Coll. Antropol. **36** (2012) 2: 651–655
Original scientific paper

Alterations in the Masseter Muscle and Plasma IL-6 Level Following Experimentally Induced Occlusal Interference and Chronic Stress – A Study in Rats

Sunčana Simonić-Kocijan, Ivone Uhač, Petra Tariba, Vesna Fugošić, Daniela Kovačević Pavičić, Vlatka Lajnert and Vedrana Braut

University of Rijeka, School of Dental Medicine, Department of Prosthodontics, Rijeka, Croatia

A B S T R A C T

This study was undertaken to examine the alteration of masseter and plasma interleukin-6 after inducing occlusal interference and chronic stress. Male Wistar rats were submitted to chronic stress procedure, exposed to occlusal interference, or exposed to both mentioned procedures. Whole blood and masseter tissue were collected to determine interleukin-6 level, measured by means of ELISA. Masseter pain was evaluated using the orofacial formalin test. Masseter interleukin-6 level was significantly higher in animals submitted to combination of occlusal interference and chronic stress than in the control group ($p<0.05$). There was positive and significant correlation between pain response and masseter interleukin-6 level ($r=0.5741$; $p<0.0003$). No significant differences in plasma interleukin-6 level were found between groups ($p>0.05$), as well as no correlation with pain ($p>0.05$). Combination of occlusal interference and chronic stress leads to strong local reaction characterized by high levels of masseter interleukine-6. High concentrations of muscle interleukin-6 and its correlation with pain point to inflammatory background of masticatory muscle pain.

Key words: *dental occlusion, interleukin-6, masseter muscle, orofacial pain, stress, psychological*

Introduction

Pain in the masticatory muscles has long been recognized as a prominent symptom of temporomandibular disorder (TMD)[1], but still the etiopathogenesis of this disorder is not well understood. There is no consensus about the involvement of occlusal alteration and psychological stressors in development of masticatory muscle pain, although many studies point to their contributing role[2–5]. Our previous study on experimental animals shows that conditions where occlusal interference and chronic stress act together are capable to induce masseter muscle pain[6]. Occlusal interference is considered to be a factor that contributes to the masticatory muscle hyperactivity causing muscle spasm and microtraumatic changes in temporomandibular joints (TMJ) characterized by high levels of proinflammatory cytokines, which implicates that muscle pain has its origin in tissue inflammation[7–10]. Interleukin-6 (IL-6) is a pleiotropic cytokine that influences immune responses and inflammatory reactions. Together with IL-1 and TNF-α, it belongs to the group of main proinflammatory cytokines[11]. IL-6 plays a relevant role in the development and maintenance of muscular hyperalgesia[12]. Patients with TMD express higher levels of chronic daily stressors, depression and other psychological disturbances which are associated with high levels of proinflammatory cytokines[13–15]. Some studies have found an association between the symptoms of TMD and the increased levels of proinflammatory cytokines in synovial fluid of temporomandibular joints suggesting their role in the development of the disorder[16,17]. While the change of cytokine level in the synovial fluid of TMJ has been described within the response to TMD, there is a lack of studies dealing with changes in the masticatory muscles despite the fact that 10 % of TMD patients have masticatory muscle symptoms[1]. The objective of this

Received for publication January 23, 2012

study was to investigate whether proposed conditions, occlusal interference and chronic stress, cause alteration in the masseter muscle and plasma IL-6 level and if there is correlation between IL-6 and pain response.

Materials and Methods

Animals. Adult male Wistar rats (weight 250–300 g) were used in this study. Animals were maintained on a 12 h light–dark cycle and allowed free access to food and water. All experiments were performed between 10 a.m. and 2 p.m. in a silent room, at a temperature of 22°C–24°C. All experimental procedures involving animals were performed in accordance with the regulations set by the Croatian related laws and rules (NN 19/99; NN 176/04) and with the guidelines set by the European Community Council Directive of 24 November 1986 (86/609/EEC). All experimental procedures were also approved by the Faculty ethical committee. The rats were randomly divided in chronic stress group (n=10), occlusal interference group (n=10), group with both occlusal interference and chronic stress (n=10) and control group (n=10) free of experimental procedures. Animals participated in the experiment for 56 days. Pain response was tested using the formalin test, and plasma and masseter muscle samples collected for further analysis. In order to exclude the possibility that changes in the IL-6 level are a consequence of formalin injection in the masseter muscle (used for pain valorization) we introduced another, absolute control group of rats (n=10), free of any experimental procedure and not subject to the formalin test.

