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Silver Releasing from Dental Alloys: Cytotoxic and Inflammatory Response in Vitro

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Abstract

Background: *Although silver used in dental alloys is not classified as mutagenic, carcinogenic, or toxic to reproduction, the European Chemicals Agency (ECHA) has listed both ionic silver and silver nanoparticles as biocidal substances. In this context, the present study aimed to assess silver release and evaluate the cytotoxic and pro-inflammatory potential of various silver-containing dental alloys from the manufacturers operating within the European Union.*

Methods: *Eight dental alloys, containing between 6.4% and 58% silver, were subjected to standardized extraction tests using a racemic lactic acid and sodium chloride solution at 37 °C for 7 days. In parallel, cytotoxicity was assessed using the MTT/MTS assay on L929 fibroblasts and THP-1 monocytes, following ISO 10993-5 guidelines. Additionally, TNF-α secretion by THP-1 cells was quantified to evaluate potential inflammatory responses.*

Results: *Extraction tests revealed low levels of silver release, ranging from 11 µg/L to 314 µg/L. In vitro cytotoxicity assays showed minimal to no cytotoxic effects on both L929 and THP-1 cells. Interestingly, some alloys even promoted slight proliferative responses in THP-1 cells. No significant induction of TNF-α was observed following exposure to any of the tested alloys.*

Conclusions: *Despite the inclusion of ionic silver in the ECHA's list of biocidal substances, the tested silver-based dental alloys released only small amounts of silver and exhibited no relevant cytotoxic or inflammatory effects under in vitro conditions. These findings support their continued evaluation within biocompatibility frameworks.*

Keywords: Silver, Alloys, Dental Alloys, Dental Materials, Toxicity, Cytotoxicity, Biocidal, European Union.

Introduction

Silver is often recovered as a by-product from the extraction of copper, lead, zinc and gold ores. It is widely used in the manufacture of jewelry, silverware, electronic components, electrical connectors, dental alloys, surgical prostheses as well as various medical devices [1]. Silver has also been used in photography, in the manufacture of electrodes for welding and brazing, in numismatics, as well as an antibacterial agent for the disinfection of drinking water and swimming pools.

Ancient civilizations already knew the bactericidal and fungicidal properties of silver. As early as 1500 BC, the Egyptians used silver salts as astringents [2, 3]. Other cultures such as the Indians, Egyptians, Persian kings, Phoenicians, Greeks and Romans used both silver and copper to preserve food and purify water [4, 5]. More recently, silver has been used in sutures and

for infection prevention [6].

Although the antimicrobial properties of silver have been exploited since ancient times in many applications, its medical use declined sharply after the discovery of antibiotics by Fleming in the 1920s. However, soluble silver compounds, such as silver salts, have been used in the treatment of mental disorders, epilepsy, gastroenteritis, and infectious diseases such as syphilis and gonorrhea [7-9].

Several factors influence the ability of a metal to produce toxic effects on the human body, including its solubility, its ability to bind to biological sites, as well as the fate of the formed metal complexes (metabolism, storage or excretion) [10, 11]. Existing studies suggest that some forms of silver are more toxic than others [12-16]. Due to apparent differences in toxicity between soluble and insoluble forms of silver, a critical review of the scientific literature was conducted to assess potential adverse health effects, particularly in occupational exposures.

Silver Exposure: Sources and Health Effects in Humans

Humans are exposed to silver and its compounds through various environmental sources (air, dust, drinking water, etc.), as well as through food, with silver being permitted as a food additive. Exposure may also originate from everyday consumer products, such as jewelry, watches, cosmetics, natural health products, food packaging materials, silverware, drugs, dental alloys/amalgams, electronics, toys, etc [17-19].

The diversity of uses for money leads to several ways of entry into the organization. Ingestion is considered the main route of exposure to silver compounds and colloidal silver proteins. Skin contact can occur from applying burn creams or wearing jewellery [18]. Silver can also enter the body through the use of amalgams or dental alloys [19-23], acupuncture needles, catheters, or as a result of accidental puncture wounds [24, 27].

Soluble silver compounds are more easily absorbed than metallic silver or insoluble forms and are therefore more likely to produce adverse effects on the human body [28]. Small amounts of money can accumulate in the brain and muscles over time [29-32]. Prolonged inhalation or ingestion of soluble or colloidal silver compounds (such as nanoparticles) can cause conditions such as argyria or argyrosis [33-36].

Argyria and Argyrosis are the result of exposure to soluble silver compounds that generate toxic effects, including liver and kidney damage, irritation of the eyes (argyrose), skin (argyria), respiratory and intestinal tract, and changes in humans blood cells. The affected area becomes bluish-grey or ashy grey and is most visible on body sun-exposed areas People are exposed to this substance and its compounds from environmental sources (air, dust and drinking water, etc.) and food (silver being a permitted food additive), as well as from products available to consumers (jewelry, watches, cosmetics, natural health products, food packaging materials, silverware, medicines, dental alloys/amalgam, electronic items, toys etc.). Acute symptoms of overexposure to soluble silver are decreased blood pressure, diarrhea, stomach irritation, and breathing. Chronic symptoms of prolonged ingestion of low doses of soluble silver salts include fatty degeneration of the liver and kidneys and changes in blood cells.

Despite these potential effects, silver—in whatever form — is generally not considered toxic to the immune, cardiovascular, nervous or reproductive systems, and is not classified as carcinogenic. Metallic silver, in particular, appears to pose a minimal health risk [37-41]. Nevertheless, scientific research continues in order to clarify some still uncertain points in this field.

