Nickel, chromium and methyl methacrylate monomer release from orthopaedic functional appliances

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DOI 10.23804/ejpd.2021.22.02.11

Abstract

Aim This study was purposed to evaluate release of nickel and chromium ions and methyl methacrylate (MMA) monomers from functional appliances and their possible health effects.

Materials and methods Study design: Twin-block appliances and Bionators were immersed in artificial saliva and kept in a thermal incubator. Control group was established as artificial saliva without appliances. Artificial saliva was analysed after 7 days, 30 days and 90 days. Inductively coupled plasma mass spectrometry and gas chromatography–mass spectrometry was used for detection of nickel and chromium ions and MMA monomers. MTT assays and cytokine array were performed. Statistics: One way ANOVA with Tukey test and Dunnett's T3 for post-hoc analysis was used for evaluation of time-dependent changes and independent t-test was used for evaluation of MTT assay results.

Results The results revealed that metal ions and MMA monomers are released from the appliances. Metal ion detection pattern was irregular and could not be analysed. Twin-block group showed significantly larger amount of MMA release. MTT assay revealed statistically significant but minimally reduced cellular activity on Bionator and twin-block groups compared to control groups. Cytokine array showed no or less inflammatory cytokine release on Bionator and twin-block groups.

Conclusion MMA monomer release was confirmed but the cytotoxic effect of functional appliance material release is minimal or negligible. General toxicity of the functional appliance from the MMA monomer release is likely to be minimal or negligible.

KEYWORDS Release of metal ions and MMA monomers; Nickel; Chromium; Methyl methacrylate; Orthopaedic appliances.

Introduction

Functional appliances are used for modulation of jaw growth in growing children [Maspero et al., 2018b]. While several of these are fixed appliances such as the Herbst appliance, the majority are removable appliances. One of the common features of these appliances is that they are composed of stainless steel and acrylic resin.

It has been reported that wires and metal brackets used in orthodontic treatment undergo corrosion and degradation in the oral environment, thus releasing metal ions [Amini et al., 2012; Eliades and Athanasiou, 2002; Mikulewicz and Chojnacka, 2011; Mikulewicz et al., 2012; Kao et al., 2007]. Among the metal ions, nickel (Ni) and chromium (Cr) have garnered special interest due to their potentially harmful effects on human health. It has been reported that Ni and Cr ions are carcinogenic, mutagenic, cytotoxic, and allergenic [Kao et al., 2007; Eliades and Athanasiou, 2002; Kargacin et al., 1993; Magaye et al., 2014; Chervona et al., 2012; Hafez et al., 2011; Maspero et al., 2018a]. Of the many *in vivo* and *in vitro* reports that quantified metal ion release from fixed orthodontic appliances including stainless steel wires, Ni-Ti wires and orthodontic brackets, most showed that such release was significantly below the average dietary intake, while others presented concern about the harmful effects and "low dose effect" of Ni and Cr ions [Kocadereli et al., 2000; Nayak et al., 2015; Spalj et al., 2012; Amini et al., 2012; Gopikrishnan et al., 2015; Sahoo et al., 2011].

Acrylic resin used in the fabrication of orthodontic removable appliances is obtained by polymerisation of methyl methacrylate (MMA). It has been reported in many studies that residual MMA monomers can leach from the acrylic resins and that these monomers are cytotoxic, and can cause hypersensitivity and allergic reactions [Ica et al., 2014; Siqueira Goncalves et al., 2008; Kedjarune et al., 1999; Ozturk et al., 2011]. This adverse effect is not only local but also systemic, and there is a growing concern that MMA may produce genetic damage by inducing mutation [Yang et al., 2003].

In reviewing previous studies of metal ion release and MMA monomer leaching, it follows that functional appliances made of stainless steel wire and acrylic resin might also be expected to release metal ions and MMA monomers that are potentially harmful. Regarding the functional appliances used in growing children, the potentially harmful effects of such ions and monomers should not be underestimated. As a contribution to the holistic safety evaluation of functional appliances, this study was designed to evaluate the release of Ni and Cr ions and MMA monomers from the Bionator and twin-block appliance, which are believed to be the most popular types of appliance in use worldwide. *In vitro* cytotoxicity testing was also performed to analyse possible cytotoxic effect of released metal ions and MMA monomers.

Materials and methods

Twenty sets of ideal stone dental models were made from one single rubber mold. Ten sets of upper and lower dental models were mounted on verticulators with a vertical occlusal clearance of 5 mm; the other 10 sets were mounted with a 2 mm clearance. All dental models were mounted with incisor

FIG. 1 Appliances used in this study: Bionator (left) and twin-block (right).

end-on-end relative to the sagittal plane. Ten twin-block appliances (TB group) were fabricated with 5 mm occlusal clearance mounted models and 10 Bionator appliances (B group) were fabricated with 2 mm occlusal clearance mounted models. All appliances were fabricated by a single professional dental technician to approximate the same size and weight of appliances in all groups (Fig. 1). Appliance weight measurements are shown in Table 1. Stainless steel wires used were from Remanium® (Dentarum, Ispringen, Germany) and acrylic resin was from Ortho-Jet™ (Lang Dental Mfg. Co. Inc., Wheeling, IL, USA). Acrylic resin was polymerised at room temperature using the spray-on (salt and pepper) method. Compositions of the wire and resin (Table 2, 3).

