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SHORT COMMUNICATION

In vivo assessment of DNA damage induced in oral mucosa cells by fixed and removable metal prosthodontic appliances

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Abstract Given long-term effect on oral tissues due to contact with dental appliances, the biocompatibility studies of casting alloys are of great importance. It has been previously documented that metal dental appliances, due to corrosion, might induce genotoxic and mutagenic effects in cells. Therefore, the aim of presented study was to examine the genotoxicity of two dental casting alloys (Co-Cr-Mo and Ni-Cr) commonly used in fixed and removable prosthodontic appliances that are in contact with the oral epithelium for 5 years or more. For that purpose, 55 age-matched subjects were included in the study; 30 wearers of prosthodontic appliances and 25 controls. Buccal cells of oral mucosa were collected and processed for further analysis. The cell viability has been assessed by trypan blue exclusion test, while genotoxic effect of metal ions on DNA in oral mucosa cells was studied by use of alkaline comet assay. Results have shown significantly higher comet assay parameters (tail length and percentage DNA in the tail) in the group wearing metal appliances. Both subjects with Co-Cr-Mo alloy and Ni-Cr alloy showed significantly higher comet assay parameters when compared with controls. It has been confirmed that metal ions released by the two base metal dental casting alloys examined in this study, might be responsible for DNA damage of oral mucosa cells. Therefore, the results of this study emphasize the importance of the in vivo evaluation of dental materials with respect to their genotoxicity, which is of major importance to ensure long-term biocompatibility.

Keywords Prosthodontic appliances · Dental casting alloys · Oral mucosa · Buccal cells · Comet assay · DNA damage

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Introduction

Dental casting alloys are widely used in applications that place them into contact with oral epithelium, connective tissue, or bone for a period of many years. Given such long-term effect on oral tissues, it is of utmost importance to measure and understand the biocompatibility of casting alloys [1]. Indeed, dental cast alloys are subject to risk assessment studies, which include a clinical evaluation, before being allowed for the market [2]. This issue is regulated by legislative of governmental agencies in European countries. The risk assessment is regulated by the European and international standard (the previous norm for the risk analysis was EN 1441, since April 2004 is EN ISO 14971) for manufacturers of medical devices. It regulates the risk management procedures that should be strictly followed by manufacturers in order to ensure the safety of their products before placing them on the market [3].

Such approach should prevent adverse effects caused by dental appliances on oral tissues. However, no methods specified so far can predict the possible occurrence of adverse effects after long-term use of dental casting alloys [2]. It has been well documented, both in vitro and in vivo, that metal dental appliances release metal ions, due to corrosion [4]. In particular, the oral environment represents an ideal medium for biodegradation of metals due to its thermal, microbiologic, and enzymatic properties [5]. Release of metal ions causes a variety of biological responses. The biological interaction between dental alloys and the soft oral tissue may be classified as: bacterial adhesion, toxicity, subtoxic effects, and allergies [2]. Numerous studies and reports in the scientific literature address these mechanisms. Mechanical/physical irritation, such as pressure caused by dentures, can also cause local tissue reactions [6]. Individual metal ions have already been thoroughly assessed for genotoxic and mutagenic effects in prokaryotic and eukaryotic test systems [4], but only few in vivo studies reporting on metal release from fixed orthodontic appliances and their influence on DNA damage of oral mucosa cells were published so far [5, 7].

Consequently, the aim of the presented study was to expand current knowledge on dental alloys effects on oral mucosa cells. In particular, we aimed to evaluate the genotoxicity of dental casting alloys in patients with fixed and removable prosthodontic appliances, which are in contact with oral epithelium not less than 5 years. For that purpose, DNA damage in oral mucosa cells has been assessed by using alkaline comet assay.

