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Comparative studies on cytotoxic effects of dental amalgams and alternative alloys according to ISO standards *in vitro*

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Deleterious effects of dental alloys, especially those of dental amalgams, have become an important issue in current discussions on biomaterials. Cytotoxicity and further related risks of amalgams are discussed in a controversial way in the literature without leading to a final conclusion. There is still a need for basic clinical and pre-clinical research, especially with respect to the wide distribution of dental amalgams. Standardized methods of cytotoxicity testing have been established by the ISO. It was the aim of the present study to detect and compare possible cytotoxic effects of dental amalgams and alternative non-amalgam alloys *in vitro*. According to the ISO standards, direct contact tests and extract dilution tests were performed using the cell lines HeLa and L-929 as well as primary isolated human fibroblasts, a relevant cell type of the human gingiva. For direct contact tests the samples were fixed on thermanox discs. Zn and Ni-chloride in defined molar concentrations were used as positive controls in the extract dilution tests, while copper was the positive control in the direct contact tests. The tested amalgam was a Non-Gamma-2 amalgam. For extract dilution tests sixteen extraction dilutions were performed. The different cell types were incubated with the extracts in 96-well microtitre plates. MTT-testing was performed to evaluate the effects on cellular metabolism. The BrdU labelling index was determined with the help of EIA methods to analyse the effects of the extracts on the cellular proliferation at DNA synthesis level. The morphological status of the cells seeded on the materials (direct contact test) were studied with the help of light microscopy. No cytotoxic effects of formerly extracted dental amalgam was found, although fresh amalgam elicited a significant cytotoxic effect, in general the non-amalgams have to be regarded as non cytotoxic. The negative control and the non-amalgams elicited no measurable cytotoxicity in the indirect contact assays, independent of the number of extraction dilutions. This applied to all cell types studied. The tested amalgam also gave a significant cytotoxic effect in the MTT-assays, while in addition a significant reduction of BrdU incorporation after incubation with the extracts of the first dilution series, compared to the silicone control was found. The effects were reduced after an incubation with the extracts of the higher dilution series. It is suggested by the presented results that amalgams might have cytotoxic effects, especially when being freshly applied. The cytotoxic effects were no longer detectable after extraction procedures. Nevertheless, a negative effect around such amalgams must be considered. The insights provided by the present studies might be helpful for a rational choice of dental materials.

1. Introduction

Deleterious effects of dental alloys, especially those of dental amalgams, have become an important issue in current discussions on dental implant materials. Cytotoxicity and further related risks of amalgams are discussed in a controversial way in the literature [1–6], based on a wide variety of *in vitro* and *in vivo* methods, without leading to a final conclusion. There is still a need for basic clinical and pre-clinical research, especially with respect to the wide distribution of dental amalgams. Because these materials will be in direct contact with vital tissues, their cytotoxic potential must be evaluated. Cytotoxicity testing of dental restorative materials must be viewed as an assessment

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of hazards, that is the potential of the material to cause pulpal problems as well as even systemic effects [4]. Because of these potentials the use of mercury-based alloys such as dental amalgams have become the subject of political controversy despite its long history of clinical use [5].

Standardized methods of cytotoxicity testing have been established by the ISO [7] whose purpose was to define a scheme for testing medical devices to be made in a series of steps. These ISO standards gave three categories of tests for cytotoxicity evaluation: extract test, direct contact test, indirect contact test. The choice of one or more of these categories has been made dependent upon the nature of the sample to be evaluated, the potential site of its use and the nature of its use. The determination of possible cytotoxic effects was grouped into categories of the evaluation type, a) assessment of cell damage by morphological means, b) measurement of cell damage, c) measurement of cell growth, and d) measurement of specific aspects of cellular metabolism [7]. The methods for evaluation should be comparable with regard to similar devices and interlaboratory comparability. To cover the required aspects of standardized cytotoxicity with respect to the items of the ISO requests mentioned above we performed a variety of *in vitro* assays. It was the aim of the present study to detect and compare possible cytotoxic effects of dental amalgams and selected non-amalgam alloys. According to the ISO standards, direct contact tests and extract dilution tests were performed using the cell lines HeLa and L-929 as well as primary isolated human fibroblasts, a relevant cell type of the human gingiva. The assays were performed according to the ISO guidelines concerning the sample and test preparation, the test conditions, the cell lines and the culture media, as well as the cell culture conditions. For intermaterial comparisons identical test and evaluation procedures were performed on both freshly prepared amalgam as well as extracted and explanted amalgam in comparison to conventional non-amalgam alloys of different composition.