To mimic the intraoral environment, artificial saliva was synthesised and used as a medium. Chemical components of the artificial saliva were adopted from a similar study [Kotyk et al., 2014] (Table 4). The chemical components were dissolved in 1 L distilled water, and an aliquot of 150 ml artificial saliva was poured into a glass jar with glass cover and silicone sealer (WECK Tulip Jar 220 ml RR60 with lids [500762], WECK, Wierden, Nederland). Glass jars, covers, and sealers were washed and sterilised with ethylene-oxide gas before use. Each Bionator and set of twin-block appliance was immersed in the artificial saliva and then the glass jar was kept in a thermal incubator (C-IN incubator, Chang-Shin Scientific Co., Seoul, Korea) at 36.5 °C. Artificial saliva only group was set as a glass jar with artificial saliva (no appliance) kept in the thermal incubator. This group was set to confirm and detect any metal ions and monomers from the jar itself and any other possible background contamination.

Ten-mililiter artificial saliva was collected from Bionator group (n = 10), twin-block group (n = 10) and artificial saliva only group (n = 1) after 7 days, 30 days and 90 days of incubation. All samples gathered at each time point were stored in glass vials and kept in a -20 °C freezer until analysis. To minimise background contamination, all instruments used to pour and measure artificial saliva were made of glassware.

TABLE 1 Appliance weights.

Appliance	Weights (gram)
Bionator	7.3 ± 0.14
Twin-block upper piece	9.4 ± 0.29
Twin-block lower piece	3.9 ± 0.29
Mean ± standard deviation	

TABLE 2 Chemical composition of wire used for fabrication of functional appliances.

C	Si	Mn	Cr	Mo	Ni	P	S	others	Fe
0.05 -0.15	≤2.0	≤2.0	16.0 -19.0	≤0.8	6.0 - 9.5	≤ 0.045	≤0.03	N ≤ 0.11	rest
In weight %									

TABLE 3 Chemical composition of resin used for fabrication of functional appliances.

Chemical name	CAS No	Weight %
Methyl Methacrylate	80-62-6	> 95
N, N-Dimethyl-p-Toluidine	99-97-8	< 2
In weight % *CAS No: Chemical Abstracts Service Registry Number*		

TABLE 4 Chemical components of artificial saliva used in this study.

Component	Concentration (g/L)
Potassium chloride (KCl)	1.044
Monosodium phosphate (NaH2PO4)	0.681
Sodium bicarbonate (NaHCO3)	0.420
Calcium chloride (CaCl2)	0.033
Magnesium chloride (MgCl2)	0.006

Detection of Ni and Cr ions

To determine the concentration of Ni and Cr ions released into the artificial saliva, inductively coupled plasma mass spectrometry (ICP-MS) analysis was performed. A 2-millilitre artificial saliva sample was taken, to which 3 ml 70% nitric acid (Dongwoo Fine-Chem Co., Pyungtaek, Korea) and 2 ml chloric acid (Dongwoo Fine-Chem Co.) were added. After digestion, the samples were diluted with distilled water to a final volume of 40 ml and analysed using an ICP-MS spectrometer (Agilent 7700x ICP-MS, Agilent Technologies, Santa Clara, CA, USA) with quadrupole mass spectrometer and dual-mode discrete dynode electron multiplier detector. A standard calibration curve was made using standard solutions of Ni and Cr in known concentrations of 5, 10, and 50 parts per billion (ppb) (Figure 2). The limit of detection for both Ni and Cr ion concentrations was 1 ppb, equivalent to 1 µg/L.

Detection of MMA monomers

For quantification of released MMA monomer in the artificial saliva, gas chromatography/mass spectrometry (GC/MS) was adopted as the analytical method. One ml of artificial saliva sample was mixed with 1 ml of ethyl acetate and vortexed for 1 hour. After shaking, the supernatant was collected and filtered through a 0.45 µm polytetrafluoroethylene syringe. A GC/MS system of Agilent 7890A GC System with Agilent 5975C inert MSD (Agilent Technologies) was used to determine the levels of MMA monomer. Agilent HP-5 MS/UI (Agilent Technologies, 30 m * 0.25 mm * 0.25 µm) column was used at a column flow of 1.0 ml/min with helium gas carrier. Temperature conditions