Materials and methods

Subjects

Fifty-five subjects were included in this study. The experimental group comprised a total of 30 subjects (mean age 69.56). Among them, 11 were wearers of removable prosthodontic appliances and 19 were wearers of fixed prosthodontic appliances. Only those subjects wearing appliances for more than 5 years were included in the study. Removable appliances were made from Co-Cr-Mo alloy consisted of Co—64%, Cr—28.65%, Mo—5%, Si—1%, Mn—1%, and C—0.5% (Wironit®, Bego, Germany), while fixed prosthodontic appliances were Ni-Cr alloy consisted of Ni—65%, Cr—22.5%, Mo—9.5%, Si—1%, Nb—1%, Fe—0.5%, Ce—0.5%, and C<0.02% (Wiron®, Bego, Germany). The control group comprised 25 edentulous subjects (mean age 72.68) who had been without teeth for at least 5 years. Control subjects were wearers of full acrylic dentures. Both study groups comprised age-matched, infrequent alcohol consumers, taking antihypertensive agents and occasionally nonsteroidal anti-inflammatory drugs (NSAIDs). Exhaustive medical history was documented for all subjects. A pre-structured questionnaire on dietary and smoking habits, alcohol and drug intake, as well as on systemic diseases and verified allergy to known allergens and medications has been filled for each subject. Clinical examinations were performed, and pH of saliva was determined (Spezial Indikatorpapier pH 6.4–8.0, Merck, Darmstadt). Patients with oral lesions, history of malignant diseases, and tobacco users were excluded from the study. Prior to signing a written consent, each patient has been thoroughly informed about the purpose of this study. The study has been approved by the Ethical Committee, School of Dental Medicine, University of Zagreb.

Sample collection

Epithelial cells of buccal mucosa from each patient were collected according to the method of Besarti Nia et al. [8] with slight modifications. Prior to cell sampling, examinees washed out the mouth three times with tepid water to remove dead exfoliated cells. Buccal swab was taken by gentle brushing of the internal part of right and left cheek with a cytobrush. The brushes were afterwards stirred in 5 ml of RPMI (RPMI 1640 Medium, Gibco-Invitrogen, Carlsbad, CA, USA), liquid (with L-glutamine, 25 mM HEPES), fetal bovine serum (Gibco-Invitrogen, Carlsbad, CA, USA), and penicillin–streptomycin solution (Sigma-Aldrich, Munich, Germany) and transported within 30 min to the laboratory for further processing.

Alkaline comet assay

Cell suspensions were centrifuged 3 min/3,200 rpm and re-suspended in phosphate-buffer saline solution (pH 7.4). Cell viability was determined by using the trypan blue (0.4% *w*/*v*; Gibco-Invitrogen, Carlsbad, CA, USA) exclusion assay. Cell viability was always found to be above 80%. Two parallel aliquots of buccal mucosa cells from each patient were immediately re-suspended in chilled buffer pH 7.5 [0.075 M NaCl (Kemika, Zagreb, Croatia) and 0.024 M Na_2EDTA (Sigma-Aldrich, Munich, Germany). Cells were macerated on ice for 2 min. All chemicals required for comet assay were purchased by Sigma-Aldrich, Munich, Germany. Procedure described by Singh et al. [9] was followed. Eight microliters of cell suspension were mixed with 100 μl of low-melting point agarose and added to a microscope slide pre-coated with 1.0% of normal-melting point agarose. Cells were lyzed (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% Na-lauroylsarcosinate, 1% Triton X-100, and 10% dimethyl sulfoxide, pH 10) for 72 h at 4°C and denatured (300 mM NaOH, 1 mM Na_2EDTA, pH 13.0) for 10 min. Electrophoresis was performed at 0.66 V/cm, 300 mA for 16 min [10]. Following neutralization (0.4 M Tris–HCl,

pH 7.5), staining with ethidium bromide (20 μg/mL) has been performed. Slides were analyzed using a 250× magnification of Leitz Orthoplan epifluorescence microscope (Wetzlar, Germany) and Comet Assay IV image analysis system (Perceptive Instruments Ltd. Suffolk, Halstead, UK). One hundred randomly selected cells (50 cells on each of two replicate slides) of each subject were scored. DNA damage was evaluated as percentage DNA in the tail (% DNA) and tail length, which was measured from the center of the comet head.

Statistical analysis

Basic statistics and the methods of multivariate analysis were performed for the data analysis. Basic statistical parameters were used including mean, standard deviation, standard error from the mean, relative standard deviation, median, minimum, maximum, and test for normality of distribution. If the differences between groups for single predictor variables were observed, *t* test has been employed. Analysis of variance was used if testing the differences between three or more groups was performed. Newman–Keuls test was used to determine if there was a statistically significant difference at significance level $p<0.05$ between the two groups. Because the data deviated from the normal distribution before the analysis, their logarithmic transformation was performed. If normal distribution using these tests was not achievable, *t* test was replaced with the Mann–Whitney *U* test (the form of nonparametric *t* test).

Results

Table 1 presents summary of descriptive statistics for results of comet assay parameters for both studied groups.

