Abstract

OBJECTIVE: Based in the premise that Ti-6Al-4V orthodontic mini-implants can release metal ions into the body fluids, this research is aimed assess the cytotoxic effect of orthodontic mini-implant on L929 fibroblast cells. MATERIAL AND METHODS: Eighteen orthodontic mini-implants made of Ti-6Al-4V alloy were divided into 6 groups: 1 (golden colour, SIN), 2 (silver colour, SIN), 3 (Neodent™), 4 (INP™), 5 (Mondeal™), and 6 (Titanium Fix™). The mini-implants were immersed into Eagle’s minimum essential medium for 24 hours, where supernatant removal and contact with L929 fibroblasts were performed. Cytotoxicity was evaluated in four different periods of time: 24, 48, 72, and 168 hours. After being in contact with the mini-implants immersed, the cells were incubated for further 24 hours and then 100 ml of 0.01% neutral-red staining solution were added. After this period of time, they were fixed and a spectrophotometer was used for counting the viable cells. RESULTS: After the 24 hours period, statistical differences were found by comparing groups 1 and 2 to groups 3, 4, 5, and C+ (p < 0.05). After the 48 hours period, groups 1 and 2 were shown to be statistically different in relation to groups 3, 4, and C+. After the 72 hours period, statistical differences were found only in group 1 compared to groups 4, 5, 6, CC, and C+ (p < 0.05). After 7 days, no statistical differences were found between the mini-implants. CONCLUSION: Although mini-implants are made of the same alloy, there are differences in their cytotoxicity because of the different concentrations of chemical elements used for manufacturing them.

Keywords: Mini-implants. Cytotoxicity. Orthodontics.
INTRODUCTION

The control of orthodontic anchorage has been an issue of concern to orthodontists since the early existence of this specialty. A successful orthodontic treatment, in the great majority of cases, requires that anchorage to be judiciously planned, and it would not be an exaggeration to state that this is one of the determinant factors for success or failure in many orthodontic treatments.

Mini-implants are currently being used for improving those situations in which orthodontic anchorage is needed (1-4). Their use is motivated by their positioning versatility, easy removal, and low cost (4-6). Most mini-implants are made of titanium alloy, differing in shape, design, measurements, and trademark (7). The commercially pure titanium (CP Ti) is largely utilized in the manufacture of dental and orthopaedic implants since it is considered chemically inert, in addition to have adequate mechanical properties and excellent biocompatibility (8). Despite these favourable characteristics, however, CP Ti has not been the preferred material for manufacturing orthodontic mini-implants because of its low resistance to fractures and possibility of Osseo integration (9).

Fracture resistance is one of the necessary characteristics required for insertion and removal of orthodontic mini-implants in view of their reduced size and inter-radicular placement (10). In order to overcome this problem, the material chosen for confectioning orthodontic mini-implants is the Ti-6Al-4V alloy because of its higher resistance to fracture (11).

However, the metallic alloys used in orthodontics are subject to corrosion and metal ion release into the oral cavity, which may cause adverse physiological effects such as cytotoxicity, genotoxicity, carcinogenicity, and allergic effects. The choice of a certain alloy depends largely on its indications. Ti-6Al-4V alloys are composed of aluminium (Al) and vanadium (V), both found to be cytotoxic elements when released in the form of ions during essays on erosion in physiological medium (12).

Ti-6Al-4V alloy is less resistant to corrosion than CP Ti (11, 13), resulting in metal ions release. As these ions can accumulate in tissues surrounding the mini-implant (14) and even in distant tissues (15) undesirable effects on human body can occur such as osteolysis, allergic reactions, renal lesions, cytotoxicity, hypersensibility, and carcinogenesis (16). In addition, metal ions are often accounted for implant failure. Because all commercially available orthodontic mini-implants are made of Ti-6Al-4V alloy, the author aims to investigate the hypothesis that there is no difference in cytotoxicity between mini-implants from different manufacturers.