Chronic Stress procedure

The rats were stressed by restrain 1 h/day, 5 days/week for 8 weeks as previously reported[18]. Briefly, restraint was carried out by placing the animal in a 7x30 cm plastic tube. Tube was designed with inner mobile wall, so it could be adjustable in size, depending on each animal, and could disable them to move. At the far end of the tube there was a 1 cm hole for breathing.

Occlusal interference procedure

Rats remained unstressed in their cages until the 48th day, when occlusal splint was fitted. They were sedated with chloral hydrate (300 mg/kg) and occlusal splint was built-up on the maxillary molar teeth[6,19,20]. Briefly, before placing the splint, the teeth were etched with 37% phosphoric acid, washed with water and air-dried. Composite resin (Gradia direct, GC Dental products corp., Aichi, Japan) was built-up (height of 1.0 mm) unilaterary on upper right molar teeth. To prevent the reduction of material during function, antagonist right lower molar teeth were coated with fluid resin. Occlusal interference was left in place for 8 days.

Combination of occlusal interference and chronic stress procedure

To evaluate the mutual effect of occlusal interference and chronic stress on IL-6 levels, animals were exposed to both experimental procedures. First the animals were exposed to the chronic stress procedure, the same way as described above, and than, on the 48th day, occlusal interference on the right upper molar teeth was fitted. Throughout the next eight days, until the completion of the experiment, animals were exposed to both stress procedure and occlusal interference[6].

Testing procedure for the masseter muscle pain

On the 56th experimental day pain response was evaluted using the orofacial formalin test as previously reported [6,21,22]. Briefly, a volume of 0.05 ml, 5% formalin in saline was injected into the mid-region of the right masseter muscle via 27 gauge needle. The behavioral responses, characterized by rubbing orofacial region (amount of time) and flinching the head (number of head flinches), were quantified within a 45 min period. Pain response was evaluated as the sum of time spent on rubbing and flinching, expressed in seconds.

IL-6 determination

Immediately after the behavioral response was analyzed, all animals were decapitated and the masseter tissues from occlusal interference side as well as blood samples were taken. Right masseter muscle was removed, frozen in liquid nitrogen, and stored at –80°C. For cytokine assay, masseter samples were weighed and homogenized in phosphate-buffer saline (PBS, pH 7.4) containing 0.4 mol/L NaCl, 0.05% Tween-20, 0.5 bovine serum albumin, 0.1 mmol/L benzethonium chloride, 10 mmol/L EDTA, and 20 KI/L aprotinin. The homogenates were centrifuged at 2 000xg for 60 min at 4°C. The supernatant was removed and analyzed as was the plasma. Blood samples were directly centrifuged at 20.200 × g for 10 min, and the supernatant was isolated. IL-6 levels were determined using an enzyme-linked immunosorbent assay (Interleukin-6 (rat) ELISA, IBL-Hamburg, GmbH, Germany) for the quantitative detection of rat IL-6 according to manufacturer's instructions. The reproducibility of this examination was confirmed by processing all the samples twice.

Statistical analysis

GraphPad Prism 4.0 software (Graph-Pad Software, San Diego, CA) was used for the statistical analysis. Since the data were not normally distributed (Kolmogorov-Smirnov test), the differences in plasma and tissue IL-6 levels among all groups were tested using Kruskal-Wallis test followed by Dunn's method for post-hoc comparisons. Spearman's rank correlation coefficients were calculated when investigating the relationship between IL-6 level and nociceptive response. A probability level of less then 0.05 was considered to indicate statistical significance.

