PHARMACOLOGY AND CELL METABOLISM

Role of the Endocannabinoid System in Ethanol-Induced Inhibition of Salivary Secretion

Juan Pablo Prestifilippo1,2,* Javier Fernández-Solari1,3 Vanina Medina4 Valeria Rettori3 and Juan Carlos Elverdin1

1Catedra de Fisiología, Facultad de Odontología, Universidad de Buenos Aires, Buenos Aires, Argentina, 2Catedra de Fisiopatología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina, 3Centro de Estudios Farmacológicos y Botánicos, Facultad de Medicina, Universidad de Buenos Aires (CEFyBO-CONICET-UBA), Buenos Aires, Argentina and 4Catedra de Física, Laboratorio de Metodología de Radiosistemas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

*Corresponding author: Cátedra de Fisiología, Facultad de Odontología, Universidad de Buenos Aires, Marcelo T de Alvear 2142, piso 3, sector A Ciudad de Buenos Aires C1122, Argentina. Tel: +54-11-4964-1275; Fax: +54-11-4508-3958; E-mail: jprestifilippo@yahoo.com.ar

(Received 16 March 2009; first review notified 30 April 2009; in revised form 27 May 2009; 31 May 2009 and 17 June 2009; accepted 18 June 2009; advance access publication 9 July 2009)

Abstract — Aim: The aim of the present study was to determine whether the endocannabinoid system could be involved in the ethanol-induced inhibition of salivation in adult male Wistar rats. Methods: Salivary secretion induced by different concentrations of methacholine, a cholinergic agonist, and the endocannabinoid arachidonoylethanolamide (anandamide, AEA) production in the submandibular gland (SMG) were determined in rats after ethanol (3 g/kg) administration by gastric gavage. To study the participation of cannabinoid receptors in ethanol action, we evaluated methacholine-induced salivary secretion after ethanol administration when CB1 or CB2 receptors were blocked by intra-SMG injections of their selective antagonists AM251 and AM630, respectively. Additionally, we evaluated the in vitro effect of ethanol (0.1 M) on SMG production of cAMP alone or combined with AM251 or AM630. Results: Acute ethanol administration increased AEA production in SMG and also inhibited the methacholine-induced saliva secretion that was partially restored by intraglandular injection of AM251 or AM630. In addition, ethanol significantly reduced the forskolin-induced increase in cAMP content in SMG in vitro while treatment with AM251 blocked this response. Conclusion: We conclude that the inhibitory effect produced by ethanol on submandibular gland salivary secretion is mediated, at least in part, by the endocannabinoid system.

INTRODUCTION

Saliva plays a key role in the local and systemic defense of the oral cavity, the oropharyngeal region, and the upper gastrointestinal tract (Zelles et al., 1995). It participates in taste, bolus formation for swallowing (water and mucus) and initiates digestion of starch (amylase) and lipids (lipase) (Nauntofte and Jensen, 1999). Furthermore, saliva components contribute to mucosal coating and provision of antimicrobial action and defense (Pedersen et al., 2002).

The submandibular gland (SMG) is one of the major salivary glands, together with the sublingual and the parotid glands. End secretory units, called acini, are continuous with a ductal system that in rodents has four sequential segments: intercalated ducts, granular convoluted tubules, striated ducts and excretory ducts that release the saliva to the oral cavity. The secretion of saliva is controlled by the autonomic nervous system. The parasympathetic nervous system exerts its function through the activation of muscarinic receptors on salivary glands via impulses in the chorda tympani nerve that releases acetylcholine and saliva-ergic nervous system exerts its function through the activation of muscarinic receptors on salivary glands via impulses in the chorda tympani nerve that releases acetylcholine and salivation depends on the contraction of myoepithelial cells embracing the acini and intercalated ducts (Tandler and Phillips, 1998). The sympathetic nervous system induces salivary secretion by releasing norepinephrine that stimulates α- and β-adrenergic receptors in the acini (Lung, 2003).

Although under normal physiological conditions, the protective potential is sufficiently maintained by saliva, it seems to be disturbed in alcoholics (Abelson et al., 1976; Dutta et al., 1992; Proctor and Shori, 1996). Studies have shown positive associations between alcohol consumption and periodontal disease (Sakki et al., 1995; Shiruzukiishi et al., 1998; Tezal et al., 2001; Pitiphat et al., 2003). Moreover, chronic ethanol consumption is considered one of the main causes of oral cancer (Wight and Ogden, 1998).