A general regression model has been employed to evaluate influence of general characteristics of the subjects (age, gender, dietary habits, pH of saliva, alcohol and drug intake) on parameters of comet assay. In this study, two parameters were exploited for evaluation of the DNA damage level: tail length measured in micrometers and percentage DNA in the tail. None of demographic or lifestyle factors tested as possible predictors have exhibited significant influence on values of comet assay parameters regardless of the group of subjects (Fig. 1).

Mann–Whitney *U* test revealed significantly increased tail length ($p=0.0117$) and percentage DNA in the tail ($p=0.0000$) values in subjects wearing metal appliances as presented in Fig. 2.

Wearers of metal appliances were divided into two groups based on the alloy from which the appliance was made. Although higher values of tail length and percentage DNA in the tail were assessed in the wearers of Co-Cr-Mo alloy compared with Ni-Cr alloy wearers (Table 2), observed difference has not resulted to be statistically significant (tail length $p=0.4769$; %DNA $p=0.3721$). However, both, patients with Co-Cr-Mo alloy and Ni-Cr alloy, showed significantly higher values of tail length (Ni-Cr, $p=0.0385$; Co-Cr-Mo, $p=0.0174$) and percentage DNA in the tail (Ni-Cr, $p=0.0338$; Co-Cr-Mo, $p=0.0091$) when compared with the controls (Fig. 3).

Discussion

In this study, we examined genotoxic influence of dental casting alloys in fixed and removable prosthodontic appliances by using alkaline comet assay. Comet assay is considered to be a very useful tool for investigation of genotoxicity at the first site of contact such as for example

Table 1 Results of descriptive statistics for recorded comet assay parameters in buccal cells from control and experimental group

Group	Statistical data	Tail length	Percentage DNA in the tail
Control group	X	13.13	0.36
	SD	3.14	1.19
	RSD	0.24	3.32
	SE	0.07	0.03
	M	12.82	0.00
	Range (min–max)	(0.00–39.74)	(0.00–17.93)
	ND	0.00	0.00
Experimental group	X	15.85	2.07
	SD	5.76	5.33
	RSD	0.36	2.58
	SE	0.12	0.11
	M	14.74	0.28
	Range (min–max)	(0.00–67.31)	(0.00–75.13)
	ND	0.00	0.00

X mean, *SD* standard deviation, *RSD* relative standard deviation, *SE* standard error, *M* median, *min* minimum, *max* maximum, *ND* test for normality of distribution

