Cell death affected by dental alloys: Modes and mechanisms

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INTRODUCTION

Dental alloys have been widely used in porcelain-fused-to-metal (PFM) crowns, bridges and casting frames in dental restorations for decades. Although a number of metal-free materials are available, dental alloys are still popular due to their favorable properties and reasonable price. However, biocompatibility has been a critical concern regarding the clinical application. Previous reports have found continuous corrosion of alloys occurs due to oral complex environment and individual differences. As a result, metal ions can be released from alloys and induce cytotoxicity for prolonged time. This can cause chronic adverse effects on the surrounding tissues and cells, such as partial gingival discolorations and hypersensitivity. Cytotoxicity is often considered to be the first manifestation of poor biocompatibility, thus leading to cell death in the form of apoptosis or necrosis. Apoptosis is a mode of cell death that is procedural and controlled by many molecular signaling pathways, such as intrinsic or extrinsic pathway. However, the manner in which dental alloys affect the tissue around them remains unclear. Contreras et al. observed that cells death was induced by necrosis rather than apoptosis due to Co and Ni in MC3T3-E1 cells. Schedule et al. reported similar findings in L929 cells and human gingival fibroblasts cells. Conversely, Wang et al. and Au et al. reported that dental alloys induce apoptosis.

As dental materials, dental alloys are in intimate contact with the oral mucosa and affect local tissue for long terms without the ability to be removed by the patients. Therefore, knowledge about the detrimental influence of them on the oral tissues is of great importance. With the aim of identifying the mode of cell death affected by dental alloys and the biological mechanism of this process, we undertook this investigation.

MATERIALS AND METHODS

Specimen preparation

Cobalt-chromium (Co-Cr), commercially pure titanium (CP-Ti), palladium-based (Pd-based) and gold-platinum (Au-Pt) alloys were casted to fabricate 216 platy specimens (10.0 mm in height, 10.0 mm in width and 1.0 mm in thickness) under a vacuum environment with argon gas protection (ARGONCASTER-SE, Shofu, Kyoto, Japan). The Composition of these dental alloys was show in Table 1. All of the specimens were polished to a mirror-like on both sides and then ultrasonically cleaned with 95% ethanol, rinsed in deionized water, and dried with compressed air without oil or water.

Preparation of alloys’ leach liquors

Specimens with each dental alloy were placed in a centrifuge tube following autoclaving at 121°C for 20 min. According to ISO 10993, Dulbecco’s modified Eagle medium (DMEM, Gibco, Waltham, MA, USA) was added to each tube based on the specimen’s superficial area such that the ratio between the superficial area of the specimen and the volume of the medium was 3.0 cm²/mL. Next, the tubes were placed in a CO₂ incubator (Heraeus, Hanau, Germany) with 5% CO₂ at 37°C for 72 h.

Cell culture

The mouse fibroblast cell line (L929) was obtained from the Shanghai cell resource center, Chinese Academy of Sciences (Shanghai, China). The cells were cultured for 24 h in DMEM supplemented with 10% Newborn

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Table 1 Composition of the dental alloys as provided by the manufacturers

| Dental alloy* | Manufacturer | Composition (wt%)                          |
|--------------|--------------|--------------------------------------------|
| Co-Cr        | Bego, Bremen, Germany | Co: 60.2, Cr: 25, W: 6.2, M: 4.8, Ga, 2.9, (Mn, Si)<0.9 |
| CP Ti        | Dentaurum, Ispringen, Germany | Ti > 99.5, (Fe, O, H, N, C)<0.5 |
| Pd-based     | Heraeus, Hanau, Germany | Pd: 78.0, Cu: 10.8, Ga: 7.5, Au: 1.6, In: 1.3, Ru: 0.4, Zn: 0.2, Sn: 0.2 |
| Au-Pt        | Alldental, Stockholm, Sweden | Au: 89.5, Pt: 5.8, Pd: 1.6, Ag: 1.2, Ir: 0.6, Sn: 0.3, In: 0.8, Fe: 0.2 |

*Commonly used in fused-to-metal (PFM) crowns, bridges and casting frames.

Table 2 Primer sequences

| Sequences                  | Forward primer       | Reverse primer       |
|----------------------------|----------------------|----------------------|
| GAPDH (83 bp)              | 5'-GCAACTCCCACTCTTTCCACC-3' | 5'-GTCATACCAGGAAATGAGCTTGACA-3' |
| Caspase 9 (79 bp)          | 5'-CGAGGATATTCAAGGCAAGAGA-3' | 5'-CCTCCGTGTCTCAAGGCTTG-3' |
| Caspase 3 (70 bp)          | 5'-GCACCTGGATTACTATTTGAA-3' | 5'-GCATGCTGCAAAGGGACTGG-3' |

Primers were designed by DNAMEN software.

