Frequency-Dependent Fatigue Development During Electrical Stimulation in the Masseter Muscle of Pigtail Monkeys

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Low-frequency fatigue was investigated in nine female and one male adult pigtail monkeys (Macaca nemestrina) with a mean weight of 5.3 kg (range 4.3 to 6.5 kg). After sedation and anesthesia, silver electrodes were inserted into the anterior and posterior parts of the right masseter muscle. The contralateral muscle was used as a control. The masseter muscles were stimulated for 3 minutes (4 Hz, 2 ms, 100 V). After a 5-minute rest period, the stimulation was repeated with the same duration and voltage but at a higher frequency of 8 Hz. Bite forces were measured, and muscle biopsies were obtained from the central part of the right masseter and immediately frozen in liquid nitrogen. After freeze-drying, a fluorometric analysis that used enzymatic methods for measuring levels of glycogen, glucose, lactate, pyruvate, creatine phosphate, nicotinamide-adenine dinucleotide (NAD), and reduced NAD (NADH) was performed. The bite force decreased by 12% after the initial 3 minutes of work. After the second contraction the bite force decreased to 56%. Prominent substrate depletion was observed. The precontraction levels of glycogen, glucose, and phosphocreatine were all reduced. The NADH and the NAD concentrations increased. An accumulation of metabolites was evident. The pyruvate increased by 32% and lactate levels increased by a factor of 3. The male measurements were comparable to the nine female measures for each assessment. The substantial substrate depletion in combination with a prominent accumulation of metabolites may contribute to the development of low-frequency fatigue.
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The jaw muscles are relatively fatigue resistant compared to limb muscles.[1,2] Nuclear magnetic resonance (NMR) studies of human masseter muscles during endurance tasks have revealed smaller decreases of high energy phosphates (creatine phosphate) and intramuscular pH changes than expected in conjunction with unchanged adenosine triphosphate (ATP) values.[3] The electromyographic–bite force ratio does not change significantly when measured during brief maximum voluntary contractions.[4] Endurance, defined as the duration of sustained maximal clenching effort until exhaustion, has been determined by the onset of intolerable pain[2,4,5] rather than by specific metabolic changes that are disclosed by means of NMR techniques.[3] Experimental investigations of jaw muscle fatigue in various species that represent different masticatory system designs such as pigs and dogs[6,7] and rhesus monkeys[8] have all revealed a close relationship between prominent bite force reduction and the depletion of intramuscular substrates such as glycogen, glucose, and high-energy phosphates,

and an accumulation of waste products (lactate and pyruvate). These findings have been validated in experimental conditions using the NMR technique in the adult rabbit masseter muscle[9,10] as well as in human masseter muscles.[11]

Physiologic research has shown that the muscular force is regulated by the discharge rate of motor neurons during voluntary contractions,[12] which allows low-frequency motor neurons to produce slow, unfused twitches during longer periods. These fatigue-resistant motor units are recruited at low firing thresholds, before the rapidly contracting, fatigable, high-frequency motor units.[13,14] Two distinct fatigue patterns based on frequency-dependent motor unit activations are therefore recognized.[15] The first, a loss of muscle force at high activating frequencies, is known as high-frequency fatigue (HFF),[16] and the second, an impairment of force development at low-frequency pulses, is known as low-frequency fatigue (LFF).[17] A decrease in bite force production in conjunction with prominent substrate depletion and metabolite accumulation was evident in the rhesus monkey masseter muscle after two repetitive contractions at identical low frequencies (4 Hz).[8]

To the best of the authors' knowledge, the contraction behavior and the metabolic response in jaw muscles to alterations in low activation frequencies between contractions has not earlier been reported in this species.The force development at high stimulation frequencies has been characterized in the cat masseter and temporalis muscles,[18,19] in the rat masseter,[20] and in opossum jaw closers.[21] Thus, the aim of the present study was to explore the LFF process in the pigtail monkey masseter muscle by electrical stimulation; a series of different stimulation frequencies were employed in combination with bite force measurements and standardized biopsy samples.