Toxicity Generated by Exposure to Silver

In this chapter the subject can be approached in the following way:

• Concerns Professionals who are in prolonged contact with Ag/Ag salts is not discussed in our publication. However, it should be remembered that there are several occupational exposure limits and guidelines for silver, but the values for each depend on the form of the silver as well as the organization that makes the recommendations. For example, the American Conference of Governmental Industrial Hygienists (ACGIH) USA set the limit value for soluble silver at 0.01 mg/m3. In contrast, the Acceptable Exposure Limit (PEL) recommended by the Occupational Safety and Health Administration (OSHA), Mine, Safety and Health Administration (MSHA) and the National Institute for Occupational Safety and Health (NIOSH), is 0.01 mg/m3 for all forms of silver.

• Concerns the General Population and the Consumer

From a toxicological perspective, exposure to silver can be classified into two broad categories: soluble compounds of silver and insoluble or metallic forms. For the general population, ingestion—including through drinking water and food — is the main route of exposure. On the other hand, in occupational settings, inhalation represents the most significant route of exposure.

Some populations may be exposed to levels of money higher than natural levels, notably workers in industries that handle or process silver compounds, as well as members of the general population who consume water or foods containing high concentrations of silver. This includes, for example, seafood from areas close to industrial discharges or wastewater, as well as agricultural crops from soils or atmospheres where the concentration of silver is high.

In consumers, exposure may result from the frequent use of products containing silver: cosmetics, health products, jewellery, silverware, medicines, toys, etc. In this case, dermal absorption is a potential route of systemic absorption. Many health agencies have assessed the exposure risks for this population, including: US Agency for Toxic Substances and Disease Registry (ATSDR), Danish Environmental Protection Agency (Danish EPA), European Food Safety Authority (EFSA), the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the Netherlands Institute of Public Health and Environment (RIVM), the OECD Cooperative Programme for the Assessment of Chemicals, the Institute of Medicine (IOM), the US EPA's IRIS system, as well as the WHO's drinking water quality program. These assessments, like those in the OECD assessment reports (SIAR), are subject to rigorous review and approval processes, and are considered reliable references for characterizing money-related health hazards.

A thorough review of the literature reveals a considerable amount

of data, with over 16 national and international organizations involved in assessing the risks of silver exposure to populations. According to Hays et al. the majority of available toxicological studies in animals, as well as epidemiological studies in humans, are based on external doses administered through different routes of exposure (oral, dermal, or inhalation). As a result, there is no direct method to interpret the concentrations of chemical substances measured in blood or urine during biomonitoring studies to infer a health risk. It is therefore not possible to directly compare an external dose expressed in mg/kg body weight/day with blood (mg/L) or urine concentrations (mg/L or mg/g creatinine), which makes the interpretation of risks for the general population more complex [42].

Canadian Health Measures Survey (CHMS) in General Population

A rigorous and comprehensive study of the Canadian population was conducted as part of the Canadian Health Measures Survey (CHMS), a national program coordinated by Statistics Canada to collect data on the overall health of Canadians [43].

Designed to be nationally representative, the survey has a biomonitoring component, in which the concentrations of several metals—including barium (Ba), molybdenum (Mo), silver (Ag), thallium (Tl) and tin (Sn) — were measured in the blood and urine of 5,500 to 7,000 people at each cycle.

This chapter is based on data from 2016, which evaluates only the effects on human health and exposure to inorganic forms of these metals. Other elements that may be present in certain substances (such as gold) have not been taken into account. The analyzed sample covers subjects aged 3 to 79 years, excluding pregnant women and people with serious chronic diseases [44-46].

For silver, the estimation is based on the total concentration measured in blood, taking into account the following compounds: Metallic silver, silver nitrate (AgNO3), Silver chloride (AgCl) Silver bromide (AgBr), Silver sulphate (Ag2 SO4), Silver oxide (Ag2 O), et silver sulfide (Ag2 S).

Total concentrations of silver in whole blood or urine are a relevant biological indicator and an integrated measure of exposures that can occur through multiple routes (i.e., oral, dermal, and inhalation) and sources (i.e., environment, diet, or frequent or daily use of certain public consumer products) [47, 48]. So, the estimates are based on the total amounts of silver found in the blood. Results from the risk assessment revealed that silver and its compounds pose a low risk to human health, at the exposure levels analysed in the screening assessment. Thus, the concentrations found are between 0.025 -0.26µg/L in whole blood, below 0.4µg/L given as Reference Dose (DRf) by EPA, MIREC (Mater-nal-Infant Research on Environmental Chemicals.

Legislation Concerning Values of Metallic Silver

In 1966, the American Conference of Governmental Industrial Hygienists (ACGIH) established a time-weighted average exposure limit value (TLV-TWA) of 0.01 mg/m3 for soluble silver compounds, mainly based on the publication by Hill and Pillsbury [46]. However, following studies carried out after their work, a new TLV ((Threshold Limit Value–Time-Weighted Average) of 0.1 mg/m3 was set in 1980 for metallic silver.

Despite research on silver and its salts, there is currently no specific legislation or testing standards established to assess the migration or release of ionic silver and its compounds from consumer products. Nevertheless, relevant data are available and could contribute to the development of new guidelines to better protect consumers [49].

Regarding drinking water, the measured silver concentrations are generally less than 0.005 mg/L [50, 51]. On this basis, the World Health Organization (WHO) estimates the average daily intake at approximately 0.007 mg/day (or 0.12 µg/kg body weight/day). The U.S. Environmental Protection Agency (EPA) analysed several studies that concluded that silver ion concentrations up to 0.2 mg/L in drinking water had no adverse effects in experimental animals [52, 53].