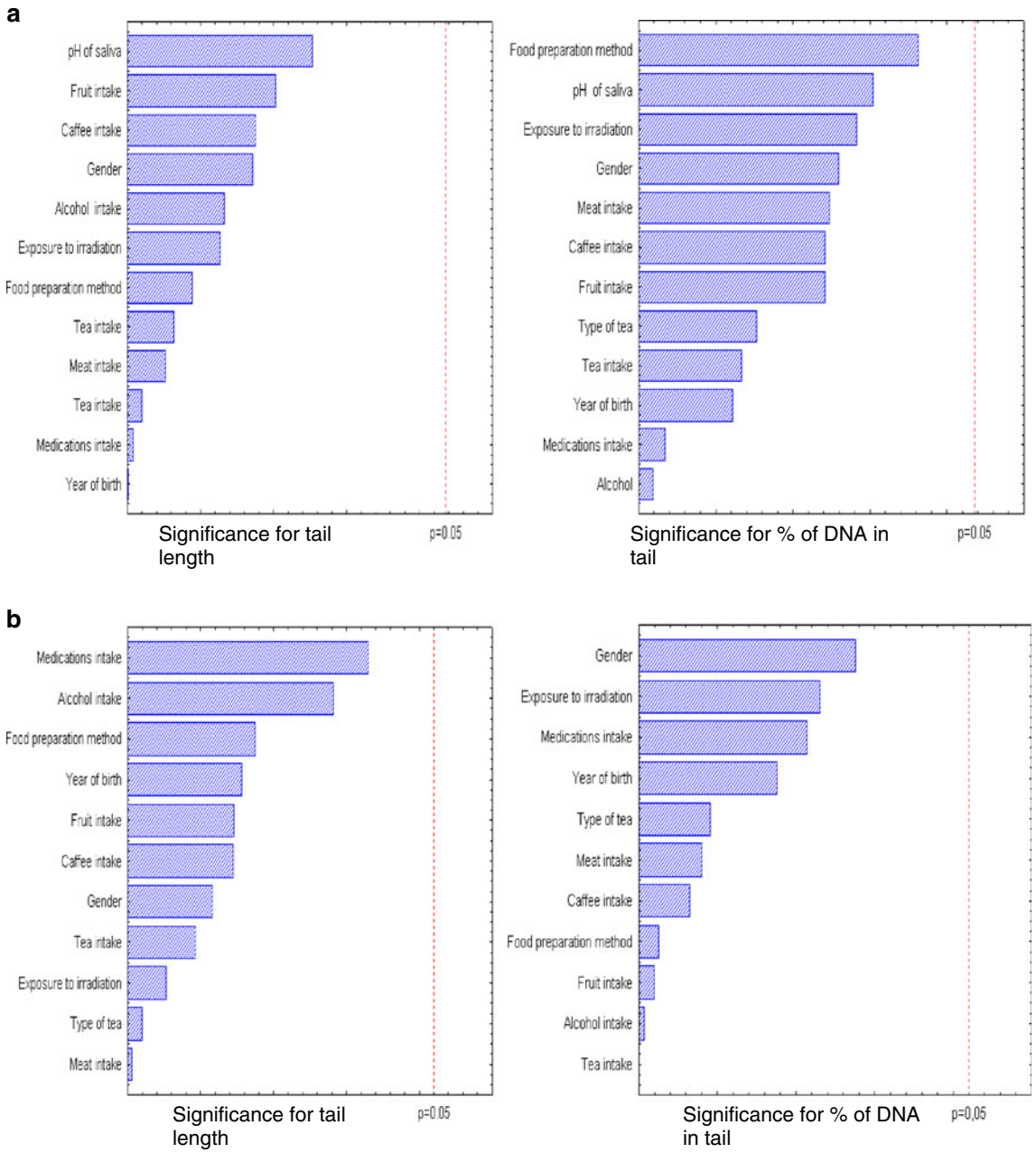

Fig. 1 Regression analysis results. Significant dependence of measured comet assay parameters (tail length and the percentage DNA in the tail) on demographic and lifestyle factors as possible predictors for control (*panel A*) and experimental group (*panel B*) are indicated

in oral mucosa. So far, only genotoxic influence of metal ions released from fixed orthodontic appliances that are present in oral cavity for a shorter period of time has been examined by comet assay. Nevertheless, these studies report ambiguous results [5, 7]. Conversely, prosthodontic appliances are placed in the oral cavity for more than few years, mostly for a decade or more, covering a large area of the oral mucosa. It might be thus assumed that DNA damage will be greater in wearers of prosthodontic appliances. Therefore, only wearers of prosthodontic appliances for more than 5 years participated in this study. Since it was difficult to gather elderly patients with natural teeth as this group of patients is quite rare, we included edentulous, full acrylic denture wearers as a control group as to ensure uniform criteria for the analyses. Although the control subjects wore full acrylic denture for a longer period of time, DNA damage of buccal cells was negligible when compared with the group with metal prosthodontics appliances. However, previous in vitro studies have shown that methyl methacrylate, a monomer of acrylic resin that acts as primary irritant and sensitizer causing allergic eczematous reaction on the oral mucosa and skin, may

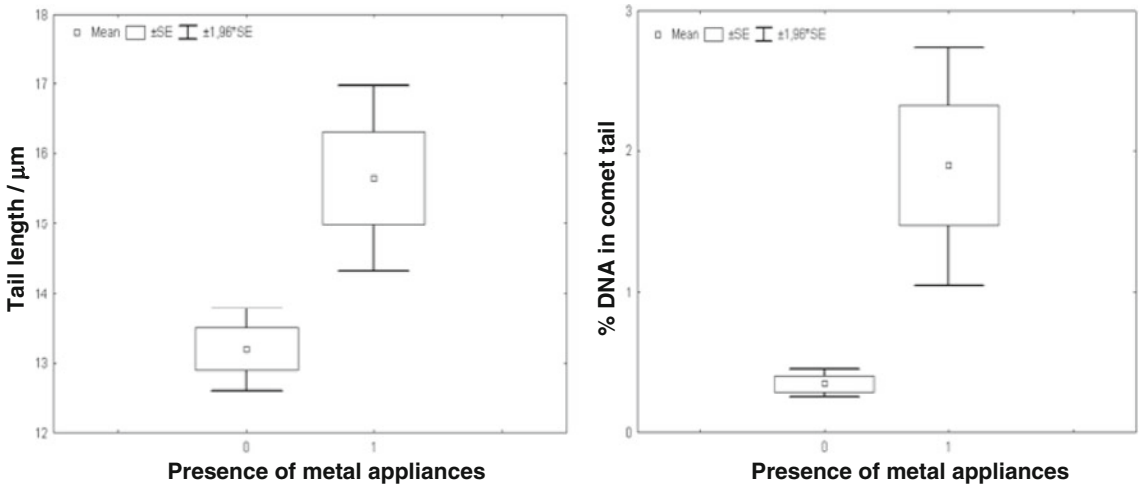

Fig. 2 Mean values and standard errors for measured comet assay parameters in control subjects (0) and metal appliance wearers (1)

cause cytotoxic and genotoxic effects in mammalian test system [11]. These results cannot however be directly correlated to our results as differences between in vitro and in vivo studies might probably be due to reparatory immune response of living tissues that are missing in the in vitro models.