Results

The results of pain response have been published in the first report from our experiment[6]. In brief, significant alteration in pain threshold was found only in animals exposed to both occlusal interference and chronic stress, when compared to the control group.

Kruskal-Wallis test revealed significant differences in the masseter IL-6 level between the groups ($p=0.0047$) (Figure 1). There were no differences in the muscle IL-6 levels after exposure to occlusal interference ($p>0.05$). Chronic stress also had no effect on IL-6 level ($p>0.05$) in comparison with the control group. The increase of masseter IL-6 was statistically significant ($p<0.01$) between the control group and the group with combination of both occlusal interference and chronic stress. There were no differences in masseter IL-6 between the control group and the absolute control group ($p>0.05$). Kruskal-Wallis test showed no significant difference in plasma IL-6 levels between groups ($p=0.1115$, Figure 2). Experimentally induced occlusal interference, chronic stress or their combination did not affect plasma IL-6 levels. Correlation analysis demonstrated that masseter IL-6 level was significantly and positively correlated to the nociceptive behavioral response ($r=0.5741$; $p<0.0003$, Figure 3). There was negative, but not significant, correlation between plasma IL-6 level and the pain response ($r=-0.2780$; $p=0.0996$, Figure 4).

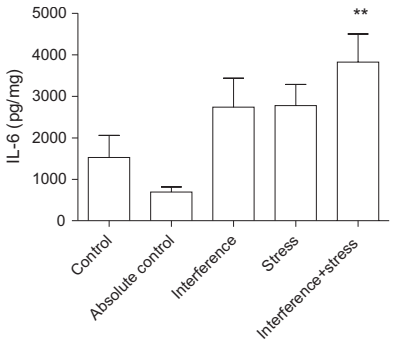

*Fig. 1. Changes in masseter muscle IL-6 level after inducing occlusal interference, chronic stress and combination of occlusal interference and chronic stress. The level of IL-6 increased significantly following combination of occlusal interference and chronic stress compared with the control group, while there were no differences between occlusal interference or chronic stress group compared with the control group. Note that there was no significant change between absolute control and control group. Data are presented as mean ±S.E.M. Asterisk shows a significant difference from the control group, **p <0.01 (Kruskal-Wallis and Dunn's post-hoc test).*

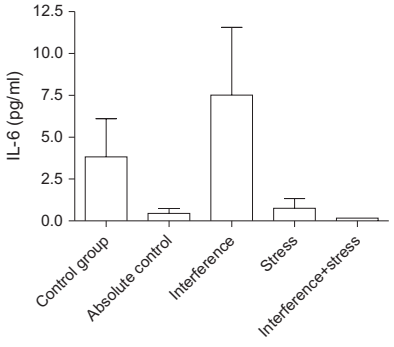

Fig. 2. Plasma IL-6 levels after inducing occlusal interference, chronic stress and combination of occlusal interference and chronic stress. There were no statistically significant differences in plasma IL-6 levels between groups. Experimental procedures do not influence plasma IL-6 level. Data are presented as mean± S.E.M. (p>0.05, Kruskal-Wallis).

Discussion and Conclusion

In this study, by inducing occlusal changes and a chronic daily stressor, local and systemic changes in IL-6 under conditions which are common in patients with masticatory muscle pain were investigated[23,24]. In order to exclude the possibility that changes in IL-6 levels are consequence of formalin injection used for valorization of pain response, another group of rats was introduced. This absolute control group of animals was free of any experimental procedure and not subject to the formalin test. Since formalin solution injected into masseter muscle did not induce changes in the tissue IL-6 level, differences have to be a consequence of experimentally indu-

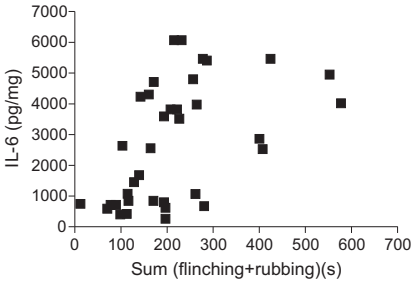

Fig. 3. Correlation between masseter IL-6 and nociceptive behavioral response. There is significant and positive association ($r=0.5741$; $p<0.0003$) between the masseter muscle IL-6 level and nociceptive behavioral response examined by Spearman's rank correlation.

