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In-vitro Assessment of Cytotoxicity from Metal Ion Release in Corroding Fixed Orthodontic Appliances

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Abstract

Aim: Metallic corrosion of orthodontic appliances occurs due to potentially damaging physical and chemical agents. The major corrosion products of stainless steel are iron, chromium, and nickel. When tissues are exposed to certain concentrations of a primary irritant for certain periods, cytotoxic effects may be observed. Hence, this study aimed to assess the cytotoxicity of nickel, iron, and chromium ions during fixed orthodontic treatment onto human keratinocytes (HaCaT).
Methods: The metal ions nickel, chromium, and iron in concentrations of 75, 150, 300, 600, 1200, and 2400 µg/ml were prepared to assess the cytotoxicity on HaCaT cells. The control group included these metal ions at 0 µg/ml. Cytotoxicity was assessed by microculture tetrazolium (MTA) assay. The half maximal inhibitory concentration (IC50) was measured and a scanning electron microscope (SEM) was used to assess the morphological changes of the HaCaT cells. The Kruskal-Wallis test followed by the Bonferroni's post hoc test was carried out to determine the difference in percentage inhibition within and between the groups at various concentrations.
Results: Nickel showed the highest cytotoxic effects in comparison with other metal ions. Iron hexahydrate, nickel hexahydrate, and chromium hexahydrate have shown an IC50 value of 552.4 µg/ml, 364.1 µg/ml, and 641.1 µg/ml inhibition in HaCaT cells respectively. Cytotoxic effects were dose dependent on the tested materials. Comparison of percentage inhibition between groups showed a p-value of 0.372.
Conclusion: The present study showed that the nickel, chromium, and iron ions induced a wide range of toxicity to human keratinocytes. The IC50 values ranged between 364.1 and 641.1. Nickel was the most toxic metal tested between the concentrations of 75 to 2400 µg/ml for HaCaT cells compared to other metal ions used in the study.

Keywords: Metal Ions, Immunologic Cytotoxicity, Corrosion, Hypersensitivity, Orthodontics

Background

Metals are a crucial part of orthodontic appliances and are exposed to physical and chemical factors that might be harmful. Due to the release of ions generated by the abrasion of toothbrushes, meals, or drinks, these substances induce metallic corrosion that happens continually

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in the mouth. Chromium, nickel, and iron are the main corrosion products of stainless steel with the potential to cause allergic, poisonous, or cancerous responses (1).

It has been demonstrated that even while orthodontic appliances are only used for a short time, the oral environment can cause their structures to corrode quickly (2). The likelihood that an alloy may produce allergic responses depends on the pattern and method of this corrosion. The oral cavity creates an optimal environment for the biodegradation of these materials due to its unique physiological, temperature, microbiological, and biochemical features (3). Electrochemical corrosion happens because of its moist environment, and a metal in an aqueous solution will be thermodynamically unstable because it tends to change from a solid state to an ionic form, which is accompanied by a loss of energy and releases metal ions into the solution (4).

The relationship between the biocompatibility of orthodontic materials and the potential dose of toxic elements released from orthodontic appliances and the response of an organism, the effect of treatment time and the type of appliance used on the amount of released metal ions because of corrosion need to be evaluated (5). Cytotoxic effects result when tissues are exposed to a sufficient concentration of a primary irritant for a sufficient period (6, 7).

The stainless-steel appliances used in orthodontics typically contain 18% chromium and 8% nickel while the nickel titanium memory wires may contain up to 70% nickel. The release of these components into the surrounding tissue may cause adverse local reactions or even systemic effects (8). Nickel is a metal widespread in the environment and an essential nutrient, but it is also a very common contact allergen. Nickel allergy occurs more frequently than allergies to other metals (2,9). According to some individualized reports, the insertion of NiTi wire alloys may occasionally lead to the formation of rashes, swelling, and painful erythematous lesions in the oral and labial mucosa (10). An allergic response is one in which certain components of the immune system react excessively to a foreign substance. The inflammatory response induced by corrosion of orthodontic appliances and subsequent release of nickel is associated with various reactions and clinical abnormalities such as gingivitis, gingival hyperplasia, lip desquamation, multiform erythema, burning sensation in the mouth, metallic taste, angular cheilitis, and periodontitis. This is manifested as Nickel Allergic Contact Stomatitis (NiACS) (11, 12).