The ingestion of a high single dose of ethanol causes alteration in saliva flow rate, electrolyte concentration and reduction in SMG protein synthesis in rats and humans (Proctor et al., 1993; Shori et al., 1994; Enberg et al., 2001). However, the mechanisms by which ethanol exerts its acute effects are not clearly understood.

The main psychoactive ingredient of Cannabis sativa, delta-9-tetrahydrocannabinol (THC), affects different physiological functions. Twenty years ago, two subtypes of G-protein-coupled cannabinoid (CB) receptors were identified: the CB1 central receptor subtype, which is mainly expressed in the brain (Devane et al., 1988; Herkenham et al., 1990), and the CB2 peripheral receptor subtype, which appears to be particularly abundant in the immune system (Munro et al., 1993). A few years later, arachidonylethanolamide (anandamide, AEA) and arachidonoylglycerol, the best-known endocannabinoids, were discovered and purified. Both endocannabinoids derive from arachidonic acid and bind with high affinity to CB receptors (Mechoulam et al., 1998). Selective antagonists have been developed for CB1 receptor, such as AM251 (Gatley et al., 1996) and SR141716A (Rinaldi-Carmona et al., 1995), and for CB2 receptor, such as AM630 (Pertwee et al., 1995) and SR144528 (Griffin et al., 1999).

Furthermore, the CB1 and CB2 receptors were described in the gastrointestinal tract. Neuronal activation of CB1 receptors reduces motility, diarrhea, pain and emesis, and induces eating, while activation of CB2 receptors, acting mostly via immune cells, reduces inflammation (Sanger, 2007).

Recently, we demonstrated that CB1 and CB2 receptors are present in the SMG exhibiting specific localizations (Prestifilippo et al., 2006). Both CB1 and CB2 receptors are functionally linked to the inhibition of adenylyl cyclase (Howlett and Fleming, 1984) and AEA binds with high affinity to both CB receptors decreasing cAMP production in the SMG.
(Mechoulam et al., 1998, Prestifilippo et al., 2006). In addition, AEA injected intraglandularly inhibited norepinephrine and methacholine-stimulated saliva secretion in rats (Prestifilippo et al., 2006). Also, it has been reported that chronic ethanol exposure increases the levels of endocannabinoids in different tissues (Basavarajappa, 2007). Therefore, since acute ethanol ingestion as well as AEA injected in the SMG reduces salivary secretion, the aim of this study was to determine whether the endocannabinoid system could be involved in the inhibition of salivary secretion produced by ethanol.

MATERIAL AND METHODS

Animals

Adult male Wistar rats (250–300 g) were kept in an animal room having a photoperiod of 12 h of light, at 22–25°C and free access to rat chow and tap water. The experimental procedures reported here were approved by the Animal Care Committee of the Center for Pharmacological and Botanicals Studies of the National Council of Scientific and Technical Research of Argentina and were carried out in accordance with the National Institute of Health (NIH) guidelines.

AEA production

The production of AEA in the SMG was assayed as described by Paria et al. (1996) with minor modifications. The SMGs (7–8 per group) were removed after 60 min of ethanol (3 g/kg of body weight in a final volume of 5 ml per rat) or vehicle (5 ml of water) administered by gastric gavage. Each SMG was homogenized in 500 μl of buffer [20 mM Tris–HCl/1 mM EDTA (pH 7.6)] and centrifuged at 2000×g for 15 min. Supernatant protein (150–100 μg) was incubated in a total volume of 200 μl of 50 mM Tris–HCl (pH 9.0) with 40 μM (0.1 μCi) of [1-14C]arachidonic acid (40–60 μCi/mmol, Perkin–Elmer, Waltham, MA, USA) and 20 mM ethanolamine (Sigma Chemicals, St. Louis, MO, USA) during 5 min at 37°C. The reaction was terminated by the addition of 400 μl of chloroform–methanol (1:1) mixture. Two additional washes of the aqueous phase with 400 μl of chloroform were performed. Organic phases were evaporated to dryness under nitrogen gas and dissolved in 40 μl of chloroform–methanol (1:1) mixture. Supernatants were collected and samples were stored at −20°C until cAMP measurements.