Currently, the European Chemicals Agency (ECHA) has included silver in the list of biocidal substances, notably in ionic form (Ag+) and in nanoparticle form. Although nano-silver has many advantages in microbiological, health and consumer fields, it is not yet classified under the REACH regulation on chemical substances in Europe [54].

In view of these elements, this subject deserves special attention and thorough research [57,58]. According to the Biocidal Products Regulation (Regulation (EU) No 528/2012 - BPR), any biocidal product must be authorised before being placed on the market or used in the European Economic Area (EEA). The process has two steps: evaluation and approval of the active substance (e.g. ionic silver), followed by authorisation of each product containing or generating this substance [55].

In this context, dental alloys classified as medical devices are not covered by these obligations. On the other hand, other materials containing money and used by consumers could be subject to the restrictions of the new European regulation, of biocidal properties. These developments are of particular concern to European importers and manufacturers of metallic silver supplying materials for the production of dental prosthetic restorations [56-59].

Silver in Direct Contact with Foodstuffs

Metals and alloys that contain silver are used in food contact materials and objects such as food processing equipment, household containers and utensils, as well as food packaging sheets. These materials are frequently used as a safety barrier between food and the environment.

The European Food Safety Authority (EFSA) has set a specific migration limit of 0.05 mg Ag/kg feed. The WHO estimates that this value corresponds to about 12.5% of the no observed adverse effect level (NOAEL) in humans.

Silver in Dental Applications

In the field of dentistry, modern materials that incorporate or are coated with silver are now widely recognized for their antibacterial properties [60]. Fixed and removable dental prostheses, implant-supported restorations, oral appliances, occlusal splints, and orthodontic devices may all contain or be treated with silver. Furthermore, the antimicrobial effectiveness of dental implant

surfaces coated with various metal elements—such as silver or silver-zinc—has been demonstrated as a means of preventing bacterial colonization and peri-implantitis.

However, a delicate balance exists between the beneficial antimicrobial or biocidal properties of silver-containing materials and their potential local or systemic adverse effects.

In light of this complex and multifaceted context, the present study was designed to investigate various silver-containing alloys used in dentistry. Although zirconia ceramics and Co-Cr-based alloys dominate current dental prosthetics—facilitated by CAD/CAM technologies, including advanced computer-controlled milling and 3D printing—silver-based alloys remain in use. It is important to note that in the oral cavity, prosthetic materials are in continuous contact with saliva and living tissues.

Dental alloys are classified as medical devices and must therefore comply with ISO 10993 standards [61]. Although no specific limits for ion release are explicitly defined in the literature, the biocompatibility assessment of such devices includes key evaluations such as cytotoxicity, sensitization, irritation, intracutaneous reactivity, acute systemic toxicity, subchronic toxicity, genotoxicity, and carcinogenicity—essential criteria for their approval as medical devices.

In this Context, our study aimed to quantify the release of silver ions from eight dental alloys with silver concentrations ranging from 6.4.2% to 58%. "In addition, we evaluated the cytotoxic behavior in accordance with the ISO 10993-Part 5 guidelines, complemented by an assessment of the inflammatory response induced by the alloys using TNF-α assays.

Materials and Methods

Our study focused on eight silver-containing dental alloys, obtained from a single supplier in the European Union market. The coding of the alloys used for extraction, cytotoxicity and TNF-α tests, as well as their chemical composition, is presented in Table 1. The alloys tested for extraction were coded from #1 to #8, with a silver content varying from 6,4% to 58% (Weight). The preparation of samples was carried out in accordance with ISO 22674:2022 and ISO 10271:2020 [62, 63].

Table 1: Chemical Composition of Dental Alloys (% weight) Tested for Extraction, Cytotoxicity and TNF -α

	#1	#2	#3	#4	#5	#6	#7	#8
Au	6.1	75.2	72	15		51	56	2
Ag	6.4	9.2	13.6	20	24.5	27	28	58
Pt		9	3					
Pd	75			52.2	61.4	7	12	32.9
Ir		< 1.0	< 1.0			< 1.0	< 1.0	
Ru	< 1.0			< 1.0	< 1.0			< 1.0
Cu		3	10.4			14		
In	5.9	1		6	2		1.4	1.5
Zn		2.5	< 1.0		2	< 1.0	2.5	3.5
Sn	< 1.0			5.5	10			2
Ga	6			1.1				

Cations Release Assessment

The sample, with a surface area of 11.3 cm was immersed in 10 ml of a 10 g/l (± 0.1) solution of 90% racemic lactic acid and 5.85 g (± 0.005) of ultrapure sodium chloride in a Falcon medical device test tube. The solution was also prepared with ultrapure quality water, conductivity (0.06 - 0.2 µS/cm) and without silicon. The extraction solutions are filtered on a 0.22µm Falcon cellulose acetate sterilized membrane. After 7 days (± 1 hour) at 37°C the sample was removed from the solution and it was analyzed by ICP OES and ICP-MS. The values given are the average of the three analyses. The samples were analysed by ICP OES (Inductively Coupled Plasma Optical Emission Spectroscopy) Optima 7300 V Perkin Elmer and ICP-MS (Inductively Coupled Plasma Mass Spectrometry) iCAPTM MSX Single Quadrupole Thermo Fisher. To increase the accuracy of measurement, we use crossover technique and the cation matrices are measured according to the following scheme:

- ICP - MS: Ba, Be, Cd, Co, Li, Mo, Nb, Pb, Sb, Sn, Zr, Zn, Ga, In
- ICP - OES : Al, Cr, Cu, Fe, Ni, Ti, V, Zn, Au, Ag, Pd, Pt
- ICP - OES / Hydrides : As, Hg, Sb, Se.