For several years, experimental studies in orthodontics have attempted to define the mechanical properties of various components of orthodontic appliances to improve bracket and orthodontic cement and shear bond strength, as well as to reduce friction of wires and brackets, increase force of elastics and achieve several other improvements. However, adverse reactions of the oral soft tissues have raised the interest of researchers in determining the biological effects of these materials, that is, their biocompatibility. Hence, the aim of this study was to assess the cytotoxicity of nickel, iron, and chromium ions during fixed orthodontic treatment.

The aim of this study was to evaluate the cytotoxicity of nickel, iron, and chromium ions at concentrations of 75–2400 µg/ml on human keratinocyte (HaCaT) cells using the MTT assay and SEM analysis.

Methods

For the present in-vitro study, the nickel, iron, and chromium ion salts were obtained from a chemistry laboratory, and the cytotoxicity was assessed at Skanda Life Sciences Private Limited (Bangalore, India).

Sample preparation (obtained from Skanda Life Sciences Pvt. Ltd., Bangalore, India):

I. Metal ions:
   1) Nickel chloride hexahydrate
   2) Chromium chloride hexahydrate
   3) Ferric chloride hexahydrate

These metal ions were tested with varying concentrations of 75, 150, 300, 600, 1200, and 2400 µg/ml HaCaT

II. The control group included these samples tested at concentrations of 0 µg/ml HaCaT
III. MTA powder (the solution was filtered through a 0.2 µ m filter and stored at 2–8°C for frequent use or frozen for extended periods)
IV. Dimethyl sulfoxide (DMSO)
V. CO2 incubator
VI. SpectraMax i3X Plate reader (Molecular Devices, San Jose, CA, USA)

Cell culture:

For cytotoxicity studies, serial two-fold dilutions (0-100 µM or 320 ug/ml) were prepared from this to carry out cytotoxic studies.

Implementation of samples into cell culture

(obtained from Skanda Life Sciences Pvt. Ltd., Bangalore, India):

All the cell lines were procured from American Type Culture Collection (ATCC). Stock cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO2 at 37°C until confluent. The cell was dissociated with cell dissociating solution (0.2% trypsin, 0.02% ethylenediaminetetraacetic acid, 0.05% glucose in phosphate buffer saline). The viability of the cells was checked and centrifuged. Furthermore, 50,000 cells/well was seeded in a 96-well plate and incubated for 24 hours at 37°C, in a 5% CO2 incubator.

Methodology

The monolayer cell culture was trypsinized and the cell count was adjusted to 1x105 cells/ml using respective media containing 10% FBS. To each well of the 96-well microtiter plate, 100 µl of the diluted cell suspension (50,000 cells/well) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once with medium and 100 µl of different test concentrations of iron hexahydrate, Nickel hexahydrate and chromium hexahydrate were added on to the partial monolayer in the microtiter plates. The percentage growth inhibition was calculated using the formula [% of inhibition=(OD of control–OD of sample)/OD of control) x 100], and the concentration needed to inhibit cell growth by 50% (IC50) values were generated from the dose-response curves for each cell line.

MTA Assay

The MTA method is simple, accurate, and yields reproducible results. The key component is (3- [4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTA, which is a water-soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTA is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells. This water-insoluble formazan can be solubilized using ethanol. The resulting purple solution is spectrophotometrically measured. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of effects caused by the test material. The results of the MTA assay are read with a plate reader at a wavelength of 590 nm.

IC50 Value

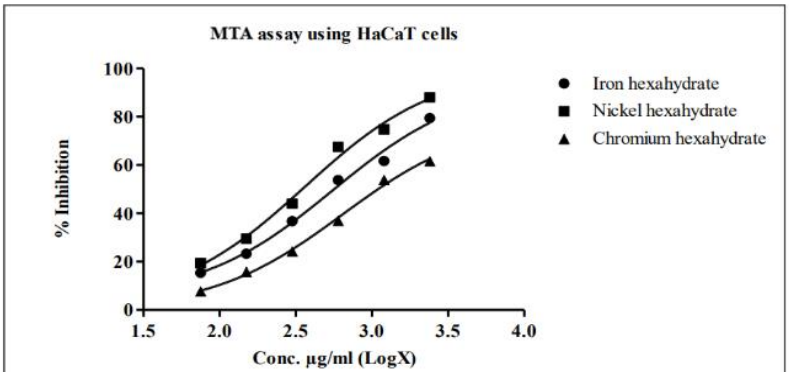
The IC50 of a drug is determined by constructing a dose-response curve and examining the effect of different concentrations of the antagonist on reversing agonist activity. IC50 values are calculated for a given antagonist by determining the concentration needed to inhibit half of the maximum biological response of the agonist. IC50 values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on the sigmoid dose response curve (variable) and computed using the software Graph Pad Prism 6 (Graph Pad, San Diego, CA, USA)