MATERIAL AND METHODS

Cell culture

The cell line used for this study was mouse L929 fibroblasts obtained from the American Type Culture Collection (TCC, Rockville, MD) and cultivated in Eagle’s minimum essential medium.
The cell culture was supplemented with 2 mM of L-glutamine (Sigma™, St. Louis, Missouri, USA), 50 µg/ml of gentamicin (Schering Plough™, Kenilworth, New Jersey, USA), 2.5 µg/ml of fungizone (Bristol-Myers-Squib™, New York, USA), 0.25 mM of sodium bicarbonate solution (Merck™, Darmstadt, Germany), 10 mM of HEPES™ (Sigma, St. Louis, Missouri, USA), and 10% of foetal bovine serum (FBS) (Cultilab™, Campinas, São Paulo, Brazil), then being kept at 37°C in 5% CO₂ environment.

**Mini-implants to be evaluated**

The sample consisted of 18 Ti-6Al-4V orthodontic mini-implants from different manufacturers divided into six experimental groups, namely, Group 1 (golden colour, SIN™, São Paulo, Brazil), Group 2 (silver colour, SIN, São Paulo, Brazil), Group 3 (Ncendent™, Curitiba, Brazil), Group 4 (INP™, São Paulo, Brazil), Group 5 (Mondeal™, Tutlingen, Germany), and Group 6 (Titanium Fix™, São José dos Campos, Brazil).

**Controls**

To verify the cell response to extreme situations, other three groups were included in the study: Group CC (cell control), consisting of cells not exposed to any material; Group C+ (positive control), consisting of amalgam cylinder; and Group C- (negative control), consisting of a stainless steel wire (nickel free) (Morelli™, Sorocaba, São Paulo) in contact with the cells.

**Assessing the cytotoxicity of the materials**

The materials were previously sterilised by exposing them to ultra-violet light (Labconco™, Kansas, Missouri, USA) during 1 hour. Next, three samples of each material were placed in 24-wells plates containing Eagles’ MEM (Cultilab™, Campinas, São Paulo, Brazil). The culture medium was replaced with fresh medium every 24 hours, and the supernatants were collected after 24, 48, 72, and 168 hours (7 days) for analysis of the toxicity to L929 cells. The supernatants were placed in a 96-well plate containing a single layer of L929 cells and then incubated at 37°C for 24 hours in 5% CO₂ environment. After the incubation period, cell viability was determined using the “dye-uptake” technique described by Neyndorff et al. (17) (1990), which was slightly modified. After the 24-hour incubation period, 100 µl of 0.01% neutral-red staining solution (Sigma™, St. Louis, Missouri, USA) were added to the medium within each well of the plates, and these were incubated for 3 hours at 37°C to allow the dye to penetrate into the living cells. After this period, the cells were fixed using 100 µl of 4% formaldehyde solution (Reagen™, Rio de Janeiro, Brazil) in PBS (130 mM NaCl; 2 mM KCl; 6 mM Na₂HPO₄·2H₂O; 1 mM K₂HPO₄, pH = 7.2) for 5 minutes. Next, 100 µl of 1% acetic acid solution (Vetec, Rio de Janeiro, Brazil) with 50% methanol (Reagen™, Rio de Janeiro, Brazil) were added to the medium to remove the dye. Absorption was measured after 20 minutes by using a spectrophotometer (BioTek™, Winooski, Vermont, USA) at a wave length of 492 nm (µ = 492 nm).

**X-ray dispersion analysis**

The metallic alloy of the mini-implants was characterised by X-ray dispersion using a JEOL scanning electron microscope (2000 FX™, Tokyo, Japan). For doing so, the mini-implants were cut with a precision sectioning machine (Isomet™, Buehler, Illinois, USA) and washed with an ultrasound equipment (Ultramet™ 2002, Buehler, Illinois, USA). Next, the samples were carefully dried and positioned for being submitted to SEM analysis.

**Statistical analysis**

Statistical analyses were performed by using a SPSS v.13.0™ software (SPSS Inc., Chicago, USA), and means and standard deviations were calculated for descriptive statistical analysis. The values for the amount of viable cells were submitted to analysis of variance (ANOVA) to determine whether statistical differences existed between the groups, and Tukey’s test was applied thereafter.
RESULTS

The results regarding the 24-hour period demonstrated that statistical differences were found when Groups 1 and 2 were compared to Groups 3, 4, 5, and C+ as well as when Group C+ were compared to the other groups (p < 0.05). Groups 1 and 2 were shown to be statistically different compared to Groups 3, 4, and C+ at 48 hours (Table 1).