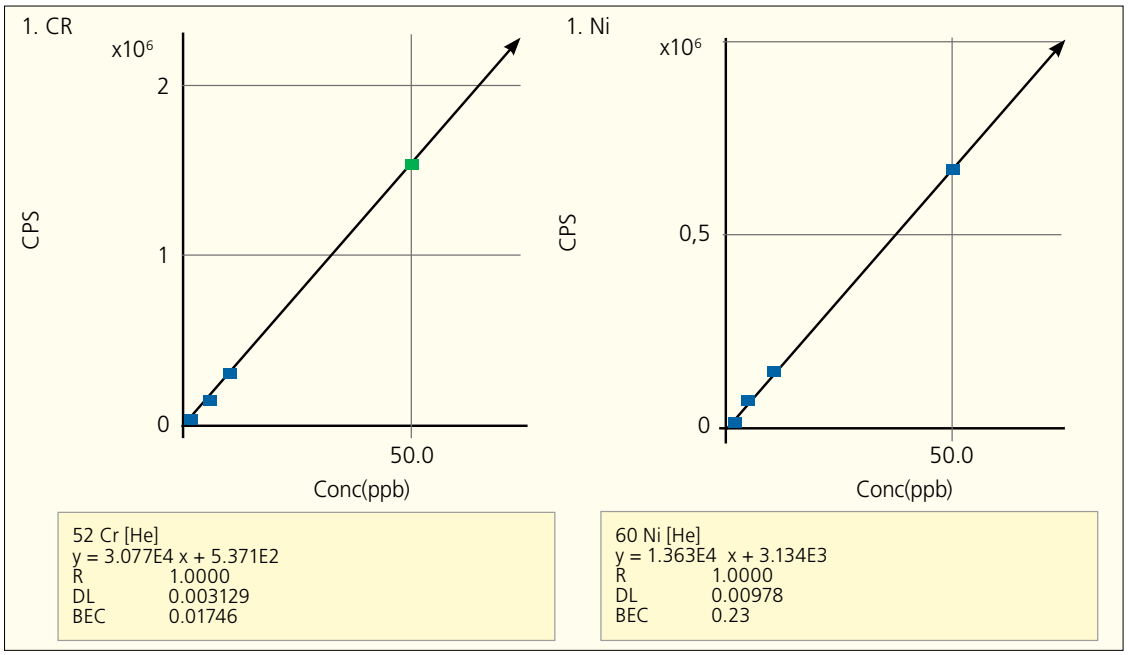

FIG. 2 ICP-MS standard calibration curve for nickel (left) and chromium (right) ion concentration.

were: isothermal for 5 min at 33 °C, increased by 25 °C/min to 250 °C, then isothermal for 3 min at 250 °C. The peak was detected in selected ion monitoring (SIM) mode of m/z 69 and m/z 100. The limit of detection of MMA in GC/MS was 0.1mg/ml (0.1 part per million, ppm). MMA standard solutions were prepared by diluting known amounts at various concentrations from which a standard calibration curve was prepared (Fig. 3).

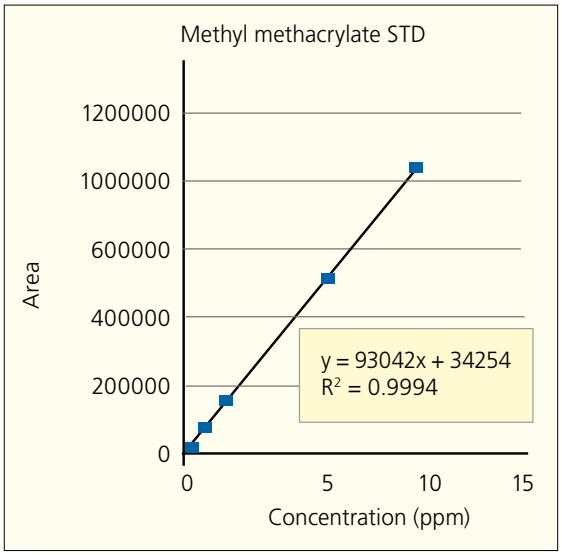

FIG. 3 GC/MS standard calibration curve for MMA monomer concentration.

Cytotoxicity assay

Cytotoxicity of the functional appliance immersed in artificial saliva soup was evaluated by the 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Ninety-day functional appliance soups (Bionator and twin-block groups) were used, with one control group using artificial saliva (no appliance) and a negative control using phosphate buffered saline (PBS pH 7.4; no appliance). In this study, human gingival fibroblasts (hGF, primary cell line) and HeLa cells (human cervical cancer epithelial cell line) were used for cytotoxicity evaluation. HeLa cells were used for evaluation of possible estrogenic effect of released MMA monomer.

Cytokine array

Again, ninety-day functional appliance soups (Bionator and twin-block groups) were used, with one control group using artificial saliva (no appliance) and a negative control using phosphate buffered saline (PBS pH 7.4; no appliance). Human gingival fibroblast cell was cultured for 3 days and harvested to undergo cytokine analysis with a human cytokine array kit (Proteome Profiler™ Array, catalogue number ARY005B, R&D systems, Inc., Mineapolis, MN, USA). The cytokine array kit used can detect 36 human cytokines including inflammatory cytokines: C5a, CD40 Ligand, G-CSF, GM-CSF, CXCL1/GRO alpha, CCL1/I-309, ICAM-1, IFN-gamma, IL-1 alpha, IL-1 beta, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-16, IL-17, IL-17E, IL-21, IL-27, IL-32 alpha, CXCL10/IP-10, CXCL11/I-TAC, CCL2/MCP-1, MIF, MIP-1 alpha/MIP-1 beta, CCL5/RANTES, CXCL12/SDF-1, Serpin E1/PAI-1, TNF-alpha, TREM-1.