Materials and Methods

Animals and Anesthesia

Ten adult pigtail monkeys (Macaca nemestrina, nine females and one male) were used. The monkeys had a mean weight of 5.3 kg (range 4.3 to 6.5 kg). All had Class I molar relations and a full complement of teeth in occlusion. The animals were fed a diet that consisted of bread and rice supplemented with carrots, apples, bananas, and other fruits, and they were allowed water ad libitum. Before the experiments the monkeys were kept in individual cages. The surgical procedure was performed under sterile conditions and general anesthesia in an operating theater within the primate laboratory. The monkeys were sedated with an intramuscular injection of 10 to 18 mg/kg body weight of Ketalar (ketamine 50 mg/mL, Parke-Davis), followed after 5 minutes by an intramuscular injection of 30 to 60 mg/kg body weight of Pentothal (pentothal sodium 50 mg/mL, Abbott). There were no muscular reactions caused by the anesthesia. Breathing, vital signs, and neurologic reflexes were continuously monitored to ensure adequate depth of anesthesia and avoid pain reactions. This project was approved by the Ethical Committee at Göteborg University. After experimentation, the animals were killed by an overdose of anesthesia and potassium chloride.

Experimental Protocol

Silver electrodes (1-mm thick) were implanted into the anterior and posterior parts of the right masseter muscles according to the technique for primates described earlier.[8] The bite force was measured using a force transducer (PM 8110 mini-recorder, Philips)[22] placed in a standardized manner between the first molars of the maxilla and mandible.[8] A continuous contraction was induced with a Grass electrical stimulator for a duration of 2 ms at a frequency of 4 Hz with a gradually increasing voltage up to 100 V. The stimulation was interrupted after 3 minutes. After 5 minutes of rest, stimulation was repeated for 3 minutes with the same duration and voltage but at a higher frequency of 8 Hz. After the stimulations, biopsies (5 $\times$ 10 $\times$ 8 mm) were obtained directly from the central part of the masseter, immediately frozen in liquid nitrogen, and stored in a deep freezer at –90°C. No bleeding or macroscopic damage caused by the electrodes could be detected in the biopsies. To establish precontraction concentrations of intramuscular substrates, biopsies were obtained from the central part of the left masseter muscle.

Analytic Methods

After freeze drying at –20°C for 24 hours, an extraction procedure was performed.[23] Glycogen, glucose, lactate, pyruvate, creatine-phosphate, nicotinamide-adenine dinucleotide (NAD), and reduced NAD (NADH) were analyzed fluorometrically by enzymatic methods.[7,8]

Statistical Methods

Standard statistical procedures were employed to calculate means and standard deviation (SD) in the

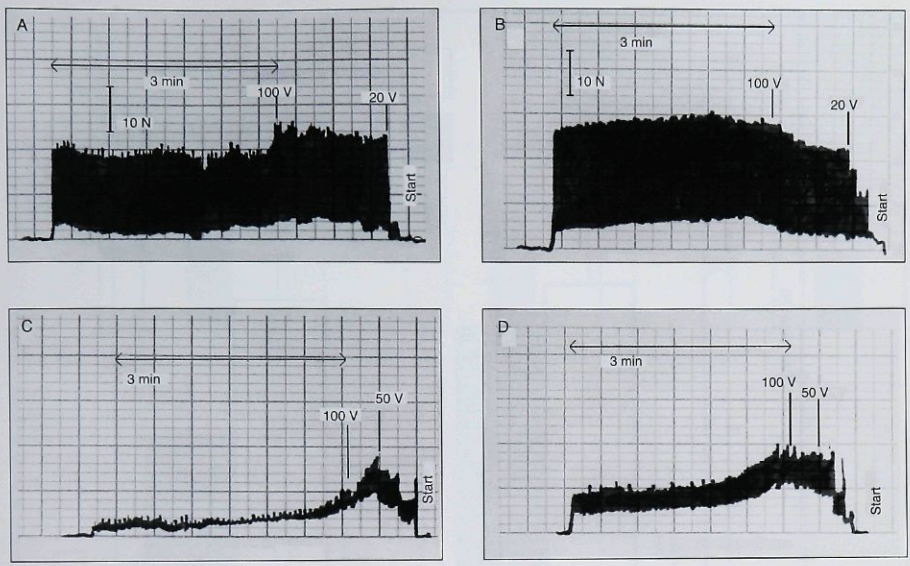


Fig 1 Original bite force curves during two stimulations (A and B = first period, 4 Hz, 2 ms; C and D = second period, 8 Hz, 2 ms) in two pigtail monkey masseters (A and C = male, B and D = female) with gradual increase of stimulation voltage up to 100 V. *Arrows* indicate maximal stimulation power (100 V) and starting position for 3 minutes of work (5 minutes recovery between stimulation events).

