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A selective laser melted Co–Cr alloy used for the rapid manufacture of removable partial denture frameworks – initial screening of biocompatibility

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Abstract: The aim of this study was to determine the cytotoxicity of a Co–Cr alloy used for the rapid manufacture of removable partial denture frameworks using murine fibroblasts L929 cell lines and three test methods: the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) assay, the agar diffusion test (ADT) and the dye exclusion test (DET). Two groups of disc specimens (5 mm diameter, 1 mm thick) were fabricated. The first group was cast using a conventional method (CM) in a Nautilus CC casting. The second group was fabricated using selective laser melting (SLM) in SLM Realiser The total cell number and viability of cells pre-incubated with CM and SLM alloys were comparable to the control sample. Differences between the growth inhibitory effects of the CM and SLM alloys in the MTT assay were below 30 %. Results of two independent agar diffusion tests with CM and SLM alloys showed neither detectable discoloration around or under the discs nor a detectable difference in staining intensity. As the cell response for both CM and SLM alloys was 0/0, the discs can be rated as non-cytotoxic. The results suggested that the F75 Co–Cr alloy used for the SLM process did not release harmful material that could cause acute effects against L929 cells under the given experimental conditions.

Keywords: dental alloys; selective laser melting; cytotoxicity; removable partial dentures.

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INTRODUCTION

Over the last decade, there has been a rapid increase in the employment of computer-aided design (CAD) and computer-aided manufacture (CAM) in dental applications. The majority of currently used CAD/CAM systems are based on a milling procedure, whereby requested forms, such as frameworks or full anatomical crowns, are fabricated by milling material from a block. Additive manufacturing (AM), on the other hand, uses a revolutionary layering additive technique, enabling the production of complex customized shapes, such as removable partial denture (RPD) frameworks.

In recent years, the term "additive manufacture" (AM) has been used to describe the fabrication of functional, end use components in a layer-by-layer manner. AM enables the fabrication of geometries unsuitable for alternative methods and can be ideal for low volume or one off production, especially in medical applications.[1,2] In dental technology, research has shown that a combination of CAD and AM may be used to replace laboratory crafting techniques [2–5]. Furthermore, the AM process, selective laser melting (SLM, Realiser GmbH/ /MTT-Group) has been used to fabricate RPD frameworks.[6,7]

The potential advantages of such a process over conventional laboratory techniques can be summarized as reduced manufacture time, inherent repeatability and elimination of inter-operator variation. In addition, CAD could provide some automation of dental processes (for example, cast analysis, undercut elimination and path of insertion identification).

The first steps towards clinical trials have been completed. A RPD framework was made by means of scanning a patient's cast followed by virtual surveying and framework design using CAD, and then CAM production using SLM technology. Conventional finishing and polishing procedures were used to complete the RPD framework.[6] The conclusions of the pilot study were that CAD/ /SLM produced frameworks that were comparable to conventional frameworks in terms of accuracy, quality of fit and function. However, this conclusion was based on a single study and no long-term results are available since there is no known data about the biocompatibility of the specific cobalt-chromium alloy used for the SLM process. Though the basic chemical elements generally match those of the conventional casting alloy, it has been stated that alterations in the composition and pre-treatment can greatly influence the cytotoxicity of an alloy.[8,9] Cell culture studies are the usual starting point when evaluating biocompatibility, providing an investigation of toxicity in a simplified system that minimizes the effect of confounding variables.[10] By using cells from the murine fibroblast cell line, the cytotoxicity of various dental materials, including dental alloys, can be determined. This study used murine fibroblasts (L929) in accordance with the requirements of the ISO standard 7405 (ISO 2008).[11] The aim was to determine the cytotoxicity of the Co–Cr alloy used for the fabrication of

an SLM RPD framework by using L 929 cell line and three test methods: the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) assay, the agar diffusion test (ADT) and the dye exclusion test (DET). To the best of our knowledge, there are no reports about the cytotoxicity of the selected laser melted Co–Cr alloy used for the rapid manufacture of RPD frameworks.