Statistical analysis

Statistical analysis was performed using SPSS version 12.0 (SPSS, Chicago, IL, USA). The results were analysed by two-way repeated measure ANOVA and the difference within (time related comparing) group was performed with Tukey test (equal variance) and Dunnett's T3 test (unequal variance) as post hoc analysis. Mann-Whitney U test was used to compare metal ions and MMA concentrations between the Bionator and twin-block groups (intergroup comparing) at same time period. Also amount of MMA monomer release per gram weight of appliance was calculated and compared between the Bionator and twin-block groups using Mann-Whitney U test. MTT assay results were compared with one-way ANOVA and Tukey test was used as post hoc analysis.

Results

Metal ions detection did not appear in a uniform manner. Ni and Cr ions were detected in some samples but not in others in the same group. In many cases, they did not show time-dependent concentration changes, and in some cases, metal ions detected in earlier sample and later sample revealed to be undetectable. While it was established that detectable Ni

Group		MMA concentration (mg/L)	MMA constituent (mg)
Control	Day 7	0.4	0.06
	Day 30	0.1	0.02
	Day 90	0.5	0.08
Bionator	Day 7	0.6 ± 0.20 a	0.09 ± 0.03 a
	Day 30	2.2 ± 1.01 b	0.31 ± 0.14 b
	Day 90	4.4 ± 3.66 b	0.58 ± 0.48 b
Twin-block	Day 7	1.8 ± 1.31 a	0.27 ± 0.20 a
	Day 30	7.3 ± 4.34 b	1.02 ± 0.61 b
	Day 90	8.8 ± 4.01 b	1.15 ± 0.52 b

*Two-way repeated measure ANOVA with Tukey test and Dunnet's T3 test was used. *p <0.05 was considered as statistically significant difference. Same superscript alphabet indicates statistically not significant different.*

TABLE 5 MMA monomer concentration and constituent level detected using GC/MS.

	Bionator (mg)	Twin-block (mg)	p-value
Day 7	0.09 ± 0.03	0.27 ± 0.20	0.012*
Day 30	0.31 ± 0.14	1.02 ± 0.61	0.004*
Day 90	0.58 ± 0.48	1.15 ± 0.52	0.019*

*Two-way repeated measure ANOVA with Mann Whitney U test was used. * p <0.05 was considered as statistically significant difference.*

TABLE 6 Analysis of MMA monomer constituent (mg) between Bionator and twin-block group using Mann Whitney U test.

	Bionator (mg/g)	Twin-block (mg/g)	p-value
Day 7	0.013 ± 0.004	0.019 ± 0.013	0.225
Day 30	0.043 ± 0.019	0.071 ± 0.042	0.059
Day 90	0.079 ± 0.065	0.080 ± 0.036	0.149

** p <0.05 was considered as statistically significant difference.*

TABLE 7 Comparison of MMA monomer constituent (mg) released per weight gram of the appliance (mg/g) between Bionator and twin-block group using Mann-Whitney U test.

Group	hGF	HeLa	
1 day culture	PBS	0.51 ± 0.04a	0.43 ± 0.02a
	Con	0.50 ± 0.03a	0.41 ± 0.01a
	Bionator	0.48 ± 0.02a	0.40 ± 0.02a
	Twin-block	0.49 ± 0.02a	0.39 ± 0.02a
2 day culture	PBS	0.67 ± 0.01a	0.54 ± 0.02a
	Con	0.64 ± 0.02a	0.53 ± 0.02a
	Bionator	0.65 ± 0.01a	0.51 ± 0.01ab
	Twin-block	0.64 ± 0.01a	0.48 ± 0.02b
3 day culture	PBS	0.74 ± 0.01a	0.62 ± 0.02a
	Con	0.72 ± 0.01a	0.58 ± 0.02ab
	Bionator	0.68 ± 0.01b	0.56 ± 0.01bc
	Twin-block	0.68 ± 0.02b	0.55 ± 0.02bc

TABLE 8 Results of MTT assay (540 nm absorbance level) of hGF and HeLa cell and intergroup comparison using one-way ANOVA and Tukey test.

and Cr ions were being released from the appliances tested, a clear pattern for metal ion release was not determined.

Both Bionator and twin-block appliance groups showed a tendency toward time-dependent increase in MMA monomer concentration in the saliva soup (Table 5). Both soups contained a statistically significantly higher level of MMA constituent (mg) at day 30 compared with day 7; however, there was no difference between the levels at day 30 and day 90, despite the higher mean value at day 90 (Table 5). The artificial saliva only group showed a minimal level of MMA monomer concentration indicating background contamination, likely originating from the commercial bottle containing the artificial saliva chemical components. This type of background contamination could not be controlled and may be considered insignificant based on the very low level compared to the appliance groups. A comparison between the Bionator and twin-block groups showed statistically significant higher levels of MMA monomer in the twin-block group at all three time points (Table 6). However, MMA monomer (mg) released per weight (gram) of appliances did not show significant difference among the appliances (Table 7).