Radioimmunoassay of arachidonic acid

AEA was resolved by using the organic layer of an ethyl acetate–hexane–acetic acid–water (100:50:20:100) mixture. The plate was exposed to an x-ray film at −70°C. After autoradiography, distribution of radioactivity on the plate was counted in a scintillation counter by scraping off the corresponding spots of the plate. The retardation factor values of AEA and arachidonic acid were 0.33 and 0.78, respectively.

In vitro studies

The animals were killed by decapitation, and SMGs were removed. SMGs (5–6 per group) were cut into halves to enhance the penetration of the different substances into the tissue and preincubated in 500 μl of Krebs–Ringer bicarbonate buffer medium (pH 7.4) containing 0.1% glucose within a Dubnoff metabolic shaker (50 cycles per minute, 95% O2/5% CO2) for 15 min before replacement with a fresh medium containing the compounds to be tested. The incubation was continued for another 30 min with forskolin (76 μM) (Sigma Chemicals, St. Louis, MO, USA), an adenylyl cyclase stimulator, forskolin plus AEA (1 nM) (Sigma Chemicals, St. Louis, MO, USA) or forskolin plus ethanol (0.1 M). In other experiments, SMGs were pre-incubated for 15 min with Krebs–Ringer alone and with AM630 (10 μM) (Tocris Ellisville, MO, USA) or AM251 (10 μM) (Tocris Ellisville, MO, USA). AM251 and AM630 were dissolved in dimethyl sulphoxide, and further dilutions were made in saline. Then, incubation was continued for another 30 min with Krebs–Ringer (control), forskolin, forskolin plus ethanol (0.1 M), forskolin plus ethanol plus AM630, AM630 alone, forskolin plus ethanol plus AM251 or AM251 alone. After the incubations, the SMGs were homogenized in 1 ml of H2O and centrifuged at 6000×g for 10 min at 4°C. Supernatants were collected and samples were stored at −20°C until cAMP measurements.

cAMP was measured by radioimmunoassay by using the highly specific cAMP antibody kindly provided by Dr A. F. Parlow (National Hormone & Peptide Program, CA, USA). The assay sensitivity was 0.061 pmol/ml. Intra- and inter-assay coefficients of variation were 8.1% and 10.5%, respectively.

Statistics

Data are presented as the means ± SEM. Comparisons between groups were performed using a one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparison test for unequal replicates or Student’s t-test, when appropriate. All analyses were conducted with the GraphPad Prism Version 4.00 software (San Diego, CA, USA). Differences with P values < 0.05 were considered statistically significant.
Role of the Endocannabinoid System in Ethanol-Induced Inhibition of Salivary Secretion 445

Fig. 1. Effect of ethanol (3 g/kg) and AEA (1 μg/50 μl) on methacholine-stimulated salivary secretion from the SMG. Data are reported as means ± SEM (6 rats per group). **P < 0.01 and ***P < 0.001 versus vehicle (ANOVA, followed by the Student–Newman–Keuls multiple comparisons post-test).

Fig. 2. Production of AEA in the SMG measured 1 h after ethanol (3 g/kg) administration. Values are means ± SEM (7–8 rats per group). ***P < 0.001 versus control (Student’s t-test).

RESULTS

Acute effect of ethanol and AEA on salivary secretion

Results indicate that ethanol administered by gastric gavage (3 g/kg) significantly inhibited SMG salivary secretion stimulated by all doses of methacholine tested [ANOVA: 1 μg/kg, F(2,15): 26.23, P < 0.01; 3 μg/kg, F(2,15): 22.57, P < 0.01; 10 μg/kg, F(2,15): 34.17, P < 0.001] (Fig. 1). The intra-SMG injection of AEA (1 μg/50 μl) also inhibited the salivary secretion stimulated by the same doses of methacholine (*P < 0.001) (Fig. 1).

Acute effect of ethanol on AEA production in the SMG

In order to investigate the participation of the endocannabinoid system in salivary responses to ethanol, we first evaluated the effect of ethanol (3 g/kg) on AEA production in the SMG. Ethanol increased AEA production (Student’s t-test, *P < 0.001) measured 1 h after administration (Fig. 2).