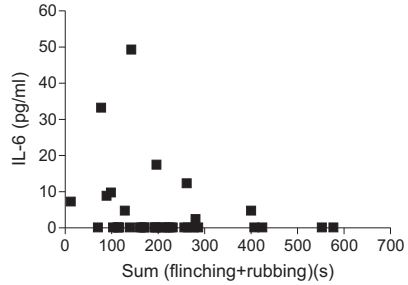

Fig. 4. Correlation between plasma IL-6 and nociceptive behavioral response. There is negative, but not significant association ($r=-0.2780$; $p=0.0996$) between the plasma IL-6 level and nociceptive behavioral response examined by Spearman's rank correlation.

ced chronic stress and occlusal interference. This result is in agreement with previous reports indicating that local injection of inflammatory substances does not induce cytokine alterations in a period shorter than one hour, with a peak at 6 hours after injection[10].

Pain is one of the common sensations which usually indicates tissue damage as a consequence of various external or internal factors[25]. From the results of our previous report it seems that masticatory muscles are capable to adapt to minor occlusal changes and chronic stress without developing the changes in pain response, but when this two proposed etiological factors work together they cause a higher pain response[6]. In the present study, similarly to pain response results, there was a lack of significant changes in both tissue and plasma IL-6 levels between the chronically stressed animals and rats with occlusal interference in comparison to the control group, while masseter IL-6 significantly increased in animals submitted to combination of experimental procedures. These results suggest that only the combination of occlusal interference and chronic stress leads to a strong local inflammatory reaction characterized by the alteration in masseter IL-6 level. Results of other studies showed increased levels of IL-6, prostaglandins, serotonin, and growth factors in muscle tissue after the contraction and muscle damage[26,27]. Also, cytokines (IL-6, IL-1, IL-8, TNF-α) and prostaglandins, when locally injected, influence nociception causing hyperalgesia[28,29]. Contrary to positive association between pain response and masseter muscle IL-6 level found in this study, in most plasma samples the IL-6 level was below the detectable limit of the assay and there were no differences in plasma IL-6 levels between groups as well as no correlation with pain. Although not significant, there was a negative correlation coefficient between the pain and blood IL-6 level. Low level of blood IL-6 found in this study, especially in the group with combination of occlusal interference and chronic stress and negative correlation coefficient regarding to pain response point to the difference between local and systemic cytokine response. It has been sugested that blood IL-6 level does not depend on its local level and in contrarary to local proinflammatory reaction, the sistemic cytokine response might be directed towards suppression of inflammatory reaction[10,30]. The differences between the local and circulating IL-6 response may be partly due to the fact that circulating cytokines have a relatively short half-life in the plasma[30]. Although cytokines can affect pain response through changes in the central nervous system[31–33], elevated levels of IL-6 in the masseter muscle and its positive association with pain as well as no changes in plasma IL-6 found in this study lead to conclusion that combination of proposed etiological factors, chronic stress and occlusal interference, influence pain response trough localized mechanisms and that IL-6 is one of the cytokines responsible for developing masticatory muscle pain. This is in agreement with previous studies which suggest that pain is mainly caused by prolonged excitation of peripheral nociceptors by IL-6[34,35].

In conclusion, this study strengthens the knowledge on inflammatory background of disorder and highlights the role of local IL-6 in masticatory muscle pain. Orofacial pain is extremely complex and further studies are needed to examine the role of local and circulating levels of other potential mediators of muscle pain and/or inflammation, as they are beyond the scope of this study.

Acknowledgements

This study was supported by grant no. 062-0650446-0403 from the Croatian Ministry of Science, Education and Sports.