The extraction values are expressed in µg/L and µg.cm-2. week-1.

Biological Evaluation: effects on cell toxicity and TN Falpha induction.

The aim of this work is to evaluate, on the one hand, the cytotoxicity of silver-containing alloys, and on the other hand, the inflammatory response induced by these alloys via the production of TNF-α (Tumor Necrosis Factor alpha).

The production of the pro-inflammatory cytokine TNF-α is particularly relevant, especially in addition to cytotoxicity tests according to ISO 10993-5. The release of TNF-α can indeed be triggered by metal particles from corrosion, by released metal ions (e.g. Ag , Cu 2), or even by irritation or cellular stress caused by non-biocompatible materials.

Cytotoxicity Tests and Tnf Induction

Tested metal specimens were in the form of a disc (11mm in diameter) with a surface metallographically polished with a 1 µm diamond paste. The samples were cleaned in a 1% detergent solution (RBS 35) with ultrasounds. Samples were washed with water and sterilized by immersing in 70% EtOH for 15 minutes at room temperature. After this, samples were rinsed with sterile water, placed into sterile plastic containers and allowed to dry. Aseptic/sterile methodology was used throughout all exper-

imental procedures. Controls were cells that were not exposed to alloys. Samples were evaluated in cytotoxicity assays according to the ISO 10993 and part 5.

Cells

L929 and THP-1 cell lines were obtained from the American Type Culture Collection (ATCC, USA). Both cell types were maintained under standard culture conditions. Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 1% penicillin-streptomycin (Pen/Strep) L929 cells are murine fibroblasts widely used in ISO-standardized cytotoxicity assays due to their sensitivity to toxic substances.

THP-1 cells are human monocytic cells that can differentiate into macrophage-like cells and are commonly used to study immune responses, particularly the secretion of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α).

Before use in experiments, both cell lines were passaged 2–3 times to ensure optimal growth and viability. Cell density and viability were monitored by trypan blue exclusion using a hemocytometer to ensure consistent experimental conditions.

Assays

The MTT/MTS assay (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, Promega) and the TNF-alpha ELISA kit (Miltenyi Biotech) were used to evaluate the cytotoxic and pro-inflammatory responses of the dental alloys, respectively. All procedures were conducted following the manufacturers' instructions.

Two cell lines were used in this study: murine fibroblast L929 cells, which are commonly employed in cytotoxicity testing, and human monocytic THP-1 cells, widely used for assessing inflammatory responses. Both cell types were exposed to the different dental alloy samples for 24 hours under standard culture conditions.

After the Exposure Period

- The L929 and THP-1 cells were assessed using the MTT/MTS assay to determine cell viability. This colorimetric assay measures the metabolic activity of viable cells based on their ability to reduce the MTS tetrazolium compound into a soluble formazan product, which correlates with the number of living cells [64, 65].
- In parallel, the inflammatory response was evaluated in THP-1 cells by measuring TNF-alpha (tumor necrosis factor-alpha) release. Following the 24-hour exposure, the cell culture supernatants were collected and analyzed using an ELISA specific for human TNF-alpha.

All tests were performed in duplicate to ensure reproducibility and reliability of the results. The MTT/MTS results were obtained directly from cells cultured in two separate wells per sample, while the supernatants from the THP-1 cells were also analyzed in duplicate for TNF-alpha quantification.

This dual-assay approach allowed for a comprehensive evaluation of both cytotoxic and inflammatory effects

Results and Discussions

Cations Release Assessment

The tests were carried out on three extracts of each dental alloy (coded #1-#8). The results presented are an average of the three values obtained (Tables 2-5). As previously mentioned, in their compositions, the amount of silver varies between 6.4% and 58%. The analyses were made by ICP MS/ICP OES. In our measurements we did not find traces of Be, Ni, Mn, Cd, Co, Li, Mo, Nb, Pb, Sb, Zr, Ti, Al, As, Hg and Se, so these elements do not appear in Figure 1 shows the quantities of silver in the alloys' compositions (% in relation to the quantities of silver released (mg/l) in the performed extraction tests.

Table 2: The Results of the Extraction Tests for the Dental Alloys Coded #1- #2

#1					#2			
	aver-age	average	St Dev			aver-age	average	St Dev
	µg/l idem	µg/cm2/ week				µg/l idem	µg/cm2/ week	
Ag	11	0,04			Ag	33	0,12	0,02
Cu					Cu			
Fe	37	0,14	0,05		Fe	0,36	0,13	0,04
Zn					Zn			
Au	0,3	0,001	0,00		Au	0,27	0,001	0,000
Cr	4,4	0,02	0,00		Cr	4.5	0,02	0,00
Ga	2,5	0,01	0,00		Ga	14.33	0,05	0,01
In	15	0,06	0,01		In	13.33	0,05	0,00
Pd	5,3	0,02	0,00		Pd	2.33	0,01	0,00
Pt					Pt			
Sn	1,1	0,004	0,00		Sn	33.33	0,06	0,00
Σ		0,22			Σ	0,45		