2. Materials and methods

2.1. Reagents and materials

Anti-BrdU antibody was supplied by ICN (Meckenheim, Germany). BCA protein assay reagent was obtained from Pierce, Rockford, USA. Bovine serum albumin (BSA) was bought from Serva Feinbiochemica, Heidelberg, Germany. 5-Bromo-3-deoxyuridine, Ethylenediamine-tetra-acetic acid (EDTA), MTT (3-[4,5-dimethylthiazol-2-yl]-2, S-diphenyl tetrazolium bromide), trypsin type III, Triton X-100 were supplied by Sigma Chemie, Deisenhofen, Germany. RPMI medium, L-glutamine, penicillin/streptomycin solution, Fungizone, Fetal calf serum (FCS), phosphate buffered saline solution (PBS) were supplied by Gibco BRL, Life Technologies, Eggenstein, FRG. Ethanol, methanol, 2-propanol, hydrochloric acid, Tris[hydroxymethyl]-aminomethane (Tris) were bought from Merck, Darmstadt, Germany. Filters (0.22 µm pore size; mixed cellulose-esters) were supplied by Millipore, Eschborn, FRG. Tissue culture ware was supplied by Becton & Dickinson, Heidelberg, Germany. Trypan blue solution was obtained from Boehringer Mannheim, Mannheim, Germany. Microplate reader, Titertek II, and software (EIA3) were supplied by ICN-Flow, Meckenheim, Germany.

2.2. Cells, reference materials and samples

Negative controls: silicone rubber tubing (polydimethylsiloxane; medical grade; supplied by Rehau, Rehau, Germany) and Thermanox tissue (pure discs, Nunc, Wiesbaden, Germany). Positive controls, Zn-and Ni-chloride, were obtained from Sigma, Germany.

Mouse fibroblast cells L-929 (CCLl; strain L, NCTC clone 929) were obtained from American Type Culture Collection, Rockville, USA and HeLa cells (ATCC code: CCLZ) from ICN-Flow, Meckenheim, Germany. Both cell types were cultured in RPMI medium with additional L-glutamine, penicillin/streptomycin and 10% fetal calf serum in a humidified atmosphere containing 5% CO_2. Human fibroblasts were isolated from the human umbilical cord and cultured as primary culture in RPMI supplemented with L-glutamine, penicillin/streptomycin, Fungizone and 10% fetal calf serum. Samples, negative and positive control materials were used as received, the tested amalgam was a non-gamma-2 amalgam (Luxalloy, Degussa, Germany) containing 70% Ag, 12% Cu and 18% Sn with mercury of dental quality (Degussa, Germany). The non-amalgams were alloys for metal-ceramic-techniques (Q 2, B 1, Q 1, Q 4, Q 1) as well as for conventional techniques (Q 4, Q 1, Q 6, Q 4, Q 5). These alloys were supplied by Qualident S.A., Geneva, Switzerland.

The samples were cleaned in a 1% detergent solution (RBS 35) with ultrasound. Both samples and controls were autoclaved at 121°C for 30 min. For direct contact tests the samples were fixed on thermanox discs with a biocompatible fixative.

2.3. Preparation of cell cultures

In order to prepare test wells, cells were detached from the culture flasks using a trypsin/EDTA solution (0.25%/0.25% w/v), and resuspended as a single cell suspension in culture medium at a density of 2×10^5 viable cells/ml (trypan blue exclusion test). HeLa, L-929 and human fibroblasts were seeded into the wells of microtitre plates and 24-well clusters, at a density of 1×10^5 cells/cm^2. After overnight incubation, the wells were emptied and refilled with the extracts, as well as their dilutions or fresh culture medium.

2.4. Extract dilution tests

The samples were extracted in culture medium for 24 h at 37 °C (1 cm^2/ml). The pH was adjusted to be between 7.2 and 7.4, the extracts were sterile filtered (pore size: 0.22 µm) and diluted 2, 4, 8 and 16 times with fresh culture medium. Positive and negative

control materials were diluted to defined molar concentrations, whereas the extract of the negative control was not diluted.