REFERENCES

1. SVENSSON P, GRAVEN-NIELSEN T, J Orofac Pain, 15 (2001) 117. — 2. CLARK GT, GREEN EM, DORNAN MR, Flack VF, J Am Dent Assoc, 115 (1987) 251. — 3. CAO Y, XIE QF, Li K, LIGHT AR, FU KY, Pain, 144 (2009) 287. DOI:10.1016/j.pain.2009.04.029 — 4. KIRVESKARI P, ALANEN P, JAMSA T, J Prosthet Dent, 67 (1992) 692. DOI:10.1016/0022-3913(92)90173-8 — 5. PALLEGAMA RW, RANASINGHE AW, WEERASINGHE VS, SITHEEQUE MA, J Oral Rehabil, 32 (2005) 701. DOI:10.1111/j.1365-2842.2005.01503.x — 6. SIMONIC-KOCIJAN S, UHAC I, BRAUT V, KOVAC Z, PAVICIC DK, FUGOSIC V, UREK MM, Coll Antropol, 33 (2009) 863. — 7. CUNHA FQ, POOLE S, LORENZETTI BB, FERREIRA SH, Br J Pharmacol, 107 (1992) 660. — 8. KANEYAMA K, SEGAMI N, NISHIMURA M, SUZUKI T, SATO J, Br J Oral Maxillofac Surg, 40 (2002) 418. DOI:10.1016/S0266-4356(02)00215-2 — 9. BASBAUM AI, WOOLF CJ, Curr Biol, 9 (1999) 429. DOI:10.1016/S0960-9822(99)80273-5 — 10. WATANABE M, GUO W, ZOU S, SUGIYO S, DUBNER R, REN K, Neurosci Lett, 382 (2005) 128. DOI:10.1016/j.neulet.2005.03.002 — 11. YAMANO S, ATKINSON JC, BAUM BJ, FOX PC, Clin Immunol, 92 (1999) 265. — 12. MANJAVACHI MN, MOTTA EM, MAROTTA DM, LEITE DF, CALIXTO JB, Pain, 151 (2010) 345. DOI:10.1016/j.pain.2010.07.018 — 13. ZHOU D, KUSNECOV AW, SHURIN MR, DEPAOLI M, RABIN BS, Endocrinology, 133 (1993) 2523. — 14. MUSSELMAN DL, MILLER AH, PORTER MR, MANATUNGA A, GAO F, PENNA S, PEARCE BD, LANDRY J, GLOVER S, MCDANIEL JS, NEMEROFF CB, Am J Psychiatry, 158 (2001) 1252. — 15. COSTELLO NL, BRAGDON EE, LIGHT KC, SIGURDSSON A, BUNTING S, GREWEN K, MAIXNER W, Pain, 100 (2002) 99. DOI:10.1016/S0304-3959(02)00263-4 — 16. TAKAHASHI T, KONDOH T, FUKUDA M, YAMAZAKI Y, TOYOSAKI T, SUZUKI R, Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 85 (1998) 135. DOI:10.1016/S1079-2104(98)90415-2 — 17. KANEYAMA K, SEGAMI N, SUN W, SATO J, FUJIMURA K, Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 99 (2005) 276. DOI:10.1016/j.tripleo.2004.03.020 — 18. GAMEIRO GH, GAMEIRO PH, ANDRADE AS, PEREIRA LF, ARTHURI MT, MARCONDES FK, VEIGA MC, Physiol Behav, 87 (2006) 643. DOI:10.1016/j.physbeh.2005.12.007 — 19. NISHIDE N, BABA S, HORI N, NISHIKAWA H, J Oral Rehabil, 28 (2001) 294. DOI:10.1046/j.1365-2842.2001.00568.x — 20. YABUSHITA T, ZEREDO JL, FUJITA K, TODA K, SOMA K, J Dent Res, 85 (2006) 849. DOI:10.1177/154405910608500914 — 21. OKAMOTO K, IMBE H, TASHIRO A, KUMABE S, SENBA E, Neurosci Lett, 367 (2004) 259. DOI:10.1016/j.neulet.2004.06.017 — 22. ROVERONI RC, PARADA CA, CECILIA M, VEIGA FA, TAMBELI CH, Pain, 94 (2001) 185. DOI:10.1016/S0304-3959(01)00357-8 — 23. GLAROS AG, WILLIAMS K, LAUSTEN L, J Am Dent Assoc, 136 (2005) 451. — 24. PEDRONI CR, DE OLIVIERA AS, GUARATINI MI, J Oral Rehabil, 30 (2003) 283. DOI:10.1046/j.1365-2842.2003.01010.x — 25. WODA A, PIONCHON P, Rev Neurol, 157 (2001) 265. — 26. ONO T, MAEKAWA K, WATANABE S, OKA H, KUBOKI T, Arch Oral Biol 52 (2007) 479. DOI:10.1016/j.archoralbio.2006.10.025 — 27. BRUUNSGAARD H, GALBO H, HALKJAER-KRISTENSEN J, JOHANSEN TL, MACLEAN DA, PEDERSEN BK, J Physiol, 499 (1997) 833. — 28. DINA OA, GREEN PG, LEVINE JD, Neuroscience, 152 (2008) 521. — 29. CHICHORRO JG, LORENZETTI BB,