Involvement of the endocannabinoid system in ethanol inhibition of salivation

We evaluated the effect on salivation of the selective antagonists for CB1 and CB2 receptors, AM251 and AM630, respectively. The inhibitory effect of ethanol (3 g/kg) on methacholine-induced salivary secretion [ANOVA: 1 μg/kg, F(2,15): 8.616, P < 0.05; 3 μg/kg, F(2,15): 32.17, P < 0.01; 10 μg/kg, F(2,15): 24.52, P < 0.01] was completely prevented by the intra-glandular injection of AM251 (15 μg/50 μl) at 1 μg/kg of methacholine dose, and was partially reversed when higher doses of methacholine were injected (#P < 0.05 versus ethanol) (Fig. 3A).

Injection of AM630 (15 μg/50 μl) partially prevented (1 μg/kg and 3 μg/kg, *P < 0.05 versus ethanol; 10 μg/kg, ###P < 0.001 versus ethanol) the decrease in salivation exerted by ethanol at the three methacholine doses used [ANOVA: 1 μg/kg, F(2,15): 34.17, P < 0.01; 3 μg/kg, F(2,15): 22.02, P < 0.01; 10 μg/kg, F(2,15): 31.96; P < 0.001] (Fig. 3B). The injection of AM251 and AM630 alone did not modify methacholine-induced salivation (data not shown).

Effect of ethanol on cAMP production in the SMG: participation of the endocannabinoid system

Since activation of both CB1 and CB2 receptors inhibits adenylate cyclase activity, we investigated whether ethanol could modulate forskolin-increased cAMP production through the activation of CB receptors in vitro. Results showed that ethanol (0.1 M) as well as AEA (1 nm) significantly blocked [ANOVA: F(3,33): 7.310, P < 0.05] the forskolin (76 μM)-induced increase in cAMP content (P < 0.001). (Fig. 4A). In order to
ethanol has been shown to modulate a variety of functions (Mukherjee et al., 2008; Harper, 2009), it does not seem to have a specific receptor site for its action. It is known that acute ethanol ingestion alters salivary flow rate and saliva composition (Shori et al., 1994; Enberg et al., 2001). It was reported that alcohol modifies membrane lipid composition; however, these alterations do not explain the changes observed in saliva composition and secretion. In addition, it was previously described that acute alcohol intake not only did affect the autonomic innervations but also produced different responses in rat parotid, sublingual and submandibular gland function (Proctor et al., 1993).

We hypothesized that the increased AEA production observed in the SMG after 1 h of ethanol administration may be responsible for the reduction of salivation. We have previously reported that CB1 or CB2 receptors were expressed in the main cellular structures involved in saliva production and secretion that are acini and ducts. Most CB1 receptors were present in ducts, while CB2 receptors were located in ducts and acini (Prestifilippo et al., 2006). To further investigate the involvement of AEA in the ethanol-induced inhibition of salivation, we employed CB receptor antagonists. Results demonstrated that ethanol effect on salivary secretion was partially prevented by blocking CB1 and CB2 receptors.

The CB1 and CB2 receptors belong to the family of the seven transmembrane-spanning receptors, and are coupled to G\textsubscript{i/o} proteins (Howlett and Fleming, 1984). Since the mechanism of action of endocannabinoids acting on its receptors is by inhibiting adenylyl cyclase with the consequent decrease in cAMP and taking into account the importance of adenylyl cyclase activity for salivary function, we measured the effect of ethanol on forskolin-induced cAMP levels in SMG slices. Ethanol markedly reduced forskolin-induced cAMP levels in SMG slice level and this effect was blocked by the CB1 but not by CB2 receptor antagonist, indicating that ethanol actions were mediated preferentially by CB1 receptors in vitro. Coincidently, only the CB1 receptor antagonist completely reversed the inhibitory effect of ethanol on methacholine-induced salivary secretion at the lowest dose of the sialogogue (1 μg/kg).