2.5. MTT-test

20 µl of a 1% MTT solution was added to each well of a 96-well microtitre plate. The plates were shaken briefly and incubated for a further 1 h (4 h in case of human fibroblast experiments) at 37 °C. After careful aspiration of the supernatants, 100 µl of 2-propanol were added to each well. After complete solubilization of the MTT formazan, the absorbance at $\lambda = 590$ nm of each well was measured after transferring the supernatants into fresh microtitre plates [8].

2.6. BrdU labelling index

To each well, except for the negative control, 10 µl of a BrdU solution (final concentration 10 mM) were added. After incubation for a 120 min period of time (24 h in case of human fibroblast experiments) the cells fixed in methanol/ethanol (1 : 2 v/v) and washed using PBS. The labelling index was determined by EIA methods after incubation of the cells with an anti-BrdU antibody (9).

Mouse fibroblasts L-929 were obtained from American Type Culture Collection, USA and HeLa cells from ICN, Germany. Human fibroblasts were isolated from the human umbilical cord and cultured in primary culture. All cell types were cultured in RPMI 1640 culture medium with L-glutamine, penicillin/streptomycin and 10% fetal calf serum.

2.7. Direct contact tests

Zn and Ni- alloys as well as copper were used as positive controls in the direct contact tests. Silicone was the negative control. Freshly prepared, formerly extracted (see extract dilution method) as well as amalgams, which were formerly removed from patients and the non-amalgams described above were the samples for the test procedure. Cells, human primary isolated fibroblasts, were seeded on the materials and incubated under standard culture conditions for a 24 h period. Samples were fixed with methanol/ethanol and were stained with hematoxylin/eosin following standard protocols. The evaluation of cytotoxic effects was performed with the help of transmission light microscopy (in the case of silicone) or dark field microscopy in the case of metal alloys.

2.8. Evaluation and statistics

Data were evaluated using Student's t-test/f-test. P-values of less than 0.01 were considered to indicate statistically significant differences and calculated with the assistance of Microsoft Excel.

3. Results

3.1. Extract dilution method

As it is illustrated in Fig. 1 silicone gave no significant effects in the MTT assays, while the positive control (copper extract) elicited cytotoxic effects in every dilution of the series (1x-8x). Freshly prepared amalgam elicited a significant cytotoxic effect as well as explanted amalgam in the first dilution. Higher dilutions