Statistical analysis:

Statistical analysis was performed using the software SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA). The descriptive statistics were presented as mean±standard deviation. The Kruskal-Wallis test followed by the Bonferroni's post hoc test was carried out to determine the difference in percentage inhibition within and between the groups at various concentrations.

Results

The results of the MTA assay are shown in Table 2 for all the given metal ions used in the study. Metal ions induced reproducible toxic effects in HaCaT cells. Cytotoxic effects were dose dependent on the tested material. There was a significant difference between the cytotoxic effects (IC50 values) of nickel, chromium, and iron ions.



	Iron hexahydrate	Nickel hexahydrate	Chromium hexahydrate
IC50	552.4	364.1	641.1

Figure 1. MTA analysis for nickel, chromium, and iron ions

HaCaT				
Compound name	**Conc. µg/ml**	**OD at 590nm**	**% Inhibition**	**IC50 µg/ml**
Control	0	0.742	0.00	
Iron hexahydrate	75	0.628	15.36	**552.4**
	150	0.569	23.32	
	300	0.469	36.79	
	600	0.343	53.77	
	1200	0.284	61.73	
	2400	0.152	79.47	
Nickel hexahydrate	75	0.598	19.41	**364.1**
	150	0.523	29.51	
	300	0.415	44.07	
	600	0.241	67.52	
	1200	0.187	74.80	
	2400	0.089	88.01	
Chromium hexahydrate	75	0.685	7.68	**641.1**
	150	0.625	15.77	
	300	0.562	24.26	
	600	0.468	36.93	
	1200	0.342	53.91	
	2400	0.285	61.59	

Figure 2. The concentration-dependence of mitochondrial dehydrogenase activity

The percentage inhibition of various metal ions at increasing concentrations can be observed in Fig. 2. It shows the concentration dependence of mitochondrial dehydrogenase activity (MTA) for the HaCaT cell line for 24 hours.

Based on the results of the current study, it can be observed that nickel ion is the most cytotoxic among the three metal ions used. The IC50 values for iron hexahydrate, nickel hexahydrate, and chromium hexahydrate inhibition in HaCaT cells were 552.4 µg/ml, 364.1 µg/ml, and 641.1 µg/ml, respectively.

Table 1 illustrates the percentage inhibition concentrations between groups at various concentrations. Among nickel, chromium, and iron at a concentration of 75 µg/ml, the average percentage inhibition is 14.15. Similarly, at a concentration of 2400 µg/ml, the average percentage inhibition is 76.36.

The Kruskal-Wallis test, followed by the Bonferroni's post hoc test was carried out to determine the difference in percentage inhibition within and between the groups (iron hexahydrate, nickel hexahydrate, and chromium hexahydrate) at various concentrations. Statistical significance was set at $p \leq 0.05$ and the confidence interval was set as 95%. Thus, regardless of concentration, Table 2 indicates no statistical difference between the percentage inhibition of all three metal ions.

Figure 3-10 shows the microscopic changes of the control group and nickel, chromium, and iron ions at different concentrations. These metal ions induced morphological changes in the HaCaT cell line, which is concentration-dependent. Thus, increased concentrations show significant changes under the microscope.

