Available online at www.sciencedirect.com

SciVerse ScienceDirect

British Journal of Oral and Maxillofacial Surgery 51 (2013) e250–e255

BRITISH Journal of Oral and Maxillofacial Surgery

www.bjoms.com

Influence of sleep deprivation on expression of MKK4 and c-fos in the mandibular condylar cartilage of rats

Jinlong Chen [a,b,1], Gaoyi Wu [c,1], Guoxiong Zhu [c], Peihuan Wang [c], Hongyu Chen [a,b], Huaqiang Zhao [a,*]

[a] *College of Stomatology, Shandong University, Number 44, Wen Hua Xi Lu, Jinan City, Shandong Province 250012, China*
[b] *Shandong Provincial Key Laboratory of Oral Biomedicine, Number 44, Wen Hua Xi Lu, Jinan City, Shandong Province 250012, China*
[c] *Department of Stomatology, Jinan Military General Hospital, Number 25, Shi Fan Lu, Jinan City, Shandong Province 250031, China*

Accepted 20 June 2013
Available online 13 July 2013

Abstract

The aim of this study was to investigate the changes in expression of mitogen-activated protein kinase kinase 4 (MKK4) and c-fos in the mandibular condylar cartilage of rats that had been subjected to sleep deprivation. One hundred and twenty female Wistar rats were randomly divided into 6 groups with 20 in each: sleep deprivation for 2 days, 4 days, 6 days, and 8 days, large-platform controls, and cage controls. After sleep deprivation by the modified multiple platform method the sleep-deprived rats were killed. The large-platform and cage control rats were killed at the same time as the rats deprived of sleep for 8 days. Haematoxylin and eosin were used to record the morphological changes in cartilage, and immunohistochemistry and real-time quantitative polymerase chain reaction (PCR) were used to detect the expression of MKK4 and c-fos. Pathological alterations were apparent after 6 and 8 days of sleep deprivation. Compared with control groups, the expression of MKK4 in the sleep-deprived groups was lower, while that of c-fos was higher. As the duration of sleep deprivation increased, the expression of MKK4 decreased. These results indicate that the variation in expression of MKK4 and c-fos may be correlated with pathological changes induced by sleep deprivation in mandibular condylar cartilage in rats.

Keywords: Sleep deprivation; Temporomandibular disorder; c-fos; MKK4

Introduction

Temporomandibular disorders (TMDs) are a group of illnesses that include pain in masticatory muscles and temporomandibular joints (TMJs), abnormality of mandibular movements, and joint sounds.[1] The pathogenic mechanisms of TMD, albeit controversial, are commonly thought to be correlated with occlusive and psychological factors.[2,3] Studies conducted by Selaimen et al.,[4] Smith et al.,[5] and Yatani et al.,[6] have all shown that sleep disorders play an important part in the development of TMD. Sleep deprivation is becoming more prevalent in modern life, but the mechanisms by which it causes pathological alterations to the TMJ are still not clear.

Mitogen-activated protein kinase kinase 4 (MKK4) is a component of the mitogen–antigen protein kinase signalling pathway, which phosphorylates and activates c-Jun N-terminal kinase (JNK) in response to various stimuli.[7,8] Activation of JNK results in phosphorylation of c-jun, which, together with c-fos, may act as a protein complex at the binding site of activator-protein 1.[9] The proto-oncogene c-fos, despite its presence at low concentrations in most common cells, could get over-expressed because of this reaction.[10]

* Corresponding author at: Department of Oral and Maxillofacial Surgery, College of Stomatology, Shandong University, Number 44, Wen Hua Xi Lu, Jinan City, Shandong Province 250012, China. Tel.: +86 13506402199; fax: +86 531 82950154.
E-mail address: zhaohq@sdu.edu.cn (H. Zhao).
[1] Jinlong Chen and Gaoyi Wu contributed equally to this paper and should be regarded as co-first author.

0266-4356/$ – see front matter
http://dx.doi.org/10.1016/j.bjoms.2013.06.010

The c-fos protein is involved in cellular proliferation, differentiation, and apoptosis of osteoclasts.[11]

In the present study we have used the modified multiple platform method (MMPM) to induce sleep deprivation experimentally, and have investigated the correlation between morphological changes and the expression of MKK4 and c-fos in mandibular condylar cartilage. We also explored the pathological mechanisms by which sleep deprivation may cause TMD.