Calf Serum (NBCS, Gibco), penicillin (100 U/mL) and streptomycin (100 U/mL) in a humidified incubator with 5% CO₂ at 37°C. Next, the culture medium was replaced with the leach liquors of the different alloys. And cells cultured in DMEM without metal ions in the same condition were considered negative group.

**Flow cytometry (FCM) assay**
The L929 cells were collected after treatment with one of the four leach liquors for 48 and 72 h. Next, the cells in each group were treated with the Annexin-V-FLUOS-Staining-Kit (Roche) according to the manufacturer’s protocol. Cells cultured containing 0.64% phenol were used as positive group. By using Annexin-V and PI staining, FCM can distinguish early apoptosis from late apoptosis/cell necrosis. In the early stages of apoptosis, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment and thus can be bound by Annexin V. At the meantime, the plasma membrane excludes viability dyes such as PI, so these cells will only stain with Annexin V and serve as an early marker of apoptosis. However, in the latest stages of cell death resulting from either apoptotic or necrotic processes, the cell membrane loses integrity, allowing Annexin V to access PS in the interior of the cell, as well. Hence, PI can be used to differentiate these late apoptotic cells or necrotic cells (Annexin V and PI double positive) from the early apoptotic cells (Annexin V positive, PI negative). The stained cells were observed by fluorescence microscope (IX71, Olympus, Tokyo, Japan) and analyzed *via* flow cytometry (FACSCalibur, BD Biosciences).

**Real-time quantitative PCR (Real-time qPCR) assay**
Cell treatments were the same as described above. The cells in each group were treated with Trizol (Invitrogen, Waltham, MA, USA) for total RNA extraction. The RNA concentration was measured by the spectrophotometer (Nanodrop2000, Thermo, Waltham, MA, USA) at the wavelength of 260 nm. The purification of the RNA was determined by the ratio of the absorbance at 260 and 280 nm (A260/280) and agarose electrophoresis. The level of the Caspase 9 and Caspase 3 mRNAs were analyzed with real-time quantitative PCR assays (7500 Real-Time PCR Systems, Applied Biosystems, Waltham, MA, USA). GAPDH was used as the internal reference. The primers pairs for Caspase 3, Caspase 9 and GAPDH were previously constructed and are shown in Table 2. The cycle thresholds (Cts) for Caspase 3, Caspase 9 and GAPDH were gathered via real-time quantitative PCR assays. The relative quantities of the Caspase 9 and Caspase 3 mRNAs for all groups were calculated according to the following formula:

Relative Quantity=\[2^{-\Delta \Delta Ct}}\];

\[\Delta \Delta Ct=\frac{(Ct_{target}-Ct_{GAPDH})_{test\ group}}{(Ct_{target}-Ct_{GAPDH})_{negative\ group}}\].

**Statistical analysis**
All trials were repeated 3 times and the results are expressed as mean±SD (standard deviation). One-way ANOVAs and student *t*-test were performed with SPSS.
19.0 software (Chicago, IL, USA) and followed with the SNK (Student-Newman-Keuls) test. \( p < 0.05 \) was considered to be significant.

**RESULTS**

*Flow cytometry (FCM) assay*

As shown in Fig. 1A, under fluorescent vision, the cells with green staining only are mainly those undergoing early apoptosis, while those with green and red staining represent late apoptotic cells and necrotic cells. Morphotologically, in the normal vision of same area (Fig. 1B), early apoptotic cells were shown characteristic membrane blebbing, cell shrinking and nuclear condensation. Meanwhile, the necrotic/late apoptotic cell present the loss of cell membrane integrity.