Table 1. Table 1. Mean percentage inhibition (±SD) of HaCaT cells at different metal ion concentrations

Concentration	Mean	SD
75 µg/ml	14.15	5.96
150 µg/ml	22.87	6.88
300 µg/ml	35.04	10.02
600 µg/ml	52.74	15.32
1200 µg/ml	63.48	10.55
2400 µg/ml	76.36	13.48

Table 2. Comparison of percentage inhibition between groups (materials)

Group	Mean ± SD	Test Statistic	p-value
Iron hexahydrate	45.07 ± 24.33	1.977	0.372
Nickel hexahydrate	53.89 ± 27.08		
Chromium hexahydrate	33.35 ± 21.37		



Figure 3. human keratinocytes

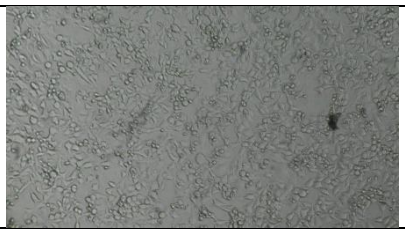


Figure 4. human keratinocytes

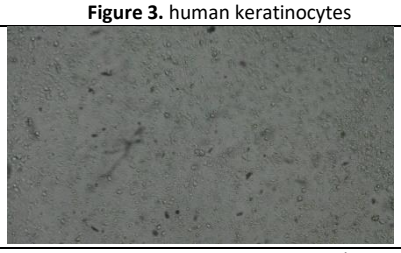


Figure 5. Nickel chloride (75 µg/ml)

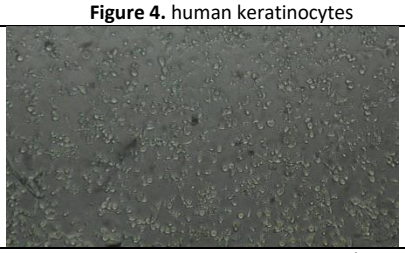


Figure 6. Nickel chloride (2400 µg/ml)

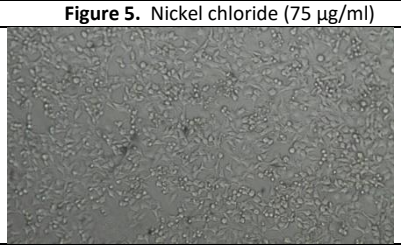


Figure 7. Chromium chloride (75 µg/ml)

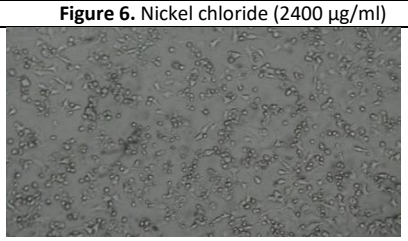


Figure 8. Chromium chloride (2400 µg/ml)

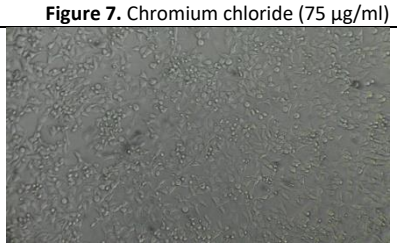


Figure 9. Ferric chloride (75 µg/ml)

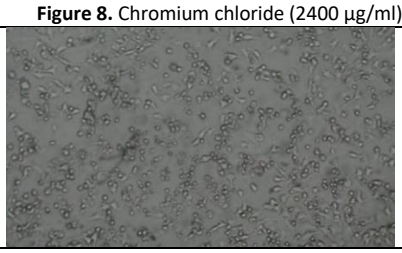


Figure 10. Ferric chloride (2400 µg/ml)

Discussion

According to the current literature, orthodontic bracket corrosion has been found to influence orthodontic therapy by increasing surface roughness, which affects sliding mechanics by increasing friction, plaque accumulation, and metal ion release, potentially with a toxic effect. During orthodontic therapy, orthodontic brackets are exposed to harsh intraoral environmental challenges such as humidity, pH, temperature

changes, mechanical stresses, and plaque buildup. Due to frictional forces and corrosion processes, they could cause the metals to degrade (13). Iron, chromium, and nickel are the primary corrosion products of stainless steel and thus corrosion of the bracket can result in uptake of these metal ions into the body (1). As a result, all these elements have the potential to cause adverse effects, such as allergic, toxic, or carcinogenic reactions (1).

In an in vivo study conducted on the evaluation of genotoxicity and cytotoxicity of metal ions, it was revealed that the buccal mucosa cells of patients treated with fixed orthodontic equipment exhibited large increases in nickel, chromium, iron, as well as considerable DNA damage and a decrease in cellular viability (14).