EXPERIMENTAL

Sample preparation

a) Conventional method (CM) samples. The composition of the commercially available alloy Remanium GM380+ (Dentaurum, Ispringen, Germany) used in this study is presented in Table I.

TABLE I. Composition (mass %) of the Remanium GM 380+ and Sandvik Osprey F75 alloys

Alloy	Co	Cr	Mo	Si	Mn	N	C	Fe	Ni
Remanium 380+	64.6	29	4.5	< 1	< 1	< 1	< 1	–	–
Sandvik Ospreys F75 alloy	Balance	27–30	5–7	<1	<1	–	<0.35	<0.75	<0.5

The alloy is a non-precious Co–Cr alloy containing no Ni, Be or Fe, which is widely used as a cast partial denture alloy. Five disc specimens (5 mm diameter, 1 mm thick) were obtained from wax patterns that were invested and cast according to the manufacturer's instructions. The investment used was Rema dynamic (Dentaurum, Ispringen, Germany), and vacuum casting was performed using a Nautilus CC (Bego, Bremen, Germany). After casting, the discs were divested and blasted with 100 μm aluminium oxide particles, then polished with silicon carbide papers in the sequence 320, 400, 600, 1200, 1500, and 2000. Final polishing was performed using oxide pastes.

b) Selective laser melting (SLM) samples. Five disc specimens (5 mm diameter, 1 mm thick) were first built in a virtual environment (Magics, Materialise, Belgium). The physical specimens were produced using an SLM Realiser (MTT-Group, UK). The powdered alloy used in the process was a Co–Cr type alloy, the composition of which contained a maximum of 0.5 % Ni (F75 alloy, Sandvik Osprey Ltd., UK, Table I.). After the build, the supporting structures were removed, and then the samples were prepared as described above for the cast samples.

Cell lines

L-929 cells were grown in a suitable culture medium, supplemented with antibiotics to prevent the growth of opportunistic microorganisms. The cell population was divided twice a week and placed in fresh media to stimulate growth and development. The tissue was broken into a suspension of single cells by enzymatic digestion in the presence of a chelating agent. The cell lines were cultured in 25 cm^3 flasks at 37 °C in 100 % humidity and 95 % air, 5 % CO_2. Only cells in the rapid or exponential growth phase of development were used for the assays. The cell number and percentage of viable cells were determined by a dye exclusion test (DET) with Trypan Blue.[12] The viability of the cells used in the assay was over 90 %.

Cytotoxicity tests

The cytotoxicity was measured as a percentage of cell growth inhibition using the three tests described below.

a) *Dye exclusion test (DET)*. Petri dishes (50 mm; Falcon, Becton Dickinson and Comp., Franklin Lakes, NJ, USA) containing CM or SLM alloy discs were plated with viable mouse cells and incubated for 24 h at 37 °C in 95 % air and 5 % CO_2. Control samples were also incubated but contained no alloy discs. After incubation, a single cell suspension was obtained by trypsinization. Cell number and viability were assayed by Trypan Blue staining in a counting chamber.[12] (Dead cells take up Trypan Blue whilst living cells do not.). Over 90 % of the cultured cells were viable (*i.e.*, no uptake of Trypan Blue) when assayed.

b) *MTT assay*. Growth inhibition was also evaluated by the tetrazolium colorimetric MTT assay (ISO 2009).[13] The assay depends on the cleavage of the tetrazolium salt MTT to purple formazan crystals by mitochondrial dehydrogenases in viable cells.

Cells (L929) were cultured in Petri dishes containing CM or SLM alloy discs and incubated for 24 h at 37 °C, in air containing 5 % CO_2. The control samples contained no discs. After incubation, the cells were detached from the alloy discs using enzymatic digestion, and counted in a counting chamber using Trypan Blue stain. New cells were cultured for 48, 72 or 96 h at 37 °C in 95 % air and 5 % CO_2. These cells were then cultured for 3 h with yellow MTT solution and the purple formazan product was isolated and dissolved in 100 µl of 0.04 M hydrochloric acid in 2-propanol. The cytotoxicity was expressed as the percentage growth inhibition.

c) *Agar diffusion test (ADT)*. L-929 cells were incubated for 24 h at 37 °C in 95 % air and 5 % CO_2 after plating on Petri dishes (10 mm). The concentration of cells was 10 ml; 2.5×10^5 cells ml^{-1}. Sterile agar was heated and a nutrient medium added. The mouse cells were combined with the agar-nutrient mixture and allowed to solidify over 30 min. The cells were stained with a neutral red solution (or toluylene red, Basic Red 5, or C.I. 50040; IUPAC name: toluylene red) and kept in the dark for 15 min. Two samples of CM or SLM discs were placed into each Petri dish and the dishes incubated for 24 h at 37 °C in 95 % air and 5 % CO_2.

Any interaction between the metal and the cells, causing the cells to die and lose the red dye, was noted using an inverted microscope, Reichert-Jung, Biostar, model 1820E. It is well known that living cells retain the red dye. Thus, the decolourised zones of dead cells were measured using a ruler and analysed according to ISO standards (2008).[11] The results were evaluated according to the zone and lyses index and rated for the severity of the cytotoxicity, as described previously.[12] Each product was tested in quadruplicate and the experiment was repeated twice.

Statistical analysis was realised using the Statgraphics Centurion program. The data were statistically evaluated by the Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Rounded, disc-shaped experimental samples are shown in Figs. 1 and 2 (CM and SLM samples, respectively). The unpolished SLM samples exhibited very fine surface roughness, caused by the transition of the laser beam during the manufacturing process. The polished samples correspond to the state of the final alloy under oral environmental conditions.

Fig. 1. CM Samples (left – cast and sandblasted, right – polished).

Fig. 2. F75 SLM Samples (left – untreated, right - polished). Note the fine surface roughness of the untreated sample caused by the laser beam.

The L929 fibroblasts were pre-incubated in culture medium with CM or SLM alloys for 24 h and then the survival rates of the pre-treated cells were evaluated by the standard procedure for the DET or MTT assay. The results of the DET and MTT assays are presented in Figs. 3 and 4, respectively.

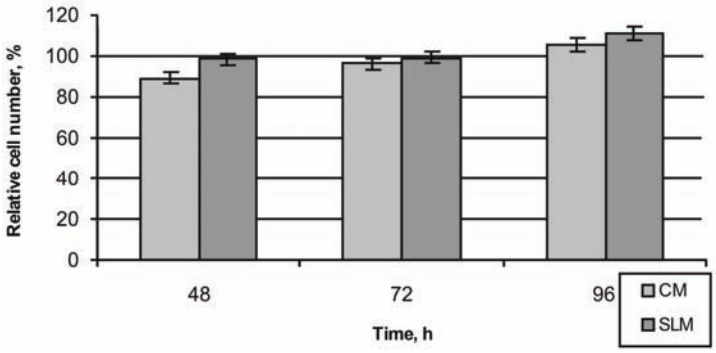

Fig. 3. Dye exclusion test (DET). Recovery of L929 cells pre-incubated with CM and SLM alloys for 24 h. The results show the relative cell number obtained from two independent experiments completed in triplicate. Data are shown as the mean and the bar indicates the standard deviation ($p > 0.05$).

The variation in the cell numbers of pre-incubated cells compared to the control sample was small: 8 percent below and 15 percent above the control value for both the CM and SLM alloys, respectively. The viability of each sample was 99 %.

The cell number steadily increased during the recovery period for both CM and SLM alloys (48–96 h), which indicated that no cytotoxic effects were registered in the several cell generations. There were no statistical significant differences between treatments regardless of the recovery period.

In the MTT assay during the same recovery period, no cytotoxic effects of the CM or SLM alloys against L929 cells were detected (Fig. 4). Differences between the growth inhibitory effects of CM and SLM alloys were found but the growth inhibition level was not statistically significant ($p > 0.05$). Therefore, both alloys can be rated as non-cytotoxic.

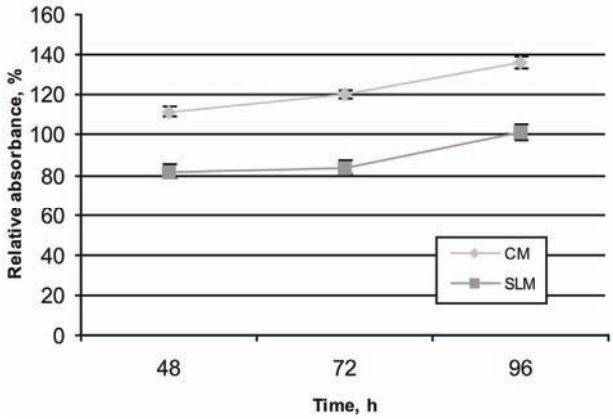

Fig. 4. MTT Assay. Survival of L929 cells pre-incubated with CM and SLM alloys for 24 h. The results represent the relative absorbance of the pre-treated cells obtained from two independent experiments, completed in quadruplicate. The data are shown as the mean and the bar indicates the standard deviation ($p > 0.05$).

The results of two independent ADT with CM and SLM alloys showed a detectable discoloration neither around nor under the discs, or a detectable difference in the staining intensity. As the cell response to both the CM and SLM alloys was 0/0, the discs can be rated as non-cytotoxic.

DISCUSSION

Super-alloys, such as Co–Cr, are suited to the SLM process as the material properties facilitate the process physics, such as melt-pool and temperature gradient control. However, alloys available for AM processes, such as SLM, routinely include nickel. When the specifications for these alloys were developed, there were allowable levels of various elements, which permitted recyclers more flexibility in making low-cost alloys. The alloy used for the tests reported herein contained a maximum of 0.5 % nickel. AM alloys containing a maximum of 0.1 % nickel can be obtained but this adds considerably to the cost.

Among other elements, the use of nickel in dental alloys has often been attacked because of its potential side effects.[14] Severely cytotoxic Co–Cr alloys contained high amounts of Ni, although no general correlation between the overall alloy composition and cytotoxicity was detected.[15] While Co and Cr undergo redox-cycling reactions, the primary Ni route is depletion of glutathione and bonding to sulph-hydryl groups of proteins.[16] However, it can be stated that Ni showed a negative effect in combination with certain other metals and, therefore, does not necessarily contribute to a toxic or allergenic potential.[17]. Generally, element release is not simply proportional to its abundance in the bulk alloy, but is also highly dependent on the inter-ionic interactions within the alloy.[10]

Cytotoxicity tests can implement several cellular parameters, such as cell viability, DNA/RNA/protein synthesis, membrane integrity, *etc*. In this study, cytotoxicity was assessed by three methods, addressed to different ends, *i.e*., viability and cell survival. Viability was determined by short-term (24 h) ADT and DET assays, and cell survival, after cell pre-treatment with the alloys, by DET and MTT assays. Although only the ADT assay has been prescribed as an ISO standard (ISO 2008),[11] the use of different test methods is highly advisable.[18] While the DET and ADT methods rely on the breakdown of membrane integrity, the MTT method focuses mainly on the mitochondrial function (dehydrogenase activity).[19] Although the last test showed a slightly worse outcome for the SLM alloy, cellular proliferation in the subsequent period (48, 72 and 96 h), which covered several cell cycles *i.e*., cell generations, showed no significant damage to the cell function. Replication during an extended contact period with potential toxic substances, however, showed good biocompatible properties of the chosen SLM alloy. Furthermore, membrane lyses was not detected in the ADT or DET assay when L929 fibroblasts were exposed to the examined alloys. The negative effect decreased with time for both examined substances.

The results suggested that the alloys did not release harmful material that could cause acute effects against L929 cells under the given experimental conditions.

In an oral environment, the intimate contact between the alloy and tissues can create microspaces with a high concentration of released metal ions. Alloy surface properties can be of decisive importance in such situations; a point supported by findings suggesting less biocompatibility in under as-cast alloy conditions compared to its highly polished state.[20] Enhanced contact might lead to local adverse tissue reaction.[21] Ensuring that cast or AM-produced frameworks are appropriately finished and that their porosity is low remains dependent on human subjectivity.

The murine L929 fibroblast assays represent sufficient screening models for an investigation of the *in vitro* toxicity of metal cations. They exhibit a similar

response with gingival fibroblasts; hence, it can be assumed that the SLM alloy also does not have cytotoxic effect on gingival tissue.[20,22,23]

Generally, corrosion is characterized by electrochemical phase boundary reactions which cause the liberation of metal ions.[14] The amount and nature of released cations varied depending upon the type of alloy and other parameters, *e.g.*, type of corrosion, composition and chemical characteristics of the corrosive solution-such as pH and ionic composition, artificial saliva, cell culture medium, serum, e*tc*.[25–27] In one study, ion release from cast and SLM Co–Cr alloy was compared.[28] The main ion released was cobalt, as, due to the passivating behaviour or chrome, only a small amount of chromium and molybdenum was detected. Due to the low releases of ions, the corrosion was influenced almost completely by the surface. The SLM test specimens showed lower emissions than the cast specimens did because the laser molten material is more homogeneous, contains fewer pores and has a finer microstructure. However, almost no difference was detected after two weeks between the different variants examined, having concentrations below the detection limit of the analyzing method.

However, oral mucosa could present only an increased resistance towards the leakage of cytotoxic agents, as it becomes keratinized and has a protective mucin layer. On the other hand, it should be emphasized that the oral environment includes severe biological factors, plus interactions such as saliva composition, pH status, *etc*. Nevertheless, based on the obtained data, the SLM alloy shows promising potential to withstand environmental conditions and have a life span comparable to the currently used cast alloy when biocompatible properties are concerned.

Although the lost wax procedure has been a central technique in RPD framework production for a very long time, AM with its link to information technology might be of great interest in dentistry in general.[24] Linking intra-oral scanning to CAD and AM has the potential to remove laborious laboratory techniques and improve accuracy and repeatability.

Future research on the mechanical properties, as well as *in vivo* tests of the SLM or other AM produced dentures are necessary to show whether in reality a revolution is at hand, as it appears.

CONCLUSIONS

Based on the results of the MTT, ADT and DET tests employed in this study, it can be concluded that the Co–Cr alloy routinely in use in AM technologies such as SLM, does not exhibit cytotoxic potential. Further clinical trials should be performed to show the *in vivo* behaviour of this alloy under oral environmental conditions.

ИЗВОД

СЕЛЕКТИВНО ЛАСЕРСКО ТОПЉЕЊЕ Co–Cr ЛЕГУРЕ ЗА СКЕЛЕТИРАНЕ ПРОТЕЗЕ – ИНИЦИЈАЛНА ПРОЦЕНА БИОКОМПАТИБИЛНОСТИ

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Циљ студије је да одреди цитотоксичност F75 Co–Cr легуре, која се употребљава током компјутерског процеса производње скелетиране парцијалне протезе, коришћењем L929 ћелијске линије мишјих фибробласта и три методе: MTT теста, агар дифузионог теста (ADT) и теста губљења боје (DET). Направљене су две групе узорака (5 mm пречник, 1 mm дебљина). Прва група је изливена од легуре кобалт–хром конвенционалном методом (CM) у пећи за ливење Nautilus CC. Друга група је направљена коришћењем методе селективног топљења ласером (SLM) у апарату SLM Realiser. Укупан број ћелија, преинкубираних са CM и SLM легуром, као и њихова одрживост упоређени су са контролним узорком. Разлике у инхибиторном ефекту на раст CM и SLM легуре у MTT тесту биле су мање од 30 %. Резултати два независна агар-дифузиона теста са CM и SLM легуром не показују приметно обезбојавање око или испод дискова, нити приметну разлику у интензитету пребојавања. Како је ћелијски одговор за CM и SLM легуру био 0/0, дискови се могу окарактерисати као не-цитотоксични. Резултати сугеришу да F75 Co–Cr легура, која се користи у SLM процесу добијања скелетираних протеза не отпушта штетне материје, које могу проузроковати акутне ефекте на линију L929 ћелија под датим експерименталним условима.

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