Results of the MTT assay revealed cellular toxicity from the 90-day soup made from both Bionator and twin-block appliances (Table 8, Fig. 4). After 3 days of incubation with functional appliance soups, hGF cells showed reduced absorbance, while HeLa cells showed reduced absorbance after 2 days of incubation (Table 8, Fig. 4). Also among the hGF cultures, the negative control (PBS) and control group both showed a time-dependent increase in absorbance level; because the Bionator and twin-block groups showed no difference between days 2 and 3 of incubation, this indicated that hGF cellular activity and proliferation was retarded after 2 days of incubation (Fig. 5). Unlike hGF, HeLa cells showed a time-dependent increase in absorbance level in all four mediums tested (Fig. 6) while Bionator and twin-block soup showed reduced absorbance level than the PBS and control medium.

Cytokine array showed negative signals for the most cytokines but for the CXCL12/SDF-1, Serpin E1/PAI-1 and MIF (Fig. 7). All 3 cytokines showed higher value in the PBS and control group. Cytokine array was not duplicated and therefore statistical analysis was impossible. However, at least, 90-day soup does not elicit inflammatory reaction to the human gingival fibroblasts.

Discussion

Based on previous studies reporting predictable Ni and Cr release from stainless steel orthodontic metal brackets and wires, it was surprising that this study did not reveal a clear pattern of time-dependent increase in concentration of metal ions into artificial saliva. The detectable-undetectable mixed result may have been due to re-adsorption of metal ions to the MMA resin or to the wall of the glass vials. However, to the best of the author's knowledge, there have been no reports on metal ion adsorption to dental resin that could support this hypothesis. Nor have there been other reports on the detection of metal ions released from metal and MMA resin complexes like those shown in this study. Instead, authors could find articles describing chelating resin adsorb metal ions [Kawamura et al., 1993; Urano et al., 1981]. In addition, several articles describing the chelation activity of copper and gallium ions with other metal ions of MMA could be found [Varadharaj et al., 1996; Crawford et al., 2015; Janus et al., 1992]. It is not clear whether dental MMA resin has chelation capability, but

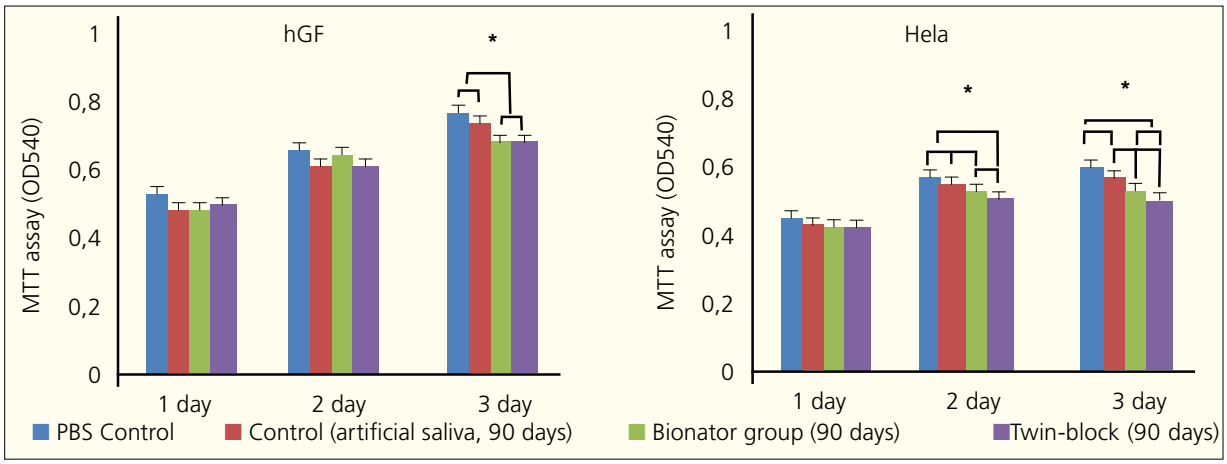

FIG. 4 MTT assay result and comparison upon different medium. * indicates statistical significant difference between the groups.