According to Issa's research, nickel is more cytotoxic than chromium at concentrations of 817.5 on human oligodendroglial cells and 827.9 on human gingival fibroblasts (15, 16). Human gingival fibroblasts suffered severe morphological changes when exposed to ions produced by nickel and chromium alloys, (15, 16). For this investigation, we used a human keratinocyte (HaCaT) cell line because keratinocytes are the most prevalent cell type in the epidermis and are biologically relevant targets for surfactants once they pass through the stratum corneum. As a result, there is a strong relationship between metal ion release, the shape of normal cells, and cytotoxicity. The MTA results of the current study demonstrated that metal ions can cause variable levels of reproducible cytotoxicity in HaCaT. This study shows that the attack of cytotoxic substances changes the structure of the cells, and these changes can be seen using electron microscopy.

In a previous study, cytotoxicity and apoptosis induced by mercuric chloride (HgCl2) on the human oligodendroglia MO3.13 cell line were studied (17). It has been shown that HgCl2 can induce apoptotic cell death in MO3.13 cells at concentrations of 25M and lower when applied for 24 hours. However, there is a lack of data in the literature that shows cell viability and cell death with various concentrations of metal ions. Hence, this study was conducted using increasing concentrations of metal ions (75 µg/ml to 2400 µg/ml) to assess the cytotoxicity on HaCaT cell lines.

As a consequence of the findings of this investigation, it is possible to conclude that different concentrations of the test substances generated diverse percentage inhibition values, as well as variations in their optical densities. The IC50 of nickel hexahydrate seems more toxic at a concentration of 364.1 µg/ml followed by iron hexahydrate and chromium hexahydrate.

In vitro exposure of cells to metal ions or corrosion products released by dental alloys has been shown to reduce cellular viability, alter cell proliferation, inhibit various enzymes in cultured cells, induce cell membrane distraction, and decrease DNA and RNA synthesis (18-22). In a recent study conducted on the cytotoxic effects of the nickel release from the stainless steel brackets it has been shown that mild DNA damage occurs on exposure to 1.18 µg nickel alloy solution for 72 hours (23). In the present study, nickel was found to be the most toxic with an IC50 value of 364.1 between the concentrations of 75 and 2400 µg/ml. Below the concentration, 70 µg/ml was found to be non-cytotoxic.

Park and Shearer reported an average release of 40 µg of nickel and 36 µg of chromium from a simulated orthodontic appliance. The release of nickel is not necessarily related to the alloy's nickel content. The amount of nickel released can increase during stress. The quantities released may be negligible from a toxicological point of view but could conceivably be of significance for patients with a high degree of hypersensitivity to nickel (24).

Allergies in orthodontic patients can occur for a variety of reasons, including nickel allergy, allergy to the acrylic resins used during treatment, latex products, and so on. Identification of patients with allergies, as well as knowledge of materials that may cause them, is required for safe and effective treatment (25). The toxic reaction may arise due to the repeated or constant influence of toxic agents in low concentrations over long periods. Such reactions are most frequently localized to the contact zone with the toxic agent (25,26). Thus, the above studies have shown that coated wires or implants can reduce the risk of metal ion release, reducing the corrosion and cytotoxic effects on the oral mucosa (25, 26). Hence, coated wires should be preferred over uncoated wires with biological stability and economical.

According to the findings of this study, increasing concentrations of metal ions induce variable cytotoxic effects. This study shows that the attack of cytotoxic substances changes the structure of the cells, and that these changes can be seen using an electron microscope. Thus, in conclusion, interactions of orthodontic materials with tissues in the oral cavity and with other dental materials can cause cytotoxicity and allergic reactions in both patients and doctors. The understanding of interactions will enable the orthodontist to make appropriate material selections that will provide adequate treatment mechanics while also preparing the clinician to deal with the complex effects of these materials in the

oral environment.

Conclusion

In HaCaT cells, metal ions produced repeatable harmful effects. The studied material's cytotoxic effects were dosage-dependent. Thus, a broad range of toxicity of nickel, chromium, and iron ions was shown to be hazardous to human keratinocytes in the current investigation. The MTA experiment indicates that HaCaT cells are more vulnerable to the toxicity of most metal salts. Among the metal ions utilized in the current investigation, nickel was the one that proved to be the most harmful to human keratinocyte cells. The range of IC50 values was 364.1 to 641.1.

Conflicts of interest: None to declare.

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