different metabolite groups. The precision of the estimation of bite force measurements was expressed as the standard error (SE) of the mean. The lactate/pyruvate (L/P) ratio was calculated. The hypothesis of no difference in bite force, substrate, and metabolic concentrations between the experimentally fatigued muscles and controls was evaluated by means of the nonparametric Wilcoxon's signed rank test. The null hypothesis was rejected at a significance level of $P = 0.05$.

Results

The bite force development for the nine females and one male monkey is shown in Figs 1 and 2. From an initial maximum peak value of 17.9 ± 2.9 N at the start of the first stimulation event, the bite force decreased by almost 12% to a value of 15.7 ± 2.2 N after 3 minutes of muscle contraction at the end of the first stimulation (not significant). After a recovery period of 5 minutes, the maximum force reached 17.2 ± 2.2 N at the start of the second stimulation. After an additional 3 minutes of muscle contraction, the bite force decreased significantly to 10.1 ± 1.1 N ($P < 0.05$), or 56% of the initial force. The bite force pattern was similar between the nine females and the male during the experiment.

Intramuscular Substrate Concentrations

After performed work the intramuscular glycogen content decreased significantly from 77.6 ± 9.0 to 41.3 ± 6.8 μmol/g muscle, a depletion of 47% ($P < 0.05$), and the glucose dropped significantly from 5.3 ± 0.9 to 3.4 ± 0.6 μmol/g masseter muscle. Thus, after the stimulation 64% ($P < 0.05$) of the initial glucose store remained. The creatine phosphate concentration decreased from 19.0 ± 2.7 to 11.6 ± 3.2 μmol/g after contraction, a decrease of 39% ($P < 0.05$). Muscle contraction elevated the NADH concentration from 0.04 ± 0.01 to 0.06 ± 0.02 μmol/g, and the NAD concentration increased from 0.18 ± 0.03 to 0.22 ± 0.03 μmol/g after contraction. Thus, the NADH content was

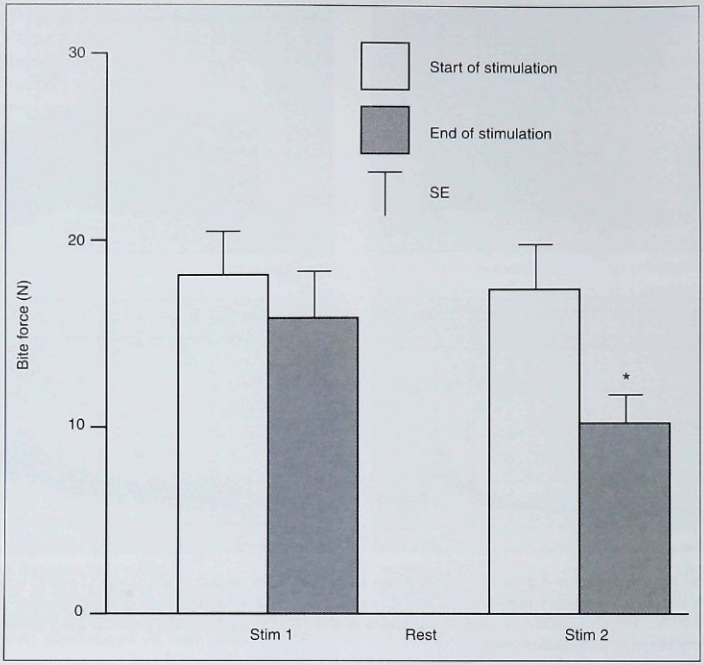
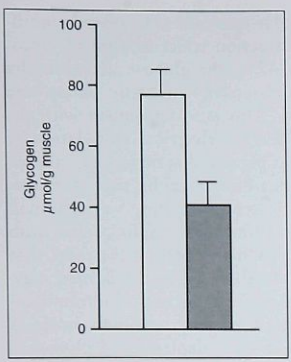