Table 1 - Statistical analysis with means and standard deviations for the groups studied

<table>
<thead>
<tr>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups 1</td>
<td>0.622 (±0.05)</td>
<td>A</td>
<td>0.510 (±0.12)</td>
<td>AC</td>
<td>0.855 (±0.09)</td>
<td>A</td>
<td>0.274 (±0.03)</td>
<td>A</td>
</tr>
<tr>
<td>Groups 2</td>
<td>0.636 (±0.09)</td>
<td>A</td>
<td>0.518 (±0.08)</td>
<td>AC</td>
<td>0.926 (±0.04)</td>
<td>AB</td>
<td>0.276 (±0.02)</td>
<td>A</td>
</tr>
<tr>
<td>Groups 3</td>
<td>0.492 (±0.06)</td>
<td>BC</td>
<td>0.719 (±0.10)</td>
<td>B</td>
<td>0.843 (±0.14)</td>
<td>AC</td>
<td>0.267 (±0.03)</td>
<td>A</td>
</tr>
<tr>
<td>Groups 4</td>
<td>0.495 (±0.06)</td>
<td>BC</td>
<td>0.741 (±0.04)</td>
<td>B</td>
<td>1.001 (±0.12)</td>
<td>BC</td>
<td>0.276 (±0.02)</td>
<td>A</td>
</tr>
<tr>
<td>Groups 5</td>
<td>0.498 (±0.08)</td>
<td>BC</td>
<td>0.681 (±0.06)</td>
<td>BC</td>
<td>1.034 (±0.07)</td>
<td>B</td>
<td>0.276 (±0.02)</td>
<td>A</td>
</tr>
<tr>
<td>Groups 6</td>
<td>0.561 (±0.07)</td>
<td>AC</td>
<td>0.646 (±0.05)</td>
<td>BC</td>
<td>1.049 (±0.15)</td>
<td>B</td>
<td>0.342 (±0.02)</td>
<td>A</td>
</tr>
<tr>
<td>C. C.</td>
<td>0.638 (±0.03)</td>
<td>AC</td>
<td>0.779 (±0.13)</td>
<td>ABC</td>
<td>1.051 (±0.07)</td>
<td>B</td>
<td>0.319 (±0.01)</td>
<td>A</td>
</tr>
<tr>
<td>C. +</td>
<td>0.285 (±0.06)</td>
<td>D</td>
<td>0.251 (±0.04)</td>
<td>D</td>
<td>0.648 (±0.04)</td>
<td>D</td>
<td>0.212 (±0.03)</td>
<td>B</td>
</tr>
<tr>
<td>C. -</td>
<td>0.538 (±0.00)</td>
<td>ABC</td>
<td>0.573 (±0.03)</td>
<td>ABC</td>
<td>0.952 (±0.05)</td>
<td>ABC</td>
<td>0.318 (±0.02)</td>
<td>A</td>
</tr>
</tbody>
</table>

Legend: M. Cel = mean values for the amount of viable cells; SD = Standard Deviation; Stat = Same letters mean no statistical difference.

After the 72-hour period, it was observed that Group 1 was statistically different from Groups 4, 5, 6, C, and C+ (p < 0.05). Statistical differences were found between Group C+ and all other groups not only at 7 hours, but also in all periods of time (p < 0.05).

Table 2 shows the percentage amount of chemical elements found in the mini-implants evaluated by using the X-ray dispersion analysis (EDX).