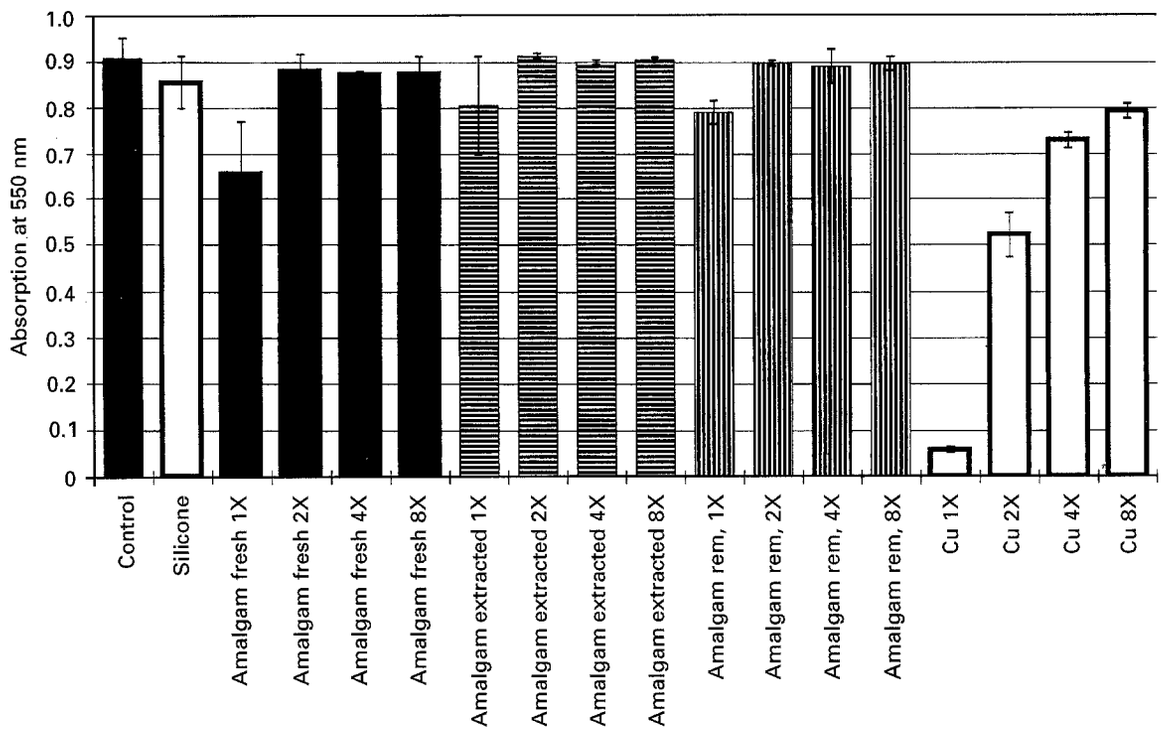

Figure 1 MTT reduction of human primary isolated fibroblasts after 24 h incubation with extracts (extract dilution series: 1x–8x, 1 : 1 v/v dilution) of amalgams (24 h medium extraction): freshly prepared, formerly extracted and removed patient's implant material. Comparison to references: negative: silicone extract and medium control, positive: copper extract dilution series. Cytotoxicity of fresh amalgam 1st dilution and patient's amalgam 1st dilution (mean/SD, $n = 4$) EIA-data, values given in relative absorbance at 550 nm.

gave no significant effects on the MTT reduction. Formerly extracted amalgam elicited no cytotoxic effects in the MTT experiments.

The non-amalgam Qualistar 5 gave no effects on MTT reduction compared to the negative control, while the MTT reduction was significantly decreased in the copper dilution series (Fig. 2).

The BrdU incorporation of HeLa-cells was significantly decreased by molar concentrations of zinc and nickel between 5×10^{-4} M and 1.25×10^{-4} M. Lower zinc/nickel concentrations elicited no significant effect on the BrdU-incorporation of this cell type, while silicone was also ineffective. Freshly prepared amalgam significantly reduced the BrdU-incorporation in the first dilution. There was also a slight but significant reducing effect in the second dilution of the extract. Further extract dilution gave no statistically significant effects (Fig. 3). Fig. 4 illustrates the effects