Cell death mode affected by dental alloys following treatment after 48 and 72 h was illustrated in Table 3. In contrast to the negative group, phenol dramatically promoted cell apoptosis in the positive group. Generally, except for Au-Pt group, the percentage of early apoptotic cells in each group increased with the incubation time \( (p < 0.05) \). At the same time, the late apoptotic/necrotic cells rate of Co-Cr and positive groups experienced a climb, but remained stable in others groups. According to a one-way ANOVA, the differences between the groups (i.e., early apoptosis, late apoptosis/necrosis) were all significant. Furthermore, Au-Pt and Pd-based groups showed no significant difference in the level of cellular effect compared with the negative group \( (p > 0.05) \). Additionally, Co-Cr alloy induced the highest rate of early apoptosis. In current study, the major effect

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**Table 3** Cell death mode affected by dental alloys following treatment after 48 h and 72 h (%; \( \bar{x} \pm SD \))

|                  | 48 h                | 72 h                |
|------------------|---------------------|---------------------|
|                  | Early apoptosis     | Late apoptosis/Necrosis | Early apoptosis     | Late apoptosis/Necrosis |
| Negative         | 0.85±0.05           | 0.49±0.16*          | 1.36±0.45           | 0.95±0.39*              |
| Au-Pt            | 1.10±0.11           | 0.59±0.16*          | 1.39±0.96           | 1.24±0.41*              |
| Pd-based         | 1.18±0.17           | 0.64±0.05*          | 3.08±0.75*          | 1.26±0.52*              |
| CP-Ti            | 3.42±0.10           | 1.19±0.44*          | 4.85±0.86           | 1.93±0.68*              |
| Co-Cr            | 5.41±0.53           | 1.47±0.28           | 7.41±0.98           | 2.32±0.38*              |
| Positive         | 20.44±2.51*         | 9.25±0.88*          | 28.22±1.47*         | 12.17±1.12*             |
| F                | 155.69              | 216.58              | 342.88              | 139.92                  |
| \( p \)          | <0.01               | <0.01               | <0.01               | <0.01                   |

* \( p < 0.05 \) versus negative control group

\( p < 0.05 \) 48 versus 72 h in each group

* \( p < 0.05 \) early apoptosis versus late apoptosis/necrosis in each group
Table 4  mRNA level of Caspase 3 and Caspase 9 affected by dental alloys following treatment for 48 and 72 h (%, x±SD)

| mRNA Level | 48 h | 72 h |
|------------|------|------|
|            | Caspase 3 | Caspase 9 | Caspase 3 | Caspase 9 |
| Negative   | 1     | 1     | 1         | 1         |
| Au-Pt      | 0.91±0.06 | 0.98±0.05 | 1.03±0.11 | 0.95±0.11 |
| Pd-based   | 0.96±0.11 | 0.94±0.04 | 1.24±0.03** | 2.88±0.16** |
| CP-Ti      | 2.50±0.09* | 3.23±0.07* | 2.86±0.05** | 3.85±0.08** |
| Co-Cr      | 0.98±0.04 | 0.61±0.06* | 3.14±0.08** | 5.00±0.10** |
| F          | 297.69 | 1389.71 | 717.03 | 875.94 |
| p          | <0.01 | <0.01 | <0.01 | <0.01 |

*p<0.05 versus negative control group
**p<0.05 48 versus 72 h

on cells caused by dental alloys was time-dependent early apoptosis.

Real-time quantitative PCR assay
As shown in Table 4, the level of the Caspase 9 and Caspase 3 mRNAs of each group followed the same trend after treatment for 48 and 72 h. According to a one-way ANOVA, the differences between the groups were all significant. Furthermore, in 48 h, the Caspase 9 and Caspase 3 mRNA levels of CP-Ti group were remarkable higher than those of others (p<0.05), and notably, the Caspase 9 mRNA level of Co-Cr group was lower than the negative group (p<0.05). It is easy to find that the levels of Caspase 9 and Caspase 3 mRNAs in each group all raised dramatically over time (p<0.05) with the exception of Au-Pt group. In general, Au-Pt group always showed no significant difference compared with the negative group (p>0.05), while Co-Cr alloy activated highest expression of Caspase 9 and Caspase 3 mRNAs. These results also corresponded to the FCM assay.

DISCUSSION
Apoptosis is another form of cytotoxic effect of dental alloys on cells, a genetically programmed form of cell death which is indispensable for development and homeostasis of multicellular organism. Although apoptotic cell death plays an important role in maintenance of the normal physiological state, it may also be responsible for diseased state of oral mucosa.

In the present study, L929 cells were used to evaluate the apoptotic effects of four different dental alloys by the FCM (Annexin-V-FLUOS-Staining). The total leach liquors of dental alloys were tested, but the contribution of individual elemental cation to cell apoptosis could not be precisely determined.

The most widely-used biological systems for in vitro toxicity testing of dental materials are cells in culture. Two types of cells are used: permanent cell lines derived from type-culture collections (L929 mouse fibroblasts) or primary cells derived from gingival or mucosal explants and established in each individual laboratory. Permanent cell lines are well defined and generally available and also appear to be more stable and reproducible compared with primary cell lines.