ZAMPRONIO AR, Br J Pharmacol, 141 (2004) 1175. DOI:10.1038/sj.bjp.0705724 — 30. LORAM LC, FULLER A, CARTMELL T, MITCHELL B, MITCHELL D, Physiol Behav, 92 (2007) 873. DOI:10.1016/j.physbeh.2007.06.015 — 31. MATSUMOKO K, KOIKE K, MIYAKE A, WATANABE K, KONISHI K, KIYAMA H, Neurosci Res, 27 (1997) 181. — 32. WATKINS LR, MAIER SF, GOEHLER LE, Pain, 63 (1995) 289. — 33. YAMAMOTO J, NISHIYORI A, TAKAMI S, OHTANI Y, MINAMI M, SATOH M, Eur J Pharmacol, 363 (1998) 131. DOI:10.1016/S0014-2999(98)00801-2 — 34. JULIUS D, BASBAUM AI, Nature, 413 (2001) 203. DOI:10.1038/35093019 — 35. VERRI WA, CUNHA TM, PARADA CA, POOLE S, CUNHA FQ, FERREIRA SH, Pharmacol Ther, 112 (2006) 116.

S. Simonić-Kocijan

University of Rijeka, School of Dental Medicine, Krešimirova 40, 51 000 Rijeka, Croatia
e-mail: suncana.simonic-kocijan@medri.hr

PROMJENE RAZINE IL-6 U MASETERIČNOM MIŠIĆU I PLAZMI NAKON EKSPERIMENTALNO IZAZVANE OKLUZIJSKE INTERFERENCIJE I KRONIČNOG STRESA

S A Ž E T A K

Cilj istraživanja bio je ispitati promjene u razini interleukina-6 u maseteričnom mišiću i plazmi nakon izazivanja okluzijske interferencije i kroničnog stresa. Muški Wistar štakori izloženi su kroničnom stresu, okluzijskoj interferenciji, ili objema spomenutim procedurama. Skupljeni su uzorci krvi i tkiva maseteričnog mišića te su iz njih utvrđene vrijednost interleukina-6, ELISA metodom. Bol maseteričnog mišića vrednovana je orofacijalnim formalinskim testom. Razina interleukina-6 maseteričnog mišića bila je statistički značajno povišena u životinja podvrgnutih zajedničkom djelovanju okluzijske interferencije i kroničnog stresa, u usporedbi s kontrolnom skupinom ($p<0,05$). Utvrđena je pozitivna i značajna korelacija između boli i razine interleukina-6 maseteričnog mišića ($r=0,5741$; $p<0,0003$). Razlika razine interleukina-6 u plazmi nije pokazala statističku značajnost između skupina ($p>0,05$), niti je utvrđena značajna korelacija s boli ($p>0,05$). Zajedničko djelovanje okluzijske interferencije i kroničnog stresa vodi snažnoj lokalnoj reakciji okarakteriziranoj visokom razinom interleukina-6 u maseteričnom mišiću. Visoka razina mišićnog interleukina-6 i korelacija s boli ukazuju da bol žvačne muskulature ima podlogu u upali.