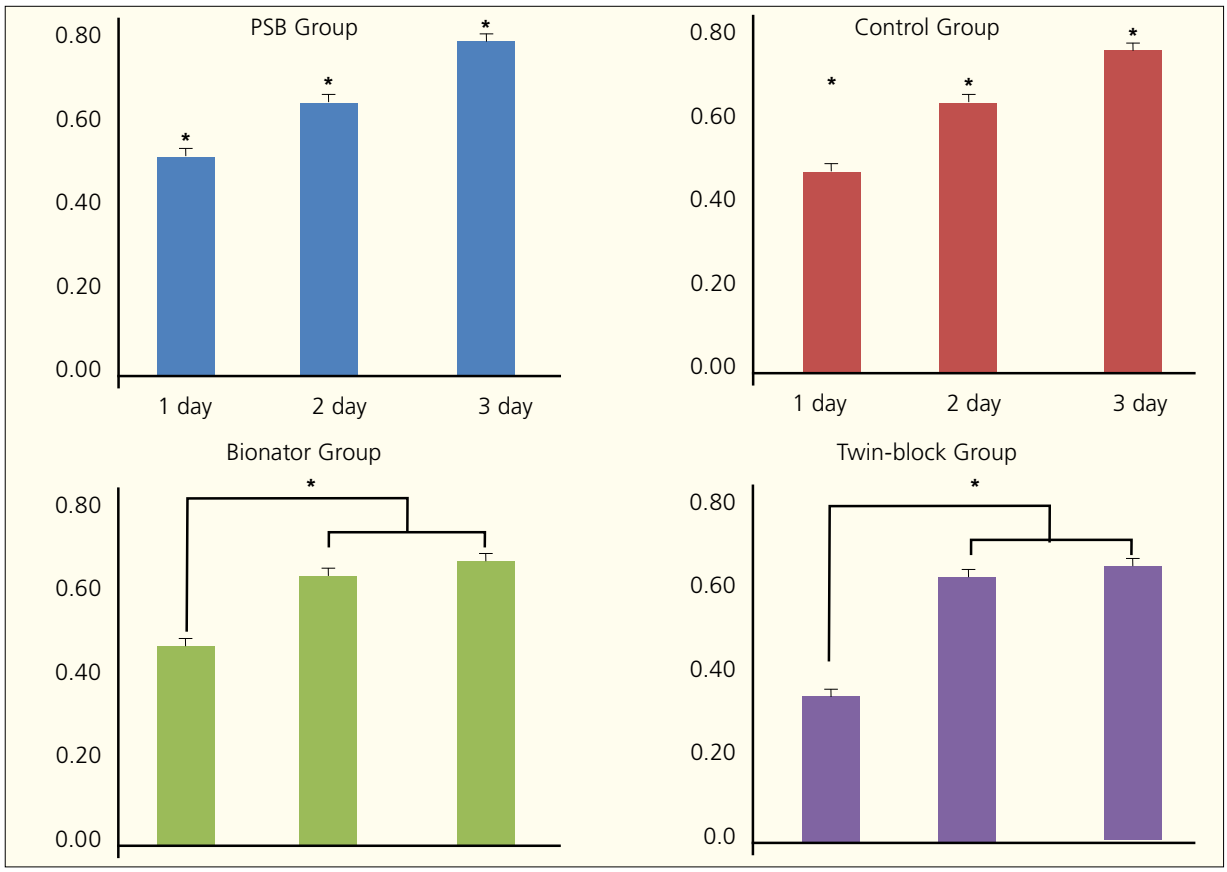

FIG. 5 MTT assay results of hGF according to culture duration. * indicates statistical significant difference between the groups.

chelation would help explain the results of this study, i.e. metal ions released from functional appliances, which are a complex of stainless steel and MMA resin, may be re-adsorbed by the MMA, thereby reducing systemic ingestion. This hypothesis requires further study.

Time has been shown to be an important factor in MMA monomer release. Ica et al. found that the monomer release rate was higher in the first 24 hours than at the 1-week and 3-month time points [Ica et al., 2014]. The results of this study showed a statistically significant increase in released MMA monomer into artificial saliva between day 7 and day 30 in both Bionator and twin-block groups, but the trend did not continue between day 30 and day 90 in either group. This result supports the notion that the rate of MMA monomer release peaks early on and decreases over time. As mentioned above, previous report indicate that monomer release peaks in the first 24 hours, but the current study result indicates a peak sometime between day 7 and day 30. These discrepancies in reporting may be the result of differences between MMA resins tested as well as other factors such as the medium used, incubation temperatures, and so on. More studies are required to fully understand the variables impacting MMA monomer release.

Acrylic resin thickness can influence residual monomer levels. Unlike most other studies investigating monomer release from MMA resins, in which acrylic resin discs were fabricated with various diameters and thicknesses, this study tested an appliance form that is used in everyday orthodontic therapy and as such aimed to mimic actual *in vivo* conditions. Thickness of the MMA