The obtained results have shown that both tail length and percentage DNA in the tail were significantly higher in wearers of metal appliances than in controls. Similarly, Faccioni et al. [5] showed that tail length and tail moment values were significantly higher in wearers of fixed orthodontic appliances, while increased percentage DNA in the tail has been reported to be non-significant. Percentage DNA in the tail is one of comet assay parameters documented to be linearly related to dose [12], and in our study, significantly higher values of DNA percentage in the tail in wearers of metal appliances might be thus explained by longer exposure of oral mucosa to the influence of metal ions released due to corrosion. Indeed, several factors that may significantly influence the type of DNA damage have been documented so far and include length of tissue exposure to harmful effect, its dose, and the reparative ability of exposed tissue [12]. Yet, no DNA damage in the study of Westphalen et al. [7] was explained by short tissue exposure to fixed orthodontic appliances. The difference in exposure period of prosthodontic and orthodontic appliances in oral cavity might explain discrepancies observed between results obtained in our study and those of Westphalen et al. [7] and Faccioni et al. [5]. Further on, different observations might be additionally explained by age differences of subject involved in these studies as reparative ability of the tissue decreases with age, making oral mucosa more permeable to noxious agents and more vulnerable to mechanical damage [13]. Similarly, other factors such as drugs, alcohol, and acidic oral environment might decrease the reparative ability of oral mucosa as well. It is very difficult to find those patients who do not take medication, occasionally drink alcohol, or consume acidic food among elderly patients. In order to minimize the influence of these factors, we created a homogeneous group that comprised subjects of similar age and lifestyle habits. Subjects including in this study were, indeed, only classified as infrequent drinkers and were not tobacco consumers. It has been reported that alcohol-associated carcinogenesis is related to acetaldehyde formation, being the first metabolite of ethanol [14]. It has been shown that smaller amounts of alcohol may exert a beneficial effect to human health [15]. Kokoshka et al. [16] have for example reported that low doses of alcohol can induce an adaptive cytoprotection effect in intestinal cells. This might probably explain that subjects involved in our study had no detectable DNA damage of buccal cells. Ishikawa et al. [17], on the other side, showed how habitual alcohol drinkers exert a higher number of chromosome aberrations in peripheral blood lymphocytes than the infrequent drinkers. It is also known that combined effect of drinking and smoking affect the results of comet assay [12], and Reis at al. [18] showed that even excessive alcohol consumption alone may induce effective alteration on oral mucosa. At last, subjects involved in this study consumed only antihypertensive agents and occasionally NSADs. However, studies focused on effect of antihyper-

Table 2 Comet assay parameters (mean values±SD) regarding composition of the metal appliances

Presence of alloy	Tail length±SD	%DNA (tail)±SD
None	13.20±1.49	0.35±0.27
Ni-Cr alloy	15.38±4.04	1.69±2.73
Co-Cr-Mo alloy	16.12±3.16	2.25±1.62