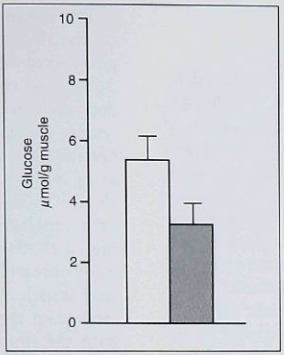
Fig 2 Bite force development during two stimulations in 10 pigtail monkey masseters with 5 minute recovery period between contractions. Stim 1 = 100 V, 2 ms, 4 Hz, 3 min; Stim 2 = 100 V, 2 ms, 8 Hz, 3 min. Values are given as mean and standard error of the mean (SE). $^*P < 0.05$.

elevated by 50% ($P < 0.05$), and the NAD concentration concomitantly increased by 22% after the fatiguing tasks (not significant) (Fig 3). No differences in substrate use between the male and the female monkeys could be identified.

Metabolite Accumulation

The pyruvate concentration increased from 0.37 ± 0.08 μmol/g masseter muscle tissue at the beginning of the stimulation to 0.49 ± 0.07 μmol/g after the second contraction, an elevation of 32% ($P < 0.05$). The precontraction lactate level increased from 7.7 ± 0.4 μmol/g at the first stimulation to 23.3 ± 3.9 μmol/g at the end of the second contraction. Thus, the intramuscular lactate content increased by a factor of 3 ($P < 0.05$).The poststimulus L/P ratio increased 2.4 times ($P < 0.05$) (Fig 4).The metabolite findings in the male masseter muscle were comparable to the nine female measures.





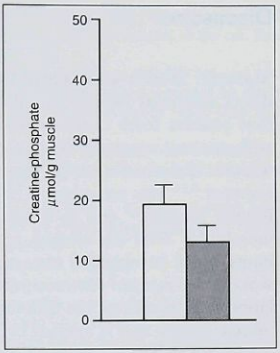
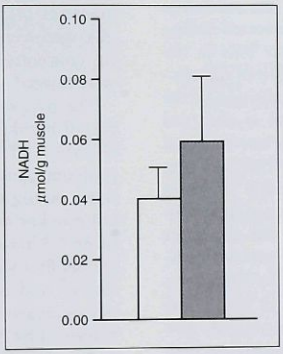
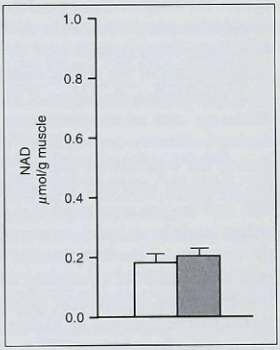
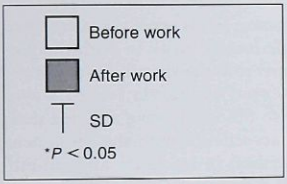


Fig 3 Contents of intramuscular substrates (μmol/g muscle) from central masseter biopsies from 10 pigtail monkeys. SD = 1 standard deviation. All values except NAD levels are significant at the $P < 0.05$ level.







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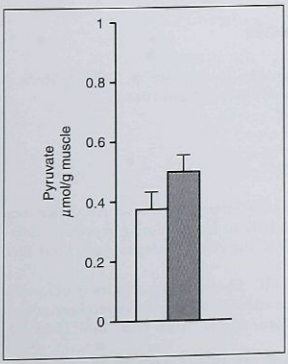

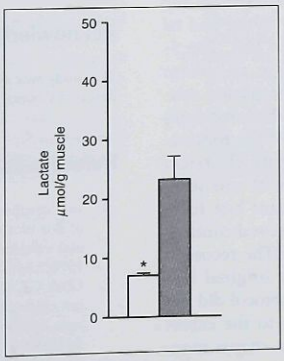

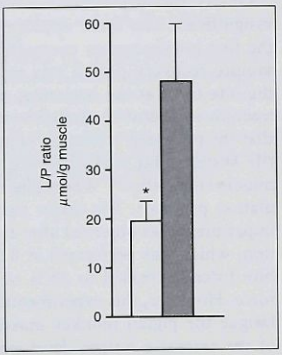

Fig 4 Waste product accumulation (μmol/g muscle) in central masseter biopsies from 10 pigtail monkeys. SD = 1 standard deviation; L/P ratio = lactate/pyruvate ratio. Lactate levels and L/P ratio are significant at the $P < 0.05$ level.