Table 3: The Results of the Extraction Tests for the Dental Alloys Coded #3 - #4

#3					#4			
	average	average	St Dev			aver-age	average	St Dev
	µg/l idem	µg/cm2/ week				µg/l idem	µg/cm2/ week	
Ag	55	0,20	0,01		Ag	59	0,22	0,04
Cu	92	0,34	0,01		Cu	63	0,23	0,10
Fe	64	0,24			Fe	41	0,15	0,10
Zn					Zn	47	0,18	0,07
Au	1,0	0,004	0,000		Au	1,3	0,005	0,00
Cr	4,8	0,02	0,01		Cr	6,4	0,02	0,01
Ga					Ga			
In	0,4	0,001	0,001		In	10	0,04	0,01
Pd	2,2	0,01	0,00		Pd	2,6	0,01	0,00
Pt	0,3	0,001	0,00		Pt	0,4	0,001	0,00
Sn					Sn	0,8	0,003	0,00
Σ		0,82			Σ		0,90	

Table 4: The Results of the Extraction Tests for the Dental Alloys Coded #5- #6

#5					#6			
	aver-age	average	St Dev			average	average	St Dev
	µg/l idem	µg/cm2/ week				µg/l idem	µg/cm2/ week	
Ag	82	0,31	0,01		Ag	103	0,38	0,01
Cu					Cu	83	0,31	0,07
Fe	30	0,11			Fe	24	0,09	0,01
Mo	12	0,04			Zn			
Zn	30	0,11			Au	0,7	0,003	0,001
Au	0,7	0,002	0,000		Cr	4,5	0,02	0,00
Cr	3,8	0,01	0,00		Ga			
Ga					In	0,4	0,001	
In	6,3	0,02	0,00		Pd	1,4	0,01	0,00
Pd	2,0	0,01	0,00		Pt			
Pt	0,2	0,001	0,000		Sn	0,2	0,001	
Sn	0,4	0,002	0,000		Σ		0,81	
Σ		0,62						

Table 5: The Results of the Extraction Tests for the Dental Alloys Coded #7- #8

#7					#8			
	average	average	St Dev			average	average	St Dev
	µg/l idem	µg/cm2/ week				µg/l idem	µg/cm2/ week	
Ag	243	0,90	0,13		Ag	314	1,17	0,15
Cu					Cu			
Fe	60	0,22			Fe	66	0,25	0,20
Mo	10	0,04			Zn			
Zn					Au	0,7	0,003	0,00
Au	0,4	0,001	0,00		Cr	7,5	0,03	0,02
Cr	5,4	0,02	0,01		Ga			

Ga					In	5,5	0,02	0,00
In	3,9	0,01	0,00		Pd	2,1	0,01	0,00
Pd	4,4	0,02	0,00		Pt			
Pt					Sn	8,1	0,03	0,00
Sn	18	0,07	0,01		Σ		1,50	
Σ		1,28						

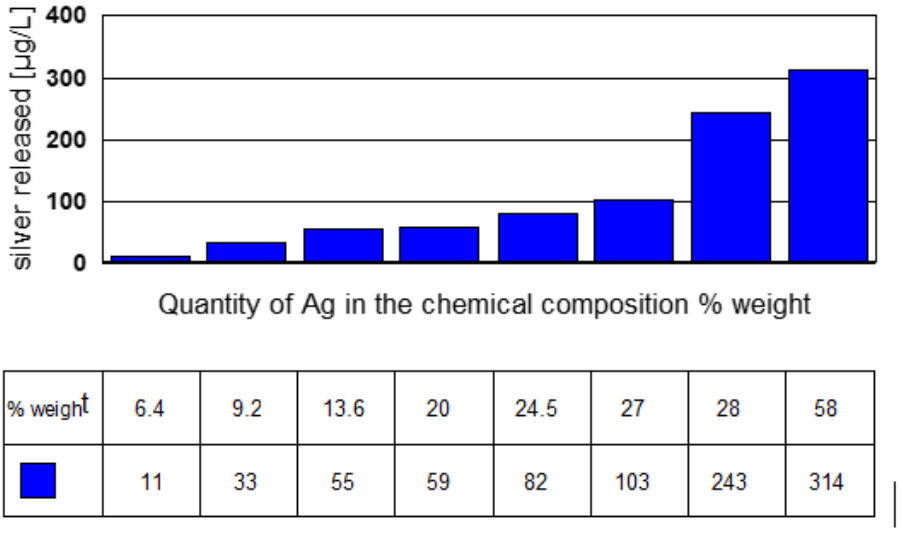

Figure 1: Silver in the Chemical Composition (%weight) and the Released Silver (µg/l) Corresponding to the Tested, Dental Alloys (coded #1- #8)

The findings of our investigation indicate that the quantities of silver released in the extraction tests performed are minimal, on the order of micrograms; therefore, it can be asserted that the tested alloys do not pose toxicity problems. Conversely, given the minimal quantities of silver released, a question can arise: can silver alloys be generally regarded to have biocidal behavior? In this context, some relevant aspects concerning the obtained results and their correlation with the findings of other studies in the specialized literature will be presented.