Table 2 - Percentage amount of chemical elements in the mini-implants tested

<table>
<thead>
<tr>
<th>Groups</th>
<th>Manufacturers</th>
<th>C</th>
<th>Al</th>
<th>Ti</th>
<th>V</th>
<th>Fe</th>
<th>Cu</th>
<th>O</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SIN (golden)</td>
<td>2.30%</td>
<td>4.27%</td>
<td>71.91%</td>
<td>2.62%</td>
<td>-</td>
<td>-</td>
<td>16.42%</td>
<td>2.48%</td>
</tr>
<tr>
<td>2</td>
<td>SIN (silver)</td>
<td>2.30%</td>
<td>4.27%</td>
<td>71.91%</td>
<td>2.62%</td>
<td>-</td>
<td>-</td>
<td>16.42%</td>
<td>2.48%</td>
</tr>
<tr>
<td>3</td>
<td>Neodent</td>
<td>-</td>
<td>5.73%</td>
<td>91.43%</td>
<td>3.94%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>INP</td>
<td>-</td>
<td>5.36%</td>
<td>90.33%</td>
<td>3.22%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Mondeal</td>
<td>1.35%</td>
<td>3.81%</td>
<td>70.39%</td>
<td>3.18%</td>
<td>0.28%</td>
<td>0.11%</td>
<td>20.87%</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Titanium Fix</td>
<td>3.05%</td>
<td>5.08%</td>
<td>87.87%</td>
<td>3.58%</td>
<td>0.27%</td>
<td>0.16%</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
DISCUSSION

The use of cell culture has been employed as part of a series of recommend tests for evaluation of the biological behaviour of materials being put in contact with human tissues. In the present study, cytotoxicity tests were conducted in order to evaluate the biocompatibility of mini-implants for orthodontic usage.

The commercially available orthodontic mini-implants are made of Ti-6A-4V alloy. The biocompatibility of Ti ions is well described in the literature, but aluminum (Al) and vanadium (V) have not been so studied. Al ions affect proliferation, metabolic activity and differentiation of osteoblasts (18). Some of the toxic effects described in the literature are encephalopathy and Alzheimer-type senile dementia (19), and Al may be associated with osteomalacia and pulmonary granulomatosis as well (20). With regard to the level of Al ions in the orthodontic mini-implants studied, Group 3 had the highest percentage of aluminium (5.73%), followed by Group 4 (5.36%), Group 6 (5.08%), Groups 1 & 2 (4.27%), and Group 5 (3.81%).

Vanadium, whose main source is food, is an essential micro-element that is present in the majority of mammalian cells (21, 22). However, this chemical element is considered highly toxic compared to other nutritionally essential micro-elements because there is a small difference between the necessary and toxic doses (23). On the other hand, V has important pharmacological and physiological effects, playing an important role in the auxiliary treatment of diabetic patients (22).

The effects of chronic and acute V intoxication are well documented. Vanadium is cytotoxic for macrophages and fibroblasts (24), binds to certain proteins (e.g. ferritin and transferrin), affecting their distribution and accumulation throughout the body (22), stimulates local and systemic allergic reactions, inhibits cell proliferation and may cause renal lesions. Urinary excretion is the main pathway for elimination of injected vanadium in human beings (22).

Similarly to the presence of aluminium, Group 3 was found to have the lowest cell viability in all periods of time. This fact may due to the greater amount of aluminium (56%) and vanadium (3.22%) found in the samples during X-ray dispersion analysis (EDX).

These differences in cell viability between the groups may be related not only to aluminium and vanadium, but also to other components such as carbon (C), titanium (Ti), iron (Fe), copper (Cu), oxygen (O), and nitrogen (N).

Despite the differences found between several orthodontic mini-implants, in fact they showed lower cytotoxicity compared to Groups CC and C-.

During the whole experiment it was observed that cell viability was higher in Group CC (mini-implant exposed to no material) and lower in Group C+, a finding that may be explained by the constant release of mercury from the amalgam – a material known to be cytotoxic (25).

After seven days, all mini-implants showed higher cell viability when compared to each other. During this period of time, no statistical differences were found between the groups studied. A drawback regarding this study was the lack of evaluation of ion content in the supernatant placed on the cells. Only ions presenting in mini-implants were evaluated. Further studies are needed to evaluate this point, which can allow us to assess any relationship between ions released by mini-implants and consequently their real cytotoxicity.

CONCLUSION

The hypothesis that no difference exists in the cytotoxicity between mini-implants made from the same alloy was not proved. Besides, small differences were observed, possibly due to the concentration of chemical elements.

CONFLICT OF INTEREST STATEMENT

The authors formally declare that there is no conflict of interest in the present manuscript.

REFERENCES


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