Discussion

Masseter muscle contractions were induced by direct electrical muscle stimulation, a technique that enables force characteristics to be obtained with minimal traumatic injury such as bleeding and without disturbance to the microcirculatory environment.[6,24] This procedure is frequently applied in human muscles[25] and in animal limb[26] and jaw muscles.[9,27] With this method, standardized muscle biopsies can be obtained after a controlled muscle work performance.[7] However, direct muscle stimulation fails to address the task-dependent nature of muscle fatigue that is exerted by the central motor command.[28] The rotation of motor unit activity within the motorneuron pool[29] and the excitation of motorneurons other than those activated during voluntary contractions may produce bias in the interpretation of the results obtained from the electrical stimulation procedure.[30] The reduction of the discharge rate of motorneurons that is observed during voluntary contractions[31,32] is not reflected during the contraction effort.[12] However, the pigtail monkeys tolerated the experimental situation well and we are convinced that the anesthetic procedure and the surgical intervention did not affect the outcome of the experiments. The present investigation focused on a fatiguing effort by applying a low-frequency stimulation protocol (4 and 8 Hz) instead of activation at higher frequencies. The major force loss during HFF is recognized at high discharge rates above 50 Hz. These unphysiologic high activation pulses are seldom identified during normal maximal voluntary contractions,[32] which suggests that HFF is not an important part of the fatigue mechanism.[15]

One of the major results of this study was the insignificant bite force impairment at the end of the first low-frequency contraction (4 Hz). The 5-minute recovery period was sufficient to restore the bite force at the beginning of the second contraction to almost the initial level, which indicates that the proposed activation failure of the contractile system that was observed during electrical muscle stimulation[33] was negligible with this stimulation protocol. The major significant bite force impairment was observed after the second contraction, which was performed at 8 Hz. The recorded bite force decreased to 58% of the original bite force. However, this experimental protocol did not fatigue the pigtail monkey masseter to the extent of the extensive fatigue developed in rhesus monkey masseters when two continuous contraction periods at 4 Hz were used.[8]

The intramuscular substrates decreased significantly; the precontraction concentration of glycogen decreased by 47%, the glucose decreased by 36%, and the high-energy creatine phosphate decreased by 39%. This is in agreement with the results presented for the electrical stimulation of jaw muscles such as pig and dog masseters,[7] as well as in NMR investigations of rabbit masseter muscles,[9,10] human masseter muscles,[11] and human limb muscles.[34] However, these results conflict with the small changes in high-energy substrates that were observed by Plesh et al[3] in the human masseter muscle. The smaller reduction of intramuscular fuel in the pigtail monkey masseter contrasts with the prominent 60% depletion of glycogen, glucose, and phosphocreatine in the rhesus monkey masseter muscle.[8] The precontraction values of glycogen were higher in the rhesus masseter, but the concentrations of glucose and phosphocreatine were lower.[8]

The pyruvate content increased by 32% in the pigtail monkey masseter muscle, compared to an elevation of 92% in the rhesus masseter.[8] The postcontraction lactate levels were elevated three times during the performed work in both the pigtail monkey masseter and the rhesus monkey masseter.[8] This increased accumulation of waste metabolites was also observed in pig and dog masseters,[7] and is in accordance with the prominent pH decrease identified in human[11] and rabbit[9] masseters by NMR techniques.

This low-frequency stimulation protocol produced a depletion of intramuscular substrates and an accumulation of toxic waste metabolites. These physiologic parameters may help to explain the decrease in bite force observed during LFF.