SD standard deviation

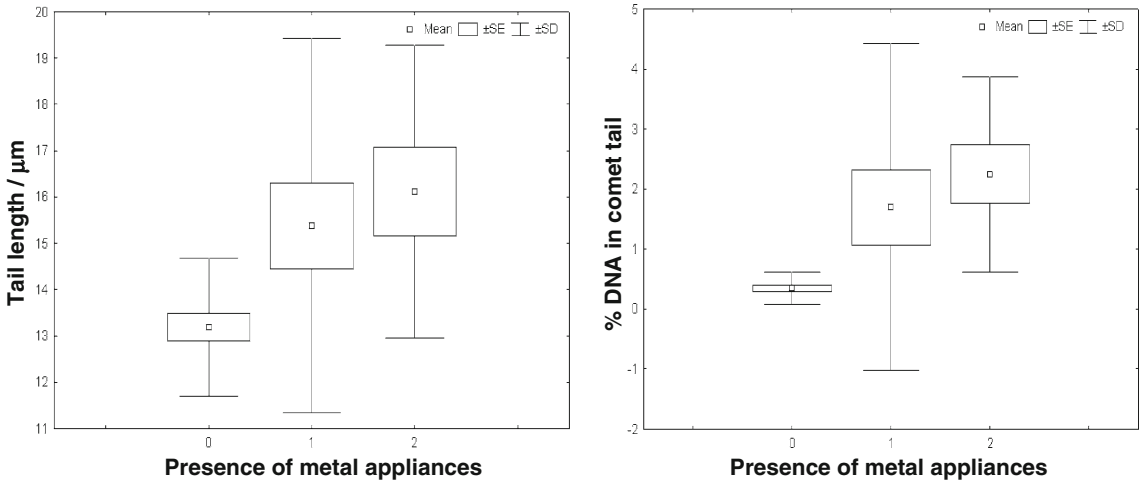

Fig. 3 Mean value, standard errors (SE), and standard deviations (SD) for measured comet assay parameters regarding the presence and alloy type (*0*—none, *1*—Ni-Co alloy, *2*—Co-Cr-Mo alloy)

tensive drugs and NSAIDs on buccal cells by using comet assay have not been reported so far. As a conclusion, and given that subjects enrolled in this study take similar or even same medications, observed DNA damage might be associated to the influence of metal ions. Indeed, significantly higher DNA damage levels were present only in this group of subjects.

Released metal ions from metal appliances in elderly patients could easily penetrate through thinned oral mucosa and cause DNA damage more than in younger patients whose oral mucosa function is preserved. This is also influenced by hyposalivation, which is caused by various diseases and drugs and therefore, frequent finding in elderly patients [19]. However, DNA damage can be induced by mechanical irritation independently of the influence of metal ions. It has been shown from the study of Sato [20] that a mechanical irritation acts as an inducer in the canceration process of tongue cancer. Sato investigated the intensity of DNA damage caused by mechanical irritation on hamster tongue by measuring the activity of Poly (ADP-ribose) polymerase (PADPRP), which is an enzyme associated with DNA damage. After carcinogenic treatment, canceration of leukoplakia and early carcinoma progressed into advanced carcinoma histopathologically by mechanical irritation only, and the activity of PADPRP increased during the scratching period. These results suggest that the mechanical irritation acts accelerative in canceration and progression of tongue cancer and causes DNA damage [20].

In our patients, greater DNA damage was detected in wearers of metal appliances, despite their intact oral mucosa. Therefore, we can assume that DNA damage in oral mucosa cells in the presence of chronic mechanical irritation could be even greater.

Although higher DNA damage has been observed in Co-Cr-Mo casting alloy wearers in comparison with Ni-Cr casting alloy wearers, no statistical significance has been proven. Co-Cr-Mo casting alloy is mostly used for fabrication of removable dentures when metal surface is placed in direct contact with oral tissues. This is not the case with Ni-Cr alloy that is mostly used for fabrication of fixed prosthodontic appliances [21]. Metal ions released underneath the metal framework of a removable partial denture toward the tissue side may not be diluted by oral fluids to the same extent as ions that are released from the opposite side of the framework. Therefore, higher metal ion concentration is expected to be found next to the tissue than in saliva [1]. Greater damage of DNA observed in this study for wearers of Co-Cr-Mo casting alloy was probably due to larger area of oral tissue in contact with metal surface of removable prosthodontic appliance and higher concentration of metal ions underneath metal framework, which were not effectively diluted by saliva flow. The absence of statistical significance might be explained by the smaller number of subjects who were Co-Cr-Mo alloy wearers in comparison to Ni-Cr alloy wearers.

The results of the present study give evidence that presence of prosthodontic appliances in oral cavity for longer period of time may cause DNA damage in oral mucosa cells. Potential genotoxic effect has been assessed for two frequently used dental casting alloys used in fabrication of metal dental appliances. However, primary DNA lesions detected by cell-repair mechanisms in vivo that are caused by metal tissue contact might be repaired error-free and do not necessarily result in formation of mutations; neither the magnitude of DNA migrations in the comet assay nor the shape of the comet can reveal the type of DNA damage [12]. In general, comet assay results might well indicate a burdening of the genome by adverse exogenic factors. Therefore, it is of utmost importance to examine the properties of dental materials as to find good

level of biocompatibility that will ensure the lowest impact on human tissues and biological processes.

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Conflict of interest The authors declare no conflict of interest.

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