On the other hand, an important center for eliciting salivary secretion exists in the hypothalamus and involves cholinergic pathways. Numerous projections exist from the lateral hypothalamus to salivary nuclei located in the brain stem (Matsuo and Kusano, 1984; Hainsworth and Epstein, 1996). Sialogogues injected through the femoral vein act not only on their receptors in the SMG but also on their receptors in the brain, stimulating efferent responses to the periphery. It was shown that THC decreased salivary flow from the SMG during electrical stimulation of the chorda tympani by a mechanism involving a decrease in the release of acetylcholine (Mc Connell et al., 1978). Acute and chronic exposure to ethanol has been proposed to induce neuroadaptations in the endocannabinoid system. The exposure of cultured neuronal cells or cerebellar granular neurons to chronic ethanol resulted in an accumulation of AEA (Basavarajappa and Hungund, 1999) and 2-arachidonoylglycerol (Basavarajappa et al., 2000). In this line, we have previously demonstrated that ethanol administration by gastric gavage (3 g/kg) increased AEA in the medial basal hypothalamus (Rettori et al., 2007). It was also reported that ethanol increased plasma AEA, which peaked 90 and 240 min after its administration (Giuffrida et al., 1999). Furthermore, the

**DISCUSSION**

It is well recognized that ethanol consumption decreases salivary secretion (Enberg et al., 2001). However, the mechanism by which ethanol exerts this noxious effect is not clearly understood. The present work shows for the first time the participation of the endocannabinoid system in the inhibition of salivary secretion provoked by acute ethanol administration.

Our results demonstrate that ethanol administration by gastric gavage increased AEA production in the SMG and also inhibited the methacholine-induced salivary secretion. Although the participation of the endocannabinoid system in the inhibition of salivary secretion exists in the hypothalamus (Enberg et al., 2001). However, the mechanism by which ethanol exerts this noxious effect is not clearly understood. The present work shows for the first time the participation of the endocannabinoid system in the inhibition of salivary secretion provoked by acute ethanol administration.

Our results demonstrate that ethanol administration by gastric gavage increased AEA production in the SMG and also inhibited the methacholine-induced salivary secretion. Although...
presence of CB1 receptors were shown in the lateral hypotha- 

lumus, which is an important area that controls salivary secre- 

tion (Matsuo and Kusano, 1984; Hainsworth and Epstein, 1996) 

and recently, we have reported that the intracerebroventricu- 

lar administration of AEA significantly decreased salivation (Fernandez-Solari et al., 2009). Therefore, ethanol administration 

by gastric gavage increases AEA production in the SMG and also in the hypothalamus suggesting that the produced AEA 

could activate not only the CB receptors in the SMG but also 

hypothalamic CB receptors and thus affecting both peripheral 

and central control of salivation.

It is known that ethanol can also alter the release of different neurotransmitters in the nervous system. It enhances the re- 

lease of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) in the hypothalamus (Lomniczi et al., 2000) that 
could attenuate autonomic neurotransmission to the SMG with 
a consequent inhibition of salivary secretion (Fernandez-Solari et al., 2009). Thus, the ethanol action on the hypothalamic sali-

vary nuclei could explain the partial blocking of the ethanol 
inhibitory effect on salivary secretion produced by the CB1 
and CB2 receptor antagonists injected in the SMG. Addition-

ally, we cannot discard a direct non-specific effect of ethanol, 

CB receptor independent, on submandibular gland function.

Therefore, we conclude that the inhibitory effect produced by 

ethanol on submandibular gland salivary secretion is mediated, at least in part, by the endocannabinoid system.

Acknowledgements — This work was supported by grants from the University of Buenos Aires (UBACYT O 007), ANPCyT (03-14264) and Fundación Alberto J. Roemmers. The 

authors are greatly indebted to Ricardo Horacio Orzuza for technical assistance.

REFERENCES

Abelson DC, Mandel M, Karmiol ID. (1976) Salivary studies in alco-


Basavarajappa BS. (2007) The endocannabinoid signaling system: 
a potential target for next-generation therapeutics for alcoholism. 

Basavarajappa BS, Hungund BL. (1999) Chronic ethanol increases 
cannabinoid receptor agonist, anandamide and its precursor N-

arachidonyl phosphatidyl ethanolamine in SK-N-SH cells. J Neu-


Basavarajappa BS, Saito M, Cooper TB et al. (2000) Stimulation 
of cannabinoid receptor agonist 2-arachidonoylglycerol by chronic 

ethanol and its modulation by specific neuromodulators in cerebel-

lar granule neurons. Biochim Biophys Acta 1535:78–86.