Acknowledgments

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Resumen

Desarrollo de una Fatiga Dependiente de la Frecuencia Durante el Estímulo Eléctrico en el Músculo Masetero de Monos Cola de Puerco

Se investigó la fatiga de baja frecuencia en diez monos (nueve hembras y un macho) cola de puerco adultos (Macaca nemestrina), con un peso medio de 5,3 kg (entre 4,3 y 6,5 kg). Luego de sedar y anestesiar a los animales, se les insertaron unos electrodos de plata en las partes anteriores y posteriores del músculo masetero derecho. El músculo contralateral fue usado como control. Se estimularon los músculos maseteros por 3 minutos (4 Hz, 2 ms, 100 V). Después de un período de descanso de 5 minutos, se repitió la estimulación con la misma duración y voltaje, pero a una frecuencia mayor de 8 Hz. Se midió la fuerza de mordida. Se tomaron biopsies musculares de la parte central del masetero derecho y estas se congelaron inmediatamente en nitrógeno líquido. Luego de la deshidratación por congelación se efectuó un análisis fluorométrico que utilizó métodos enzimáticos para medir niveles de glicógeno, glucosa, lactato, piruvato, fosfato de creatina, dinucleótido de nicotinamida y adenina (DNA), y DNA reducida (DNAR). La fuerza de mordida disminuyó un 12% después de los 3 minutos iniciales de trabajo. Luego de la segunda contracción la fuerza de mordida disminuyó a 56%. Se observó una reducción del substrato prominente. Todos los niveles de precontracción del glicógeno, glucosa, y la fosfocreatina fueron reducidos. Las concentraciones de DNA y DNAR aumentaron. La acumulación de metabolitos fue algo evidente. El piruvato aumentó un 32% y los niveles de lactato aumentaron hacia un factor de 3. Las medidas del macho fueron comparables a las de las nueve hembras en cada evaluación. La reducción del substrato substancial en combinación con una acumulación prominente de metabolitos puede contribuir al desarrollo de una fatiga de baja frecuencia.

Zusammenfassung

Frequenz-Abhängige Ermüdungsentwicklung während Elektrischer Stimulation im Musculus Masseter bei Makaken

Niedrigfrequente Ermüdung wurde bei neun weiblichen und einem männlichen erwachsenen Makaken (Macaca nemestrina) mit einem durchschnittlichen Gewicht von 5,3 kg (Bereich 4,3 bis 6,5 kg) untersucht. Nach Sedation und Anaesthesie wurden Silberelektroden in den anterioren und den posterioren Teil des rechten Musculus masseter eingesetzt. Der kontralaterale Muskel wurde als Kontrolle verwendet. Die Masseteren wurden für 3 Minuten stimuliert (4 Hz, 2 ms, 100 V). Nach einer Ruheperiode von 5 Minuten wurde die Stimulation mit der gleichen Dauer und Spannung, aber einer höheren Frequenz von 8 Hz wiederholt. Die Beisskraft wurde gemessen. Es wuren Muskelbiopsien aus dem zentralen Teil des rechten Marsseters entnommen und unmittelbar in flüssigem Stickstoff eingefroren. Nach Gefriertrocknung wurde eine fluorometrische Analyse durchgeführt, welche enzymatische Methoden verwendete, um die Niveaus von Glycogen, Glukose, Laktat, Pyruvat, Kreatinphosphat, Nikotinamid Dinukleotid (NAD), und reduziertem NAD (NADH) zu messen. Die Beisskraft verminderte sich um 12% nach den anfänglichen 3 Minuten der Arbeit. Nach der zweiten Kontraktion sank die Beisskraft um 56%. Eine hervorstechende Substratentleerung wurde beobachtet. Die Vorkontraktionsniveaus von Glycogen, Glukose, und Phosphokreatin waren alle reduziert. Die NADH- und die NAD-Konzentrationen stiegen an. Eine Akkumulation von Metaboliten war offensichtlich. Das Pyruvat erhöhte sich um 32% und die Laktatniveaus stiegen um einen Faktor 3. Die männlichen Messungen waren vergleichbar mit den neun weiblichen Messungen für jede Beurteilung. Die starke Substratentleerung in Kombination mit einer hervorstechendenen Akkumulation von Metaboliten könnte zur Entwicklung einer niedrigfrequenten Ermüdung beitragen.