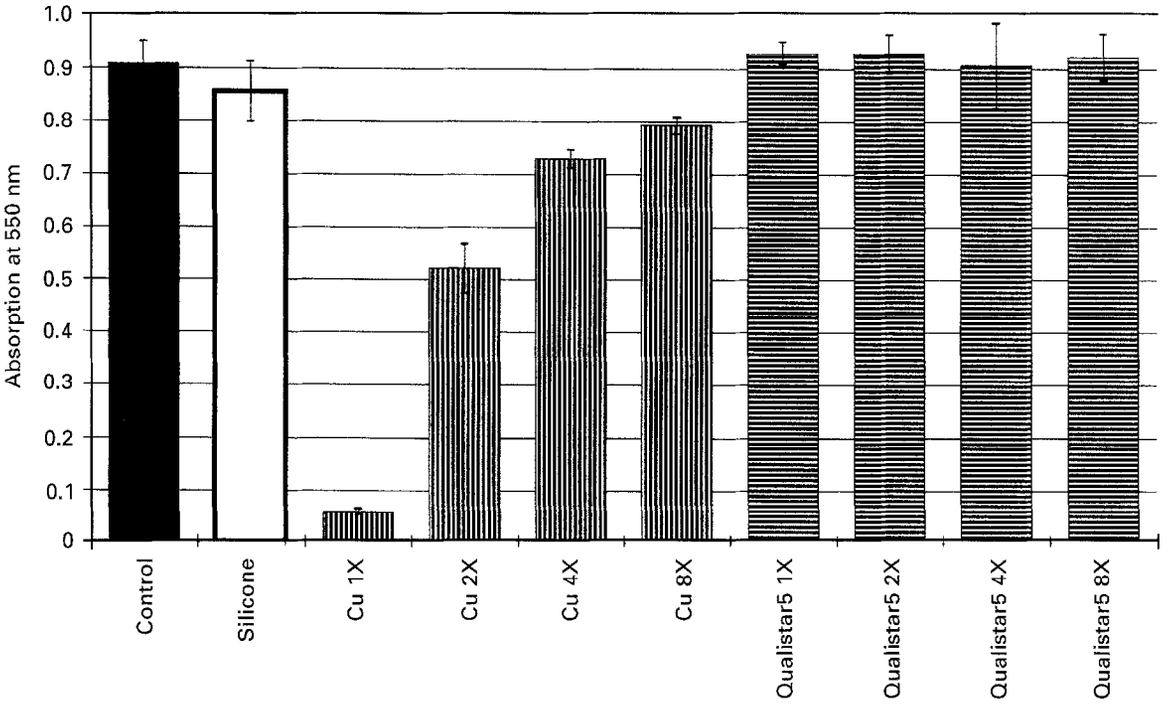

Figure 2 MTT reduction of human primary isolated fibroblasts after a 24 h incubation with extracts (extract dilution series: 1x–8x, 1 : 1 v/v dilution) of a non-amalgam alloy (24 h medium extraction). Comparison to references: negative: silicone extract and medium control, positive: copper extract dilution series. No cytotoxic effect of the alloy. (mean /SD, $n = 4$). EIA-data, values given in relative absorbance at 550 nm.

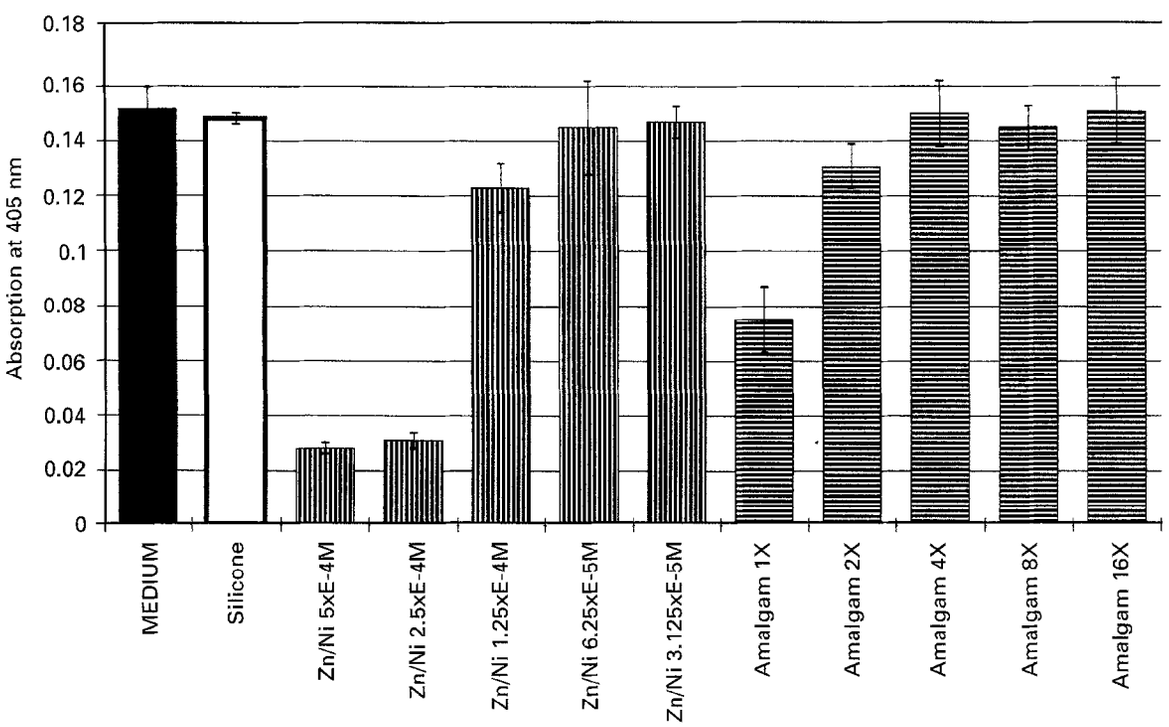

Figure 3 BrdU-incorporation by HeLa-cells after a 24 h incubation with extracts (extract dilution series: 1x–8x, 1 : 1 v/v dilution) of freshly prepared amalgam (24 h medium extraction). Comparison to references: negative: silicone extract and medium control, positive: defined molar concentrations of Zn/Ni ions. Cytotoxic effects of the amalgam in the 1st dilution (mean /SD, $n = 4$). EIA-data, values given in relative absorbance at 405 nm.

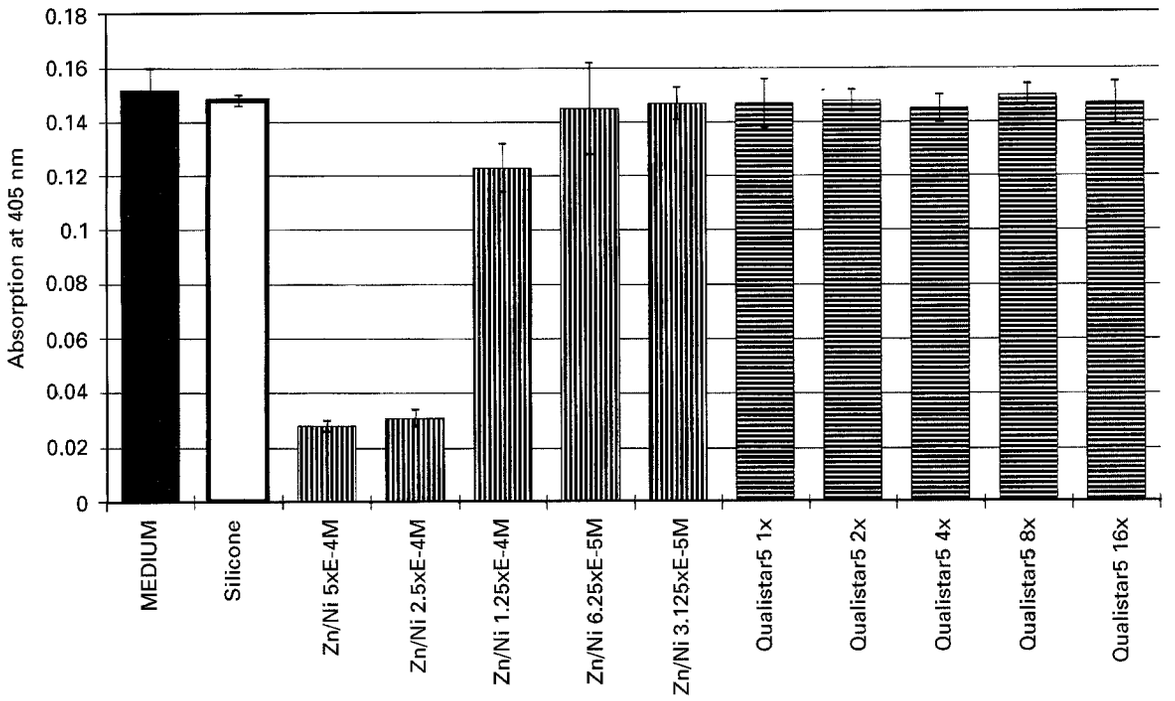

Figure 4 BrdU-incorporation by HeLa-cells after a 24 h incubation with extracts (extract dilution series: 1x–8x, 1 : 1 v/v dilution) of a non-amalgam (24 h medium extraction). Comparison to references: negative: silicone extract and medium control, positive: defined molar concentration of Zn/Ni ions. No cytotoxic effects of the tested alloy (mean /SD, $n = 4$). EIA-data, values given in relative absorbance at 405 nm.

of Zn/Ni-ions in defined molar concentrations on the BrdU incorporation of HeLa cells in comparison to those of an extract dilution series of a selected non-amalgam. It could be shown that the alloy Qualibond 4 elicited no significant effects on the BrdU incorporation of HeLa, while zinc/nickel had deleterious effects. The results of the Qualibond 4 experiments on HeLa were representative of further performed experiments on other non-amalgam alloys, in that no significant reduction of the BrdU-incorporation was found. The data were also representative of extract dilution experiments on non-amalgams using the cell line L-929, as well as primary isolated human fibroblasts, independent of the alloy which had to be tested.

3.2. Direct contact tests

Human fibroblasts elicited a monolayer-like growth when being cultured on thermanox tissue culture plates (Fig. 5), which represented the internal negative control of the direct contact experiments. Darkfield illumination of copper-wire which had been incubated with human fibroblasts under the same standard culture conditions elicited massive cytotoxic effects (Fig. 6). Thus, only some cells remained adherent on the metal surface and gave a rounded-up shape. The silicone negative control gave a different shape of cellular growth compared to the thermanox control, with a colony-like growth being found on this polymeric material (Fig. 7). Freshly prepared amalgam elicited distinct cytotoxic effects in the direct contact experiments, giving naked nuclei and rounded-up cells. However, vital cells were still found as well as areas of monolayer-like growth of human fibroblasts on the amalgam (Fig. 8).

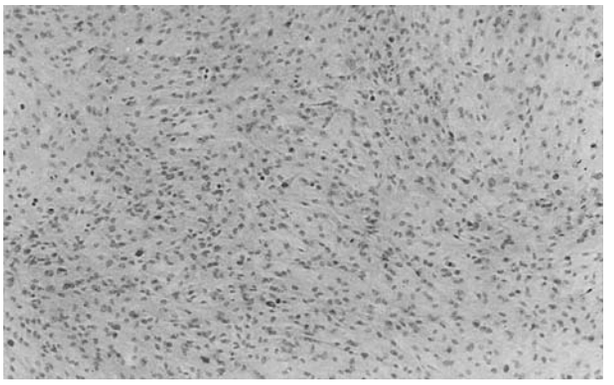

Figure 5 Negative control: Human fibroblasts cultured under standard culture conditions on thermanox tissue culture plates. Results of light microscopical analysis: unaffected, confluent monolayer structure. (magnification ×65).

Figure 6 Positive control: Human fibroblasts cultured under standard culture conditions on copper. Results of light microscopical analysis by reflected-light darkfield illumination: severe cytotoxic effects, rounded-up cells. (magnification ×130).

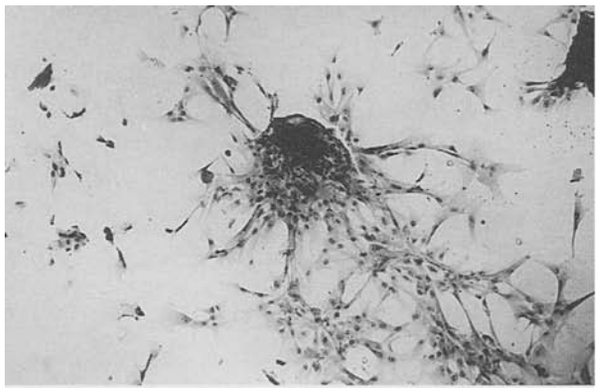

Figure 7 Silicone reference: Human fibroblasts cultured under standard culture conditions on silicone. Results of light microscopical analysis; colony-like growth, with no evidence of cytotoxicity. (magnification × 65).

Figure 8 Non-gamma 2 amalgam (freshly prepared) : Human fibroblasts cultured under standard culture conditions on amalgam. Results of light microscopical analysis by reflected-light darkfield illumination: degeneration of a significant number of cells, naked nuclei: in general significant cytotoxic effects (magnification × 130).

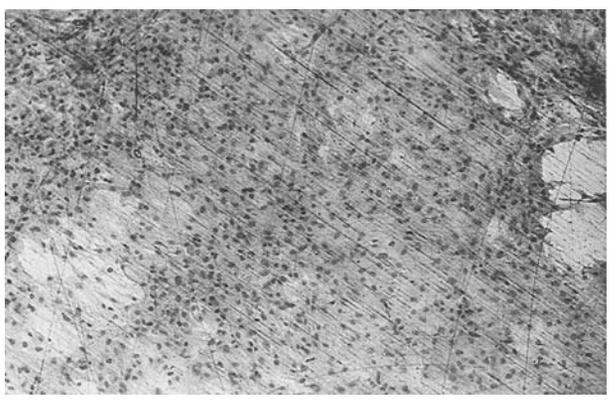

Figure 9 Non-amalgam alloy: Human fibroblasts cultured under standard culture conditions on Qualiplus 6. Results of light microscopical analysis by reflected-light darkfield illumination: monolayer growth, no evidence of cytotoxicity. (magnification × 65).

Fig. 9 illustrates the results of the direct contact tests on the non-amalgam alloy, Qualiplus 6. Human fibroblasts presented a monolayer structure after standard culture on this material, which was comparable to the monolayer found on thermanox plates. Clear cytotoxic effects were not found.

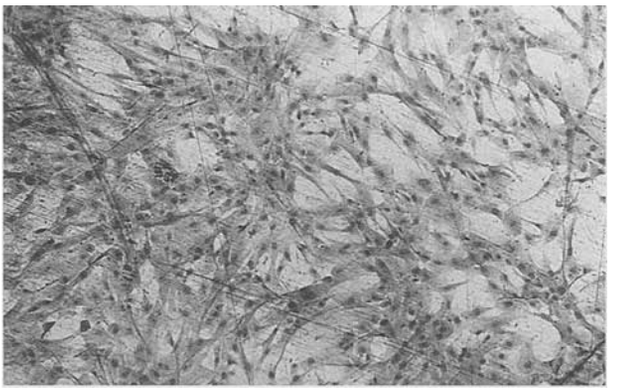

Figure 10 Non-amalgam alloy: Human fibroblasts cultured under standard culture conditions on Qualiceram 2. Results of light microscopical analysis by reflected-light darkfield illumination: monolayer growth as well as colony forming, no cytotoxicity. (magnification × 130).

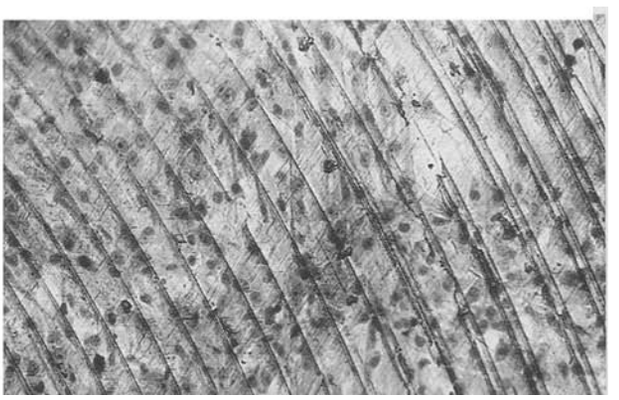

Figure 11 Non-amalgam alloy: Human fibroblasts cultured under standard culture conditions on Qualibond 4. Results of light microscopical analysis (reflected-light): monolayer growth, no cytotoxicity. (magnification × 130).

Direct contact tests on Qualiceram 2 elicited a mixture of characters of growth on this material, with both the monolayer shape, which was found on the thermanox negative control, as well as the colony-like growth which was found in the silicone experiments (Fig. 10). Again cytotoxic effects were not found.

Qualibond 4 again permitted a fibroblast monolayer to form, which is illustrated in Fig. 11. The appearance of the single cells was unaffected, so that the material has to be regarded as non-cytotoxic from the view of the direct contact test method. Further tested non-amalgam alloys also elicited no cytotoxic effects (data not shown). The results of the direct contact experiments were comparable to those presented above.

4. Discussion

It can be suggested from the presented results that amalgams might have cytotoxic effects, especially when being freshly applied. The cytotoxic effects were found to be reduced after both extraction procedures *in vitro*. Significant cytotoxic effects of the tested non-amalgams could not be found. Cytotoxicity of

amalgams is discussed controversially in the literature [10–12].

The detection of cytotoxic effects appears to depend on the detection assay, the time of exposure, as well as on the cell type which is used in the *in vitro* experiments. However, potential cytotoxic effects in general cannot be excluded when screening both the literature and our own results of the presented studies based on the ISO requirements of material cytotoxicity testing.

With respect to formerly presented studies on metal ion effects [13–15], it has to be stressed that the sensitivity of the assays employed might not be sufficient to detect deleterious effects of very low doses of metal ions. Further studies on specific pathways of cellular metabolism are necessary to detect or exclude e.g. influences on the expression of cell adhesion molecules (CAM) or the release of cytokines, both mechanisms which play an important role in the case of inflammation at sites of material implantation. It therefore needs to be stressed that the lack of detection of cytotoxic effects of non-amalgams and dilutions of amalgams in the presented experiments does not necessarily classify these materials as harmless.

The role of cytotoxicity also has to be discussed with respect to possible desirable effects of antibacterial activity [16] of alloys, which have to be regarded as cytotoxic, but which may well fit into a site of implantation where bacteria may cause injury. The insights provided by the present studies might be helpful for a rational choice of dental materials, but they do not allow a final conclusion on 'what is bad and what is good' in the interest of patient health. In addition to avoiding harm to the patient, costs to the public medical system which might be caused by a certain choice of implant materials also need to be considered. Nevertheless, we conclude that the methods which have been established by the ISO, appear to be helpful for the development of a rational, comparable and standardized test system, at least for the detection of cytotoxic effects.

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