Biancotti LG, Elverdin JC, Vatta MS et al. (1994) Atrial natriuretic 


Devane WA, Dyszar FA, Johnson MR et al. (1988) Determination 

and characterization of a cannabinoid receptor in rat brain. Mol 


Dutta SK, Orestes M, Vengulekur S et al. (1992) Ethanol and human 

saliva: effect of chronic alcoholism on flow rate, composition, 


Enberg N, Alho H, Loimaranta V et al. (2001) Saliva flow rate, 
amylase activity, and protein and electrolyte concentrations in saliva after 


injected into the lateral ventricle of the brain inhibits submandibular 
salivary secretion by attenuating parasympathetic neurotransmis-


Gatley SJ, Gifford AN, Volkow ND et al. (1996) 123Iabeled AM251: 
a radioiodinated ligand which binds in vivo to mouse brain cannabi-


Giuffrida A, Parsons LH, Kerr TM et al. (1999) Dopamine activation of 


Griffin G, Wray EJ, Tao Q et al. (1999) Evaluation of the cannabinoid 

CB2 receptor-selective antagonist, SR144528: further evidence for 

Hainsworth FR, Epstein AN. (1996) Severe impairment of heat 

induced salvia spreading in rats recovered from lateral hypothalamic 


Alcohol Alcohol 44:136–40.

Herkenham M, Lynn AB, Little MD et al. (1990) Cannabinoid receptor 


Howlett AC, Fleming RM. (1984) Cannabinoid inhibition of adenyl-

ate cyclase. Pharmacology of the response in neuroblastoma cell 

Lomniczi A, Mastronardi CA, Faletti AG et al. (2000) Inhibitory path-

ways and the inhibition of luteinizing hormone-releasing hormone 

release by alcohol. Proc Natl Acad Sci USA 97:2337–42.

Lung MA. (2003) Autonomic nervous control of myoepithelial cells 

and secretion in submandibular gland of anesthetized dogs. J Physiol 


Mc Connell WR, Dewey WL, Harris LS et al. (1978) A study of the 
effect of delta 9-tetrahydrocannabinol (delta9-THC) on mammalian 

J Pharmacol 359:1–18.

Mukherjee S, Das SK, Vaidyanathan K et al. (2008) Consequences 
of alcohol consumption on neurotransmitters -an overview. Curr 

Munro S, Thomas KL, Abu-Shaar M. (1993) Molecular characteriza-
tion of a peripheral receptor for cannabinoids. Nature 365: 

61–5.


Williams, Wilkins, 119–45.

Paria BC, Deutsch DD, Dey SK. (1996) The uterus is a potential site 

for anandamide synthesis and hydrolysis: differential profiles of 
anandamide synthase and hydrolase activities in the mouse uterus 

Pedersen AM, Bardow A, Beier Jensen S et al. (2002) Saliva and 
gastrointestinal functions of taste, mastication, swallowing and di-

Pertwee R, Griffin G, Fernando S et al. (1995) AM630, a competitive 

Pitiphat W, Merchant AT, Rimm EB et al. (2003) Alcohol consump-

Prestifilippo JP, Fernández-Solari J, de la Cal C et al. (2006) Inhibition 
of salivary secretion by activation of cannabinoid receptors. Exp 

In Preedy VR, Watson RR (eds). Alcohol and the Gastrointestinal 

Proctor GB, Shorty DK, Preedy VR. (1995) Protein synthesis in the 

major salivary glands of the rat and the effects of re-feeding and 


Rettori V, Fernandez-Solari J, Prestifilippo JP et al. (2007) Endo-
cannabinoids in TNF-alpha and ethanol actions. Neuroimmunomod-
ulation 14:188–92.

Rinaldi-Carmona M, Barth F, Heaulme M et al. (1995) Biochemical 
and pharmacological characterisation of SR141716A, the first po-
tent and selective brain cannabinoid receptor antagonist. Life Sci 

Sakki TK, Knutttila ML, Vimpuri SS et al. (1995) Association of 

Sanger GJ. (2007) Endocannabinoids and the gastrointestinal tract: 
what are the key questions? Br J Pharmacol 152:663–70.

Shizukuishi S, Hayashi N, Tamagawa H et al. (1998) Lifestyle and pe-
riodontal health status of Japanese factory workers. Ann Periodontol 
3:303–11.