Migration of Metallic Silver in Ionic Solutions

Silver in the metallic state is very poorly soluble in water; its solubility is 30ng/L. The measurements were made within the framework of the Consortium Precious Metals and Rehnium REACH Euro Métaux [66]. A first approach to the migration of silver in ionic solutions was made by Steppan et al [67]. proposing the following mechanism:

$Ag \rightarrow Ag^+$

$H_2O \rightarrow H^+ + OH^-$

$Ag^+ + OH^- \rightarrow AgOH$

$2\ AgOH \rightarrow Ag_2O + H_2O$

$Ag_2O + H_2O \rightarrow 2\ AgOH \rightarrow 2\ Ag^+ + 2OH^-$

The proposed model is for Ag-Pd alloys in an H2O medium. According to Vu et al [68]. in an Ag-Pd binary alloy, the anodic formation of palladium oxide blocks the migration of Ag+ ions in the electrolyte solution. The proposed mechanism is based on the dependence of potential energies and the pH values according to the Pourbaix diagrams, as follows:

$Ag + ½\ H_2O \rightarrow ½\ Ag_2O + H^+ + e^-$

$E0 = 1.173 – 0.0591\ pH$

$Ag_2O + H_2O \rightarrow Ag_2O + 2\ H^+ + 2\ e^-$

$E0 = 1.398 – 0.0591\ pH$

$Pd + H_2O \rightarrow PdO + 2\ H^+ + 2\ e^-$

$E0 = 0.896 – 0.0591\ pH$

Their study was conducted on two binary alloys with 15% Palladium and another that contains 30% Palladium. In other words, the formation of palladium oxide blocks the migration of silver ions and consequently the concentration of ions in solution is greatly decreased. In reality, biological media are more complex than H2O and can therefore contain chlorides (NaCl, KCl, CaCl2), phosphates (Na2HPO4), lactic acid, urea, many proteins, including enzymes, amino acids (peptides) etc. Thus, the model of silver ions migration in ionic solutions based on the diagrams of Pourbaix according to seems a bit simplistic to us.

The solubility of silver chloride in water at 25°C is approximately 1.3 x 10-5 moles per litre (or approximately 1.77 mg/L). Also, the Product Solubility (Ksp) of silver chloride at 25°C is about 1.8 x 10-10. This low Ksp indicates a very low solubility. However, the solubility of silver chloride can be affected by the presence of other ions in the solution. For example, solubility may increase in the presence of ammonia (NH3) or sodium thiosulfate (Na2S2O3), urea, lactic acid because these substances can form soluble complexes with Ag+. The electrical conductivity of silver chloride (AgCl) solid is low due to its low solubility in water. In aqueous solution, conductivity depends on the concentration of dissolved ions. The molar conductivity of Ag+ and Cl- ions in solution is 61.9 $S \cdot cm^2/mol$ and 76.3 $S \cdot cm^2/mol$, respective. In other words, it is possible to measure current flows in the range of nano- to picoamperes, which can mobilize Ag+ cations in a solution. However, conductivity may change in the presence of various chemical substances capable of forming complexes with silver ions.

The observed reality is quite different from the proposed theoretical mechanism, which is based on thermodynamic redox potential diagrams, such as Pourbaix diagrams.

Generally speaking, alloys containing silver in their composition are biphasic. We distinguish two possibilities regarding the distribution of silver in these alloys: either silver is present in a single phase, or it is distributed in both phases. This distribution significantly influences the corrosion process and the release of silver cations, which can then be very different.

As an example, we studied two alloys containing silver (see Figure 2 and Figure 3) in order to evaluate their behavior against corrosion. In the first alloy, silver is localized only in the Au-Ag phase (Figure 1a). In the second alloy, silver is divided into two phases: the Au-Ag-Cu phase and the Pd-In-Ag phase (Figure 3a).

After electrochemical corrosion tests, we observed that the corrosion was localized only in the Au-Ag phase of the first alloy (Figure 1b), while for the second alloy, the corrosion was distributed over the entire surface Figure 3b) [69].

The quantitative analysis of the electrolytes used for these tests revealed a notable difference in the concentrations of released silver: about 630 µg/L for the alloy in which the silver is distributed in two phases, against only 330 µg/L when silver is concentrated in a single phase (Table 6).

Table 6: Cation's Concentration in Composition of Fusayama Solution after first Corrosion test. Adapted from [69]

Alloy	Concentration of cations release (µg/L)					
	Au	Ag	Pd	In	Cu	Zn
11.8Au 47.7Ag 22.8Pd 6.7In 6.8Zn (Figure 1)	10	330	15	1.5	13	85
1.6Au 62.7Ag 22.5 Pd 1.8In Cu10.6 Zn0.8 (Figure 2)	53	640	1680	-	465	80

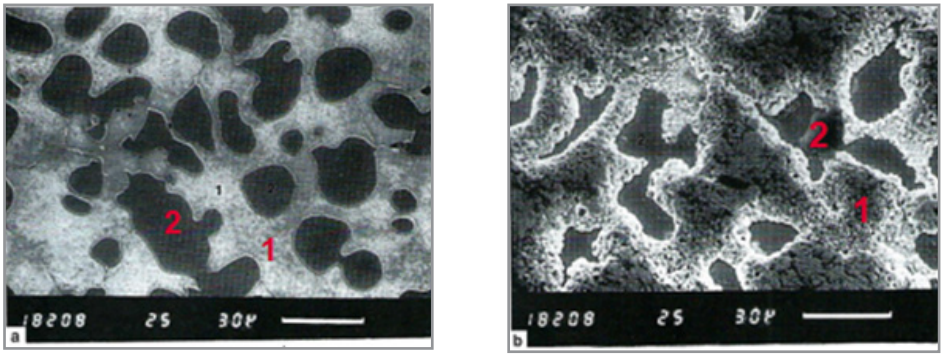

Figure 2: Alloy 11.8Au 47.7Ag22.8Pd6.7In6.8Zn: a, prior to corrosion test; b, after first corrosion test; Phase Au-Ag (1); Phase Pd-In (2)

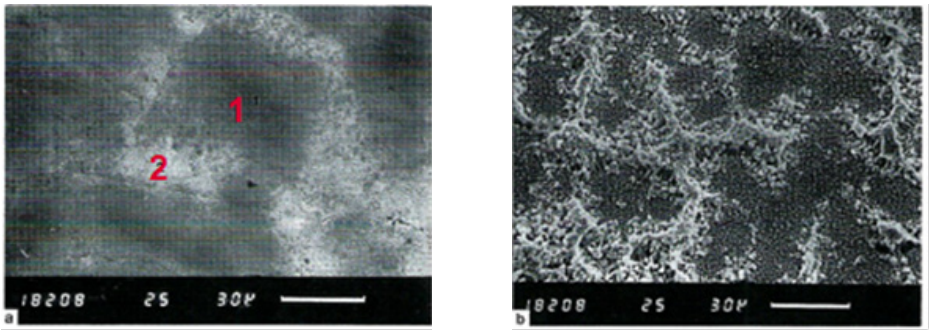

Figure 3: Alloy 1.6Au 62.7Ag 22.5 Pd 1.8InCu10.6 Zn0.8: a, prior to corrosion test; b, after first corrosion test; Phase Au-Ag-Cu (1); Phase Pd-In-Ag (2).

Considering the complexity of the topic addressed, it can be summarized that various factors could influence the migration of metallic silver, outlined as follows: alloy composition, alloying elements Au, Cu, Pd, In, Zn, Sn, Ga etc), microstructural structure and phases distribution, grains and grain boundaries, chemical environment of the solution (pH composition, oxydo-redox potential,), alloy surface (roughness, treatments), alloy tension state (mechanical stress or deformations) etc. These factors can

interact in complex ways, influencing the rate and amount of silver ion release in ionic solutions.

Effects of Dental Alloys on L929 and THP-1 Cells Evaluated by the MTT/MTS Assay and TNF-α Expression

The potential cytotoxicity of various dental alloys was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay, Promega), applied to two different cell lines: L929 (mouse fibroblasts) and THP-1 (human monocytic cells). The dental alloy samples were placed in direct contact with the cultured cells for 24 hours. Following incubation, the materials were removed, and the MTS assay was performed to evaluate cell viability and proliferation. The results are presented in Figure 4.

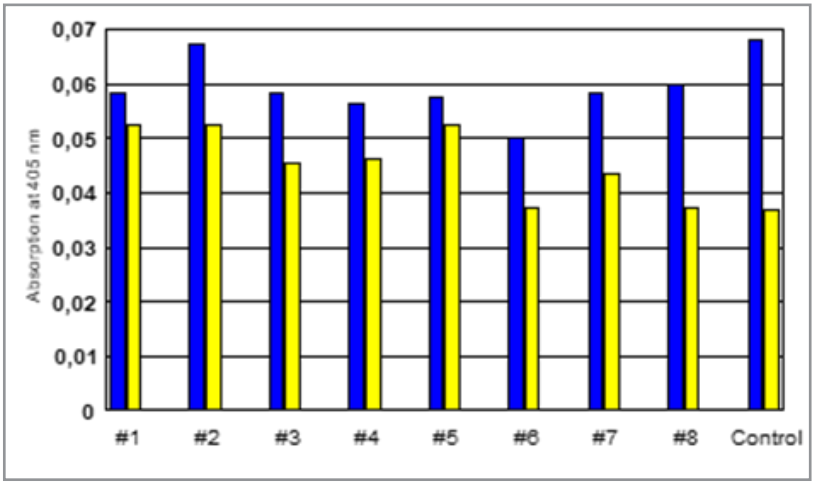

Figure 4: MTS Results of L929 and THP-1 cells Exposed to the Different Dental Alloys #1- #8

As shown in Figure 1, L929 cells exposed to the dental alloys exhibited a slight reduction in cell proliferation relative to the untreated control. However, this decrease remained within a biologically acceptable range and did not indicate significant cytotoxicity. In contrast, THP-1 cells displayed no reduction in proliferation when exposed to the same materials. On the contrary, some samples (notably samples #8 through #6) induced a slight increase in THP-1 proliferation compared to control cells.

Morphological assessment of both L929 and THP-1 cells under microscopy confirmed a typical, healthy cell appearance in all exposed groups, with no visible signs of stress, membrane damage, or abnormal detachment (data not shown). Based on the combined MTS results and morphological observations, it can be concluded that none of the tested dental alloys induced significant cytotoxic effects in either cell type.

To complement the cytotoxicity analysis, the pro-inflammatory response was assessed through quantification of tumor necrosis factor alpha (TNF-α) production in THP-1 cells. After a 24-hour exposure to the dental alloy samples, cell culture supernatants were collected and analyzed using an ELISA kit specific for human TNF-α (Miltenyi Biotec). The levels of TNF-α were compared to those in control groups, including THP-1 cells treated with lipopolysaccharide (LPS) as a positive control and untreated cells as a negative control (Figure 5).

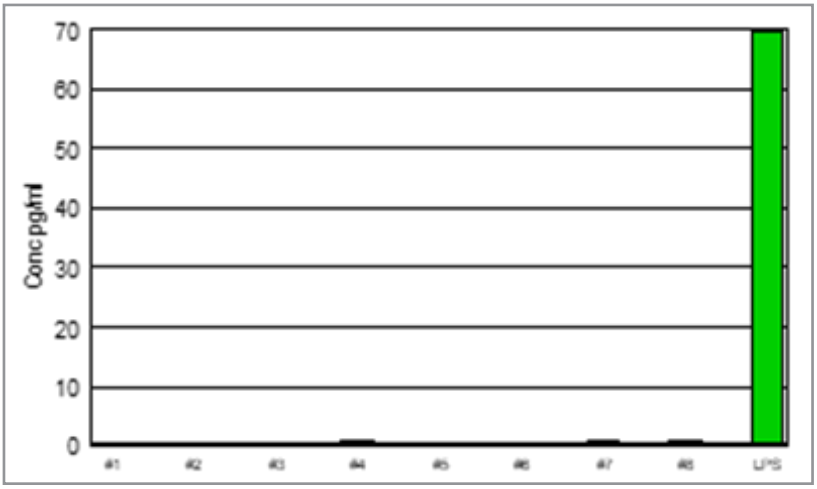

Figure 5: TNF-alpha Levels in THP-1 cells Exposed to the Different Dental Alloys #1- #8

As illustrated in Figure 2, THP-1 cells not stimulated with LPS showed no detectable levels of TNF-α, while LPS-treated cells produced a robust TNF-α response, confirming the assay's sensitivity. The majority of the dental alloys did not elicit detectable TNF-α secretion. Only samples #4, #7 and #8 induced very low levels of TNF-α, but these were far below the threshold of

biological significance. Consequently, it can be concluded that the tested dental alloys did not provoke a notable inflammatory response under these experimental conditions.

For the MTS assay, duplicate wells were analyzed for each condition and cell type, and results are reported as the mean of these two measurements. For the TNF-α analysis, only one test per sample was conducted, though each supernatant was analyzed in duplicate to ensure consistency. As such, the conclusions drawn from this study are based on low replicate numbers and are not statistically validated.

Summary and Conclusions

All samples were sterile - there was no sign of turbidity of cultures exposed to the dental alloys for 24 hours.

Cell proliferation in L929 cells was only slightly inhibited in the presence the dental alloys and in some cases with THP-1 there was a slight proliferation. No major cytotoxic effect was observed. LPS significantly induced THF-alpha production in THP-1. None of the dental samples exhibited this effect on THP-1. The results cannot be interpreted as statistically significant due to the limited number of each sample tested.

Cytotoxicity of Dental Alloys: Focus on Silver-Based Materials

The study of the cellular cytotoxicity of dental alloys has been the subject of numerous studies over the years. Craig et al. studied more than 70 noble and non-noble alloys for the conventional technique and for the ceramic-metal technique on Balb/c 3T3 cell lines. The surface analyses of the samples using ESCA, the quantification of released cations measured by atomic absorption spectroscopy and the observed cellular damage resulting from cellular metabolic activity allow the authors to draw several conclusions, as follows: cytotoxicity varies with the composition of the alloy; the surface condition (polished or unpolished) also plays an important role, the polished surfaces show better tolerance; the presence of Cu, Ni, Cd in precious alloys can lead to deterioration of biological tolerance; the non-precious alloys Co-Cr also show good tolerance, probably due to the formation of a chromium oxide layer [70, 71].

Wataha et al. have also tested more than 100 pure alloys and metals using the direct contact technique with MTT [72, 73]. This encompasses virtually all alloy systems available on the market. Based on these results, they classified the alloys according to cytotoxic response (cell viability) into four groups:

o "A", alloys that reveal cytotoxicity close to Teflon (90% to 100% cell viability). In this group are silver-palladium-based alloys, gold-rich alloys, and medium-grade gold alloys.
o "B", alloys that reveal a cellular viability of between 70-89% and that do not represent any risk of toxicity in the oral environment. In this group are classified the titanium-based alloys and silver-based alloys.
o "C", alloys that reveal a cellular viability between 45 -69%, therefore represent significant cytotoxicity, and which may represent a risk of toxicity from their use in the mouth.
o "D", alloys that reveal a cell viability < 44%, therefore have a strong cytotoxic response. In this group are classified metals such as nickel, copper and gold-nickel and gold-cadmium alloys. Furthermore, Wataha et al. also studied the toxicity of silver in cell culture, presenting the cell activity response of Balb/c mouse fibroblasts as a function of the concentration of Ag+1 ions. After the contact test, the cell layer was separated from the material and cell viability was checked; over the entire cell layer, only living cells could be stained by the MTT formazan dye in a blue color [74]. Thus, Wataha shows that silver cations (Ag+) were toxic in the cellular environment only from a concentration of 0.1 mg/L of Ag+. The findings of this study indicated that gold (Au), indium (In), and palladium (Pd) typically did not dissolve in the medium, whereas silver (Ag), cadmium (Cd), copper (Cu), gallium (Ga), nickel (Ni), and zinc (Zn) dissolved frequently. The commercial dental alloys investigated in this research showed complex and unpredictable release behavior.

More recently, the study of Gawlik-Maj et al. (2022) indicates that silver, when used in small concentrations, generally does not show significant cytotoxic effects [75]. Titanium alloy that contains silver demonstrated good stability and showed no issues related with cytotoxicity (Zhang L. et al., 2023)and the cytotoxicity of titanium-silver alloys seems to be either non-existent or mild [76, 77]. Moreover, Zhang Y et al. point out that the addition of 5 at. % Ag to titanium alloys could significantly increase the antibacterial activity but does not adversely affect the mouse osteoblastic cells [78].

On the other hand, Shao et al. (2023) recommend that we should exercise vigilance regarding the use of silver alloys, necessitating additional research into their cytotoxic effects [79]. As an example, Nimeri at al. (2021) studied the cytotoxic effects of two different silver solders used for orthodontic appliances on human periodontal ligament fibroblast cells and pointed out that the materials demonstrated different levels of cytotoxicity, and neither oxidative stress nor apoptosis was involved in the mechanism of cytotoxicity [80]. In the same line, Paqué et Özcan (2024) consider that silver is the most problematic component in gold alloy dental restorations due to its toