related to a lower number of SCs. Reduced muscle function might decrease microdamage and hence the requirement of SCs in the muscle fibres.

Introduction

Many animal studies have been performed to study the influence of masticatory muscle function on craniofacial growth as well as the response of muscles to interventions like distraction osteogenesis, surgical mandibular lengthening, mandibular repositioning by insertion of devices into the oral cavity, occlusal changes, and diet changes (Kiliaridis, 1986; Carlson *et al.*, 1978, 1989; Tüz *et al.*, 2003; Proff *et al.*, 2007), and muscle detachment (Carlson *et al.*, 1978). Gedrange *et al.* (2001, 2003) also showed an increase in slow fibres, blood flow, and vascularization.

The variation in food consistency to change the functional demands offers the opportunity to study muscle changes in an intact masticatory system. Decreased functional demands in animals fed a soft diet (SD) changed the distribution of fibre type (type IIB fibres increase) and reduced muscle mass and cross-sectional area (Kiliaridis *et al.*, 1988; Gedrange *et al.*, 2001; Langenbach *et al.*, 2003; He *et al.*, 2004; Kitagawa *et al.*, 2004; Saito *et al.*, 2004; Urushiyama *et al.*, 2004; Taylor *et al.*, 2006). In contrast, little is known about the effect of functional changes on masseter satellite cells (SCs).

SCs are myogenic adult stem cells which received their name due to their specific location between the basal lamina and the sarcolemma of the myofibres (Mauro, 1961; Muir *et al.*, 1965; Dwahan and Rando, 2005; Zammit *et al.*, 2006; Grefte *et al.*, 2007). A true stem cell is able to give rise to two cell types, i.e. a new stem cell and a differentiated cell type. In the case of a myogenic stem cell, the cell should be able to give rise to a more differentiated cell in the muscle cell lineage on the one hand and to a new SC on the other (Dwahan and Rando, 2005; Zammit *et al.*, 2006). The latter crucial property of stem cells is termed self-renewal.

Normally, SCs are quiescent in adult muscle. When activated, for example, as a consequence of injury or exercise, SCs divide to produce myoblasts that proliferate, differentiate, and fuse to form myotubes. The latter mature into myofibres (Dwahan and Rando, 2005; Negroni *et al.*, 2006; Zammit *et al.*, 2006; Grefte *et al.*, 2007). Quiescent SCs express markers such as the transcription factor Pax7, the adhesion molecule M-cadherin, and saliomucin or CD34 (Charge and Rudnicki, 2004). There are indications that SCs from the craniofacial muscles are different from those of the skeletal muscles in the rest of the body (McLoon *et al.*, 2007). The two types of muscles also respond differently to injury. Craniofacial muscles show a larger capacity to regenerate, whereas other skeletal muscles often respond with necrosis.

This might indicate that the physiological response of the SCs to injury also differs (McLoon *et al*., 2007). Further, craniofacial muscles have a higher proportion of SCs than skeletal muscles, which might enhance their response to damage (Renault *et al*., 2002; McLoon *et al*., 2007). They also lack the expression of specific transcription factors such as Pax3, while skeletal muscle SCs express both Pax3 and Pax7 (McLoon *et al*., 2007; Miura *et al*., 2006). The response of craniofacial muscles to damage and varying functional demands has mainly been studied in extraocular muscles (McLoon *et al*., 2007), but not yet in masticatory muscles.

Therefore, the aim of this study is to investigate changes in masseter muscle SC numbers in response to reduced functional demands in growing rats.

Materials and methods

Animals

All animal experiments were approved by the Board for Animal Experiments of the Radboud University Nijmegen Medical Centre in accordance to Dutch laws and regulations on animal experiments. Twelve 5-week-old male Sprague–Dawley rats (Janvier, Le Genest, France) were used for the experiments; they were divided into an SD group ($n = 6$) and a hard diet (HD) group ($n = 6$). The rats were housed under normal laboratory conditions. Before the start of the experiments, the rats had been acclimatized to the animal facility for 1 week. The rats in the HD group were fed normal rat chow and water *ad libitum*. The rats in the SD group received grinded rat chow mixed with water (1:2.5) and water *ad libitum*. The experimental model has been used in earlier studies into the effect of decreased functional demands (Kiliaridis, 1986). The body weight of the animals was monitored throughout the study.

Histology and immunohistochemistry

After 2 weeks feeding with the experimental diet, the rats were killed according to the standard CO_2/O_2 protocol. After sacrifice, the left m. masseter and the left m. digastricus (control muscle) were fixed in 4 per cent paraformaldehyde for 6 hours and processed for paraffin embedding. The right superficial m. masseter and the posterior belly of the right m. digastricus were directly frozen in optimal cutting temperature embedding compound (CellPath®, Newtown, UK) using isopentane precooled in liquid nitrogen. Five micrometres of paraffin and frozen sections were cut transversally and collected on superfrost plus slides (Menzel-Gläser, Braunschweig, Germany). Three sections per muscle per rat were taken this way. Sections were made through the centre of the muscles. Paraffin sections were stained with haematoxylin and eosin (HE, according to Delafield) for general tissue survey. Distribution of slow and fast myosin was assessed by staining paraffin sections of m. digastricus and superficial m. masseter sections from both HD and SD

with antibodies mouse anti-slow (1:1600) and mouse anti-fast myosin heavy chain (MHC; 1:1600; both from Sigma Chemical Co., St. Louis, Missouri, USA; Figure 1). Subsequently, the bound antibodies were visualized using the biotinylated secondary antibodies donkey-anti-mouse IgG (H+L; 1:500; Jackson Labs, West Grove, Pennsylvania, USA) and stained with 3,3-diaminobenzidine.

The frozen sections were double-stained with the antibodies rabbit anti-collagen type IV for staining muscle fibres (1:100; Euro-Diagnostica BV, Arnhem, The Netherlands) and mouse anti-Pax7 (1:100; Developmental Studies Hybridoma Bank, Iowa City, California, USA) for SCs as described previously (Grefte *et al*., 2010). The nuclei of all cells were visualized using 4′,6-diamidino-2-phenylindole (DAPI).

Standardized pictures were taken with the AxioCam MRc5 camera using a grid positioned over the entire cross-section. Using the centre as a reference, five and three pictures were taken on the vertical and horizontal midlines, respectively. All sections were visualized with the Zeiss Imager.Z1 using the AxioVision 4.6.3 software (Carl Zeiss Microimaging GmbH, Jena, Germany).

Quantification

The total number of nuclei, Pax7-positive nuclei, and fibres were calculated per surface area using a representative overview field of every frozen section from all muscles. The tissue surface area was determined by measuring the surface of the tissue without the empty spaces (breaks). Means and standard deviation ($N = 6$) were calculated for the number of nuclei per surface area, the number of nuclei per fibre, the number of fibres per surface area, the number of Pax7-positive cells per muscle fibre, the number of Pax7-positive cells per surface area, and the percentage of Pax7-positive cells of the total number of cells.

Statistics

The total number of cells, the number of Pax7-positive cells (SCs), and the number of muscle fibres in the HD and SD groups were compared with Student's *t*-test. Student's *t*-test was further used to compare the number of SCs per surface area, the number of SCs per fibre, and the number of SCs per total amount of all cells for both muscles. The differences between the m. digastricus and m. masseter were compared with a paired *t*-test.

Intra-observer reliability was tested with a paired *t*-test for the nuclei, the number of Pax7, and the fibre count and was 0.938, 0.979, and 0.927, respectively. This means that the reliability is very good.

Results

The mean weight of the rats in the SD group was 236 ± 12.14 and 243 ± 11.53 in the HD group. The difference was
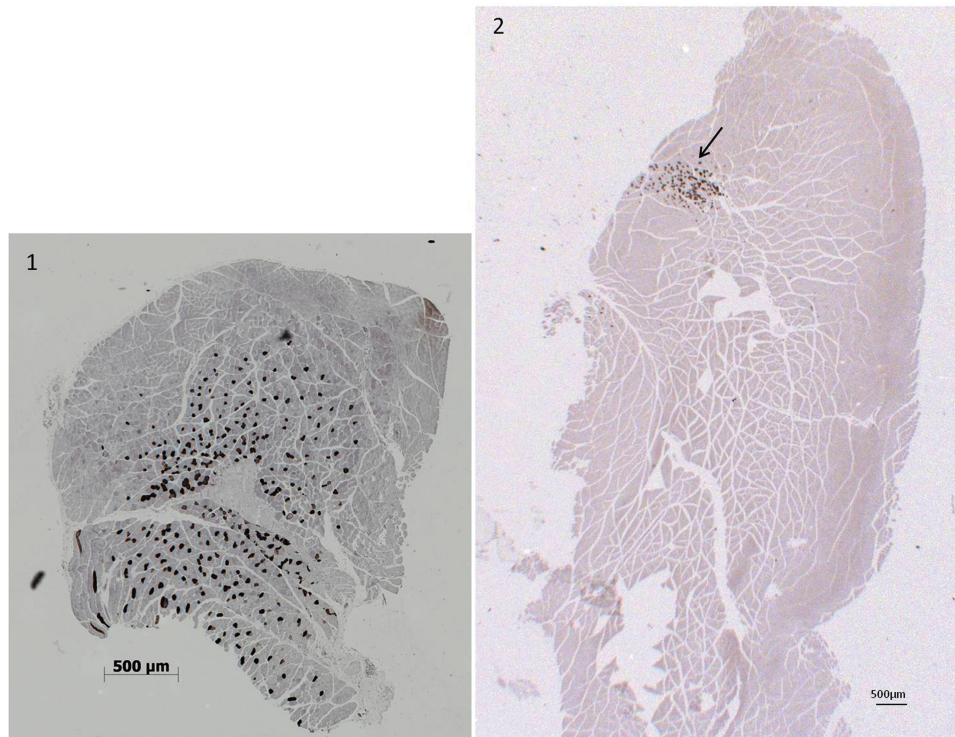
Figure 1 Immunohistochemistry of the m. masseter and m. digastricus. Paraffin sections from the m. digastricus (1) and superficial m. masseter (2) from the HD group stained for slow MHC fibres. The arrow indicates the localized area of slow fibres in the m. masseter.

not statistically significant (*t*-test). One rat in the SD group died before the end of the experiment.

General histology

Paraffin sections from the m. digastricus and m. masseter were stained with HE for a general tissue survey. The m. digastricus is a somewhat rounded muscle in cross-section with a diameter of about 3–4 mm. In contrast, the superficial m. masseter is elongated in cross-section with the largest diameter about 8 mm. Anatomically, the superficial m. masseter is covering the deep m. masseter. For the immunohistochemical studies, only the superficial m. masseter and the posterior belly of the m. digastricus were used.
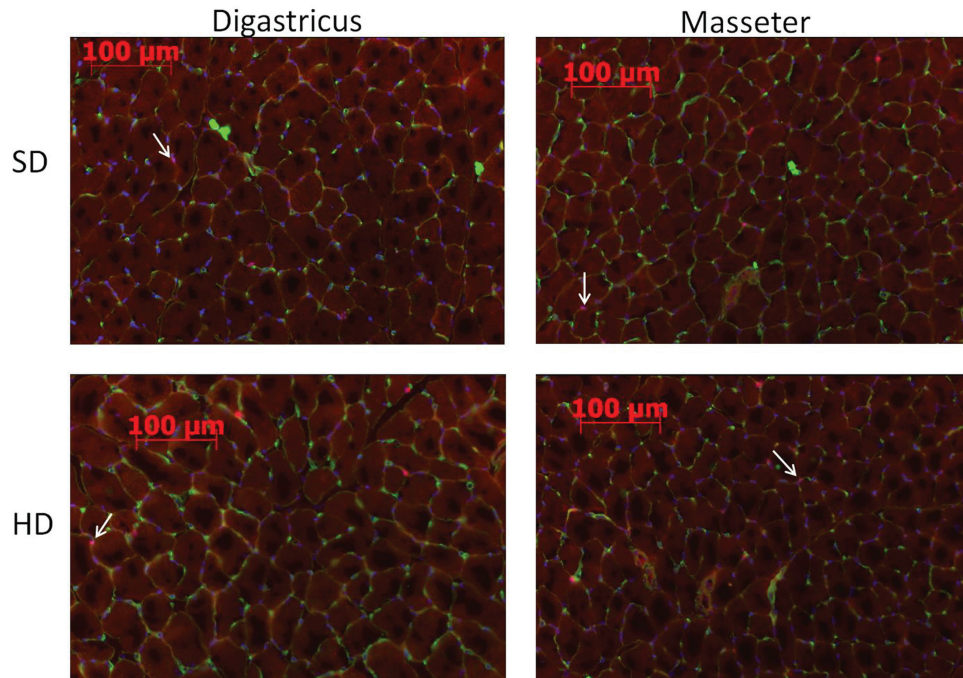
Immunohistochemistry

Paraffin sections from the m. digastricus and superficial m. masseter from both HD and SD groups were stained for slow and fast MHC fibres (Figure 1). Nearly, all myofibres in both muscles were positive for fast MHC (not shown). Only a few slow MHC-positive fibres are scattered throughout the m. digastricus muscle, but the superficial m. masseter only contains some positive fibres in one specific area, the region where the muscle spindles are localized. No obvious differences were observed in the numbers and distribution of slow MHC-positive fibres between the HD and SD group.

Frozen sections were double-stained for Pax7 and collagen type IV (nuclei; Figure 2). The collagen type IV antibody stains the basal lamina (green) surrounding every muscle fibre and thus allows the identification of single fibres. The Pax7 antibody stains the nuclei of the SCs (red), but the combination with the blue DAPI yields a purple staining. Multiple cross-sections were used to determine the number of fibres per millimetre square (Figure 3a), the number of SCs per fibre (Figure 3b), and the total fraction of SCs (Figure 3c).

No significant differences between HD and SD in any of the measurements for the m. digastricus were found. There were no significant differences for the m. masseter in any of the measurements except for the number of SCs per fibre. The m. masseter had more SCs per fibre in HD (0.093 ± 0.007) than in SD (0.081 ± 0.008; $P = 0.027$; Figure 3c).

When comparing the muscles in each diet group, some differences were found. The m. masseter had significantly more fibres per surface area than the m. digastricus in rats with an SD group (758.1 ± 101.6 and 568.4 ± 85.6; $P = 0.047$) and a HD group (737.7 ± 32.6 and 592.2 ± 82.2; $P = 0.007$; Figure 3a). The m. digastricus had more SCs per fibre than the m. masseter in the SD group (0.094 ± 0.01 and 0.081 ± 0.008; $P = 0.039$; Figure 3c). The differences between m. masseter and m. digastricus in number of nuclei per fibre in the HD and the SD were not statistically

Figure 2   Frozen sections of the m. masseter and m. digastricus with immunohistochemical staining for collagen type IV (green) to show the muscle fibres and for Pax7 (red) to show the SCs and with DAPI staining for the nuclei (blue). SCs are indicated with white arrows.

significant (Figure 3b). In both muscles, the percentage of SCs on total of nuclei was about 6.1 in HD and 5.3 per cent in SD, which was not statistically significant.

Discussion

The rat model for investigating the effects of changes in masticatory function has been used extensively (Kiliaridis, 1986; Katsaros, 2001; Bresin and Kiliaridis, 2002). These studies mainly showed changes in sutures, craniofacial skeletal growth, and muscle structure. To our knowledge, it has never been tested whether changes in diet consistency also affect the SC population in the masticatory muscles. In our study, the m. digastricus was used as a control muscle as in previous studies (Kiliaridis *et al*., 1988; He *et al*., 2004). Theoretically, the m. digastricus is not affected by changes in mastication as its function is to antagonize the masseter during opening of the jaw. We confirmed in our study that diet consistency does not affect the fibre density, cell density, nor the number of SCs of the m. digastricus. There were also no differences in fibre density in the masseter. However, the size of the fibres of the masseter muscle has been reported to increase (Kiliaridis *et al*., 1988; He *et al*., 2004). The distribution of slow fibres was also similar in HD and SD in both muscles. This last finding has already been reported by Kiliaridis *et al*. (1988). However, the reduced functional demand on the m. masseter (SD) led to lower numbers of SCs. SCs continuously react to changes in the

environment. They respond to normal changes in function as well as to injury in order to maintain muscles' integrity. After injury, SCs give rise to myoblasts that fuse with existing damaged fibres or form new fibres (Anderson, 2006; Zammit *et al*., 2006). The SCs in both skeletal muscles and craniofacial muscles respond to injury and exercise with proliferation. However, SCs in craniofacial muscles proliferate more, yet differentiate later than SCs in skeletal muscles (Kadi *et al*., 2005; Harel *et al.*, 2009; Ono *et al.*, 2010).

Previous studies on the effect of function on the SC population in skeletal muscles are based either on exercise models or on disuse models with an extra intervention. In the latter studies, disuse was generally achieved by changing the innervation (Gunderson and Bruusgaard, 2008; Legerlotz and Smith, 2008) and is therefore not completely comparable with our disuse model. These studies in skeletal muscles show a reduction of activated SCs (Gunderson and Bruusgaard, 2008; Legerlotz and Smith, 2008). Studies on the effect of function on the SC population using an exercise model mainly focus on changes during exercise itself and on changes after the cessation of exercise. Skeletal muscles adapt to some types of exercise with an increase in fibre size, the number of nuclei per fibre, and the number of SCs (Kadi *et al*., 2005; Mackey *et al*., 2007; Snijders *et al*., 2009). However, a reduction in SC number occurs when training is stopped (Mackey *et al*., 2007; Snijders *et al*., 2009). This seems comparable to our results as the rats are switched from the standard hard laboratory chow to soft powdered food.
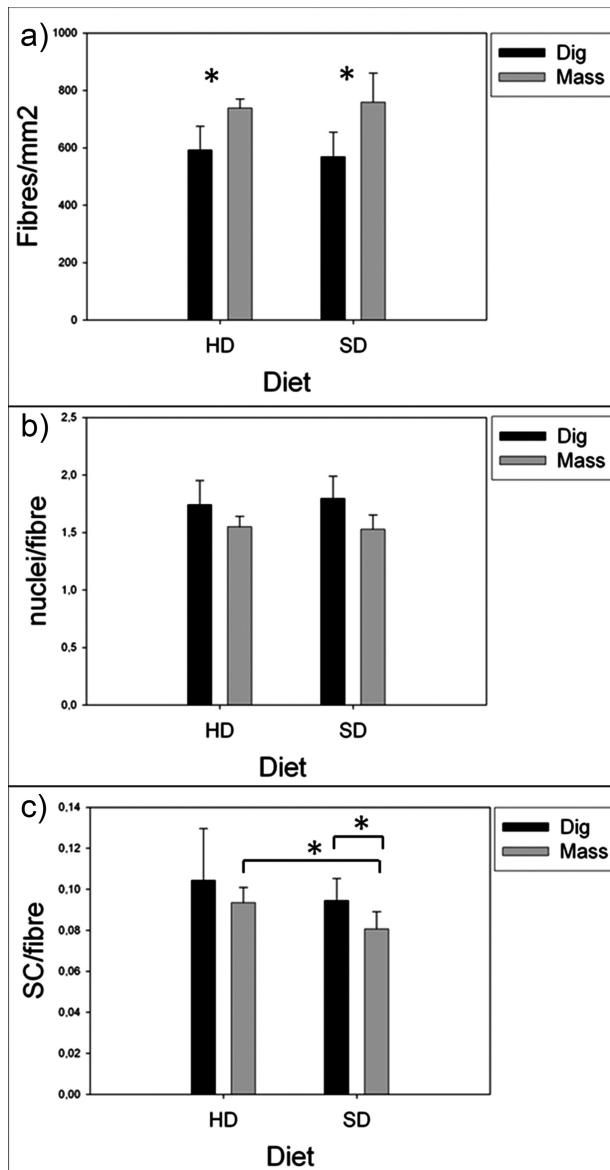
Figure 3 Quantification of the immunohistochemistry. Numbers of fibres, nuclei, and SCs were counted on frozen sections of the m. masseter and m. digastricus stained for collagen type IV (green) and Pax7 (red) and stained with DAPI. *$P < 0.05$. (a) Number of fibres per surface area, (b) number of nuclei per fibre, and (c) number of SCs per fibre for HD and SD for both the m. digastricus and the m. masseter.

In aged as well as young individuals, an increase in SC numbers was found after endurance exercises, whereas resistance training only increased SC numbers in young individuals (Snijders *et al*., 2009). It is not known whether the increase in SCs during training is related to the exercise itself or to damage induced by the exercise (Kadi *et al*., 2005). Exercise always increases the number of SCs, but does not always increase the number of fibres and fibre diameter. Exercise does occasionally increase the number of myonuclei per fibre, but this does not seem to be the standard mechanism (Kadi *et al*., 2005; Smith and Merry, 2012). To our knowledge, no previous studies on the SC population

in a masseter muscle disuse model have been carried out. The results of this disuse model suggest that the reactions are similar as in skeletal muscle disuse models. However, it needs to be further investigated whether the differences in SC numbers between SD and HD increase in time or remain stable.

In conclusion, our data show that the SC population is smaller in m. masseter with reduced function. Although this seems to show that the SCs in the masseter muscle adapt to changes in function, the exact mechanism remains to be clarified.

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