Materials and methods

Animals and model of sleep deprivation

All experimental procedures followed the University's Ethics Committee regulations and institutional guidelines. One hundred and twenty female Wistar rats (8 weeks' old, weight 220–240 g) were bought from the Animal Centre of Shandong University (Jinan, China), and the experiments were done in the animal experimental centre at Jinan Military General Hospital. All rats were acclimatised under laboratory conditions for 2 weeks before the experiment and had free access to food and tap water. The temperature was controlled at 21–23 °C, and they had a 12:12 h light–dark cycle. The rats were then randomly assigned to 6 groups with 20 in each: sleep deprivation for 2 days, 4 days, 6 days, or 8 days, large platform controls, and cage controls. The 2 latter groups were regarded as the control group.

Rats were deprived of sleep according to the MMPM as described in Suchecki and Tufik[12] with minimal modification. Each group of rats to be deprived of sleep was placed in a container with narrow platforms (6.5 cm in diameter), whereas large platform controls were placed in a container with large platforms (24 cm × 18 cm). Cage controls were maintained in cages in the same room as the other rats. Sleep-deprived rats were killed after 2, 4, 6, or 8 days according to their group under deep anaesthesia by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). Control rats were killed at the same time as the 8-day group.

Temporomandibular joints (TMJs) were dissected, and 10 right joints were randomly picked out from each group to be fixed in 10% buffered paraformaldehyde overnight at 4 °C. They were then decalcified for 48 h with formalin/nitric acid solution (40% formaldehyde 5 ml, concentrated nitric acid 10 ml, and distilled water 85 ml). After dehydration and embedding in paraffin, serial sections 5 μm thick were cut on the sagittal plane. One section from each joint was stained with haematoxylin and eosin for histological examination. Twenty joints from the other 10 rats in each group were frozen in liquid nitrogen after adherent connective tissue had been removed, and were stored for isolation of RNA. Two pieces of condylar cartilage from each rat were regarded as one sample to guarantee enough RNA.

Immunohistochemistry

After slides had been deparaffinised, rehydrated, and mixed with 3% hydrogen peroxide, we used the "pressure cooking" method to retrieve the antigens. The procedure was conducted using a SP-9001 HistostainTM Plus Kit (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China), according to the manufacturer's instructions. The primary antibodies were rabbit polyclonal anti-MKK4 and rabbit polyclonal anti-c-fos (Bioworld Technology, Inc., USA). Sections mixed with phosphate buffered saline (PBS) instead of primary antibodies were used as negative controls, which showed no immunoreaction. Antibodies were stained with ZLI-9031 diaminobenzidine tetrahydrochloride Kit (Beijing Zhongshan Golden Bridge Biotechnolgy Co., Ltd., Beijing, China). Sections were then lightly counterstained with haematoxylin, dehydrated, cleared, and mounted. Five randomly-chosen high power fields (magnification 400×) in each stained section were assessed by two independent clinical pathologists who were unaware of the sources of the sections.

RNA isolation, reverse transcription, and real-time quantitative polymerase chain reaction (RT-qPCR)

The joints were homogenised in a gentle MACSTM Dissociator (Miltenyl Biotech, Germany), RNA was then isolated with Trizol® Reagent (Invitrogen-Life Technologies, USA). Primer sequences were as follows: MKK4 (forward: 5′-AGAGACTGAGAACCCACAGCAT-3′ and reverse: 5′-CTACTCCGCATCACTACATCCA-3′; 247 bp, NM_001030023.1), c-fos (forward: 5′-GTCCGTCTCTAGTGCCAACTTTAT-3′ and reverse: 5′-GTCTTCACCACTCCCGCTCT-3′; 183 bp, AY780202) and GAPDH (forward: 5′-CAGTGCCAGCCTCGTCTCAT-3′ and reverse: 5′-AGGGGCCATCCACAGTCTTC-3′; 595 bp, BC059110). Reverse transcription and RT-qPCR were done with an UltraSYBR Two Step RT-qPCR Kit (With ROX) (CWbiotech, Beijing, China) according to the manufacturer's instruction. RT-qPCR was done in an Eppendorf realplex 4 (Eppendorf AG, Hamburg, Germany) with the following settings: 10 min of pre-incubation at 95 °C followed by 40 cycles for 20 s at 95 °C and 60 s at 55 °C. The 25 μl reaction volume contained 2 × UltraSYBR mixture (with ROX), forward and reverse primers (10 μm) and template cDNA. We did a melting curve analysis using the default programme. After each reaction, the cycle threshold (Ct) was recorded when the amplification curve reflected the exponential kinetic measurements. The $2^{-\Delta\Delta Ct}$ method[13] was adopted with GAPDH as the reference gene and the cage control group as the calibrator.

Statistical analysis

Statistical analyses were made with the help of Prism 5.01 (GraphPad Software, Inc., USA). Data are reported as mean