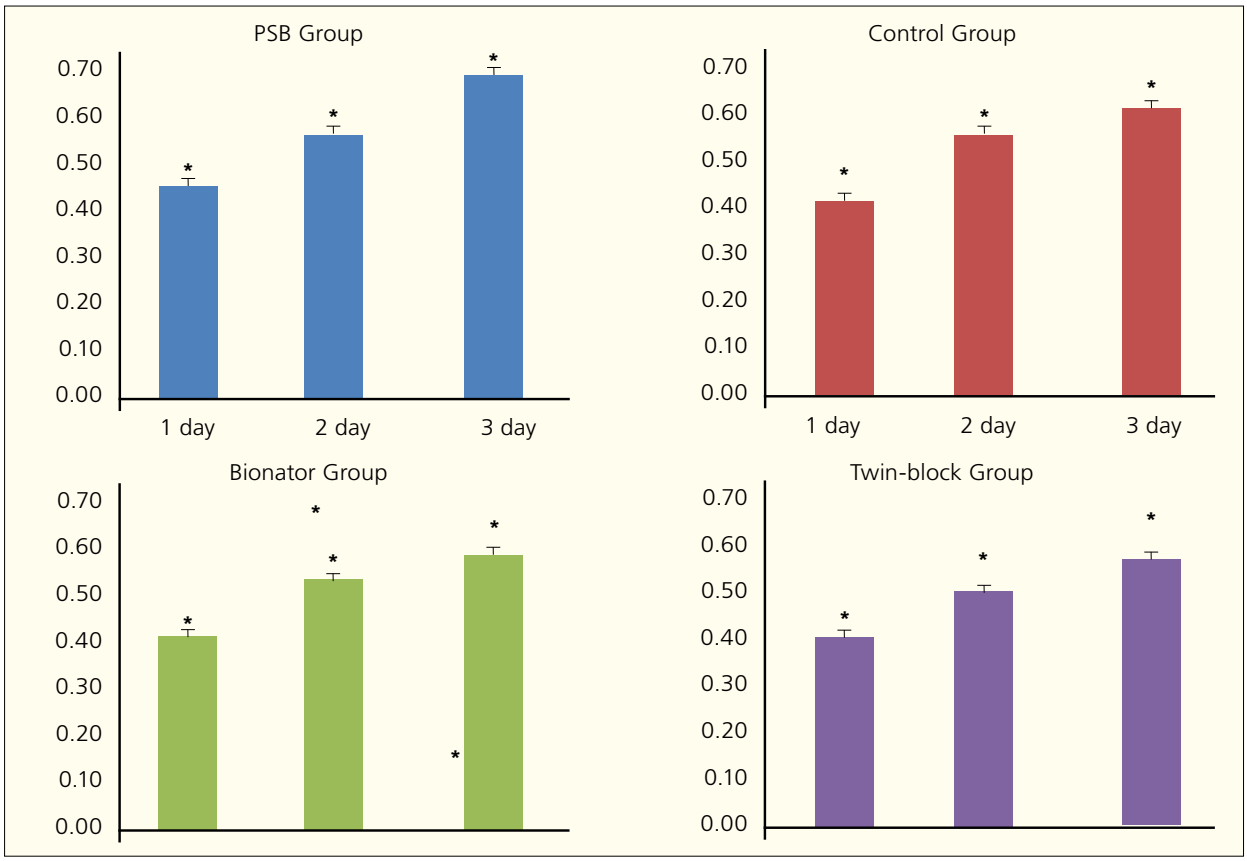

FIG. 6 MTT assay results of HeLa cell according to culture duration. * indicates statistical significant difference between the groups.

resin varies across the different parts of a single appliance and therefore uniform thickness of MMA resin does not reflect the monomer release from the appliance. The difference in MMA monomer release between the Bionator and twin-block groups can be explained by different resin thickness, different resin surface areas and different amounts of resin used. The twin-block appliance uses more resin overall as reflected by the weight of the appliance, as well as a thicker resin due to the greater occlusal clearance distance, and full coverage of the palatal surface creates a wider surface area compared with the Bionator appliance, resulting in statistically greater release in MMA monomer. However, amounts of MMA monomer released per weight gram of appliance were not different and this result can be interpreted as amount of resin used is the key factor for how much MMA monomer released from a specific appliance. Cytotoxicity of functional appliances immersed in artificial saliva was confirmed here by MTT assay. Evidence of cytotoxicity was minimal and regarding the 90 day accumulated artificial saliva was tested, which is not likely to approximate *in vivo* conditions, the actual toxicity of a functional appliance might be much lower or even negligible. According to Slatten and Dahl's classification of cytotoxicity, the current result of less than 10% decrease in OD540 absorbance can be classified into "slight" cytotoxic which is less than 30% [Sletten and Dahl, 1999]. Also the cytokine array results showed no specific inflammatory reaction of the hGF cells after 3 day culture with 90-day soup.

World Health Organization (WHO) reported that the acute toxicity of MMA is low and tolerable daily intake level for an adult is 1.2 mg/kg/day [WHO, 1998]. If we apply this standard to functional appliances, which are usually applied to children at age 10-12 years, when the typical body weight for boys and girls is approximately over 32 kg, a simple calculation using the data presented herein will show that MMA monomer release from the functional appliance is far lower than the daily tolerable level. One should remind that the WHO reported tolerable level are intended for adults and therefore cannot be applied to growing children and should be approached more conservatively. Even if this is taken into account, the amount of MMA shown in the current study (0.58 ± 0.48 mg for Bionator and 1.15 ± 0.52 mg for twin-block after 90-day) are very small.

It is possible that MMA monomers and metal ions are released at higher levels in the *in vivo* oral cavity than the *in vitro* experiments shown here. Extreme changes in pH and temperature affect corrosion of the metal and degradation of MMA resin. In addition, mechanical stress from the occlusion force would accelerate reactions. Absence of the complex intraoral flora and accumulation of plaque and its byproducts further distinguish the *in vitro* from *in vivo* condition. It is quite likely that higher levels of MMA monomers and metal ions are released in the oral cavity, but would still be expected at far lower levels than the daily tolerable intake. Furthermore, human *in vivo* oral tissue cells might be more resistant to chemicals than *in vitro* cells because they are continuously supplied with nutrients through abundant blood vessels. MMA monomers that are absorbed into the human body are rapidly metabolised to methacrylic acid and excreted without accumulation. Therefore, total MMA monomer released from the functional appliance for a year (which is the general treatment period for functional orthopedic treatment) might be larger than that measured here, but not expected to have more than a negligible impact on health. However, there still exists possibility of allergic and hypersensitivity reaction to those released monomer.