Therefore, L929 mouse fibroblast cell line was investigated.

The FCM results certified that, Co-Cr, CP-Ti groups caused significantly higher rates of early apoptosis compared to the negative group (p<0.05), among which the Co-Cr group was the highest, sharing the similar result with some researchers. Apart from Au-Pt alloy, the major cell death of dental alloys was time-dependent early apoptosis rather than late apoptosis/necrosis (p<0.05). The primary reason might be the metal cations released from the dental alloys into the leach liquors. Through there was no significantly apoptotic effect caused by Pd-based alloy (p>0.05), the cell apoptotic rate increased from 48 to 72 h, so it is hard to determine whether Pd-based would induce large apoptotic cells in the future. Therefore, FCM analysis indicated that the Au-Pt alloy was more biocompatible and Co-Cr, CP-Ti, Pd-based alloys had different levels of apoptotic effect on cells.

Apoptosis is tightly controlled by two major pathways, extrinsic and intrinsic. The extrinsic pathway is triggered by cell surface receptors, like tumor necrosis factor (TNF-α) family while the intrinsic pathway involves the mitochondria pathway. The activation of caspase plays a critical role in the execution of apoptosis and Caspase 3 is the main form active in apoptosis. Additionally, Caspase 9 is the caspase initiator of the intrinsic pathway. Thus, in this research, the activity of
Caspase 3 and Caspase 9 mRNA were also detected with Real-time qPCR to further investigate the mechanism of apoptosis induced by four dental alloys.

The result showed that only Au-Pt alloy had no effect on the mRNA level of Caspase 3 and Caspase 9 which was corresponded to the excellent biocompatibility indicated by the FCM assay.

In other three groups, the expression of Caspase 3 and Caspase 9 mRNA shared the same trend and also had an increment with incubation time. Therefore, it implied that dental alloys can induce the apoptosis of L929 cells via the intrinsic pathway in a time-dependent manner.

As for Pd-based alloy, consistent with the FCM, the Caspase 3 and 9 mRNA level was quite low. It might be attributed to one of components, Zinc, a potent inhibitor of the apoptotic protease. Nonetheless, in 72 h, the early apoptotic rate and Caspase mRNA level of the Pd-based group were both higher than those of the negative groups (p<0.05). This is probably a consequence of copper in Pd-based alloy, which has been reported to be the cause of cell apoptosis.

Regarding the CP-Ti alloy, the early apoptotic rate was significantly higher than that of the negative group and so as the activation of Caspase 9 and Caspase 3 mRNA. Due to the previous report about the favorable corrosion resistance of CP-Ti alloy, we tentatively put forward that the reason for the apoptosis in the CP-Ti group might not be the ions released from the CP-Ti alloy. An alternative potential reason could be that titanium particles impaired cell adhesion, which led to cell apoptosis by impeding the focal adhesion assembly as reported by Zhao et al.

The highest level of Caspase 3 and Caspase 9 mRNA in 72 h demonstrated that Co-Cr alloy was poor biocompatible, which agreed with results of FCM analysis. Two theories are available regarding the possible causes of the Caspase-9 and Caspase-3 activations. The first is the DNA damage induced by the Co and Cr. The second is the reduction reactions in the alloy leach liquors as reported by Haeri et al. As a result, Co-Cr alloy might be much easier to induce cell apoptosis than other three alloys via intrinsic pathway.

The biocompatibility of certain dental alloys may be improvable as long as the apoptotic effect is controlled. Furthermore, regulation via the intrinsic pathway could be a valuable research direction for improving the biocompatibility of dental alloys so as to exhibit excellent performances. The further study would be fulfilled by determining the concentrations of the specific ions released from the alloys and identify which specific ions were responsible for the observed problematic cell phenomena. In addition, it has been reported that inappropriate apoptosis (either too little or too much) is a factor in many oral diseases including autoimmune disorders such as pemphigus. The process of apoptosis may cause exacerbation or speeding of the bulla formation. Whether dental alloys could increase the sensitivity of apoptosis-related oral diseases also needs further research.

CONCLUSIONS

This investigation demonstrated the following:
1. Au-Pt alloy had no apoptotic effect on cells, presenting favorable biocompatibility.
2. Time-dependent apoptosis was the major mode of cell death that was affected by the dental alloys.
3. Dental alloys might induce cell apoptosis via the intrinsic pathway.

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