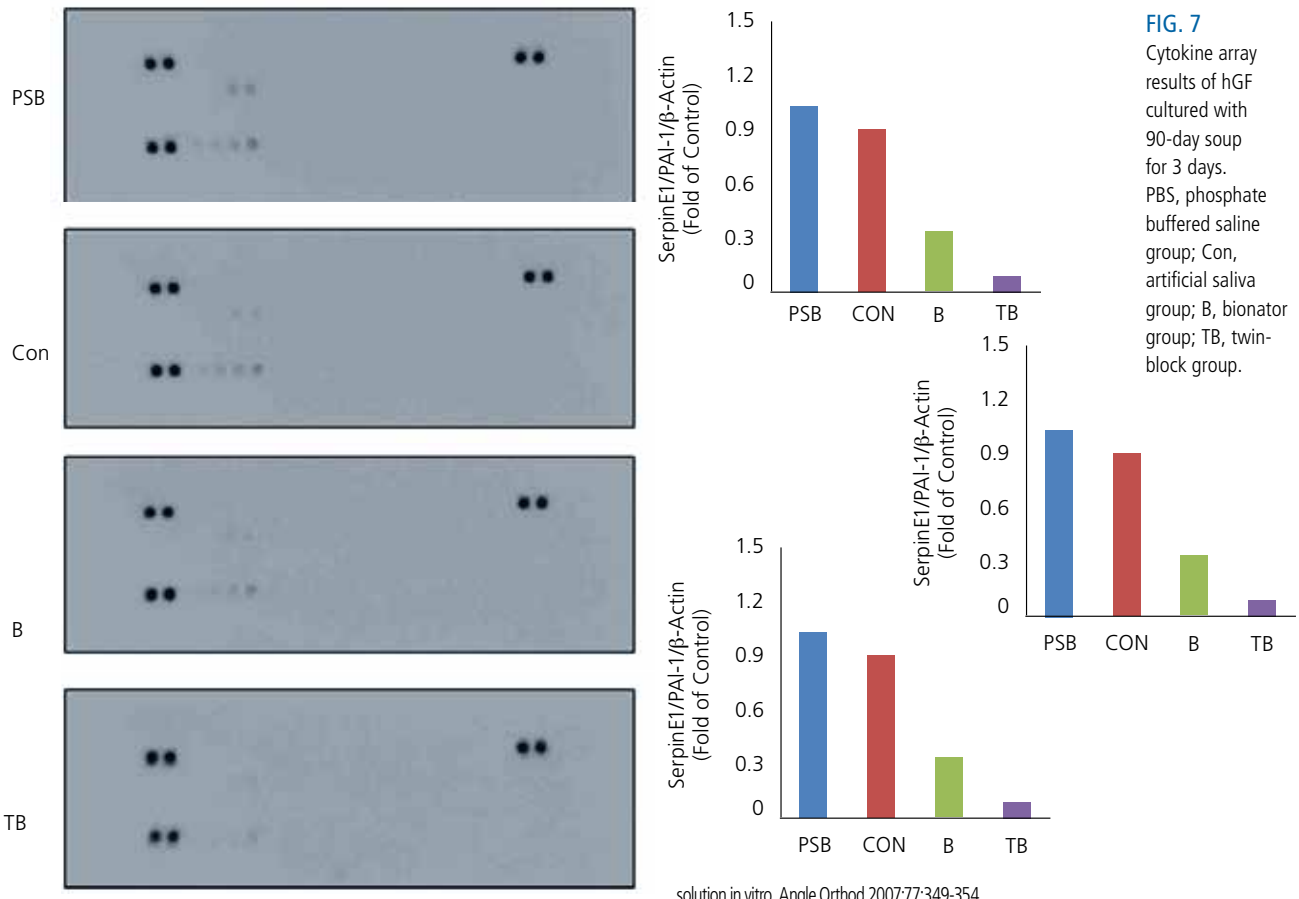

FIG. 7
Cytokine array results of hGF cultured with 90-day soup for 3 days. PBS, phosphate buffered saline group; Con, artificial saliva group; B, bionator group; TB, twin-block group.

Conclusions

Ni and Cr ion release from Bionator and twin-block functional appliances was not clear and its concentration could not be obtained. MMA monomer release was confirmed and a time-dependent increase until around 30 days of immersion was observed. Cytotoxicity of the appliance-immersed artificial saliva was shown but the level of cytotoxicity was minimal. These results suggest that the cytotoxic effect of functional appliance material release is minimal or negligible. Taken with the WHO reported tolerable daily intake level of MMA, general toxicity of the functional appliance from the MMA monomer release is likely to be minimal or negligible.

Acknowledgement

We thank professor Suk-Won Lee for providing primary human gingival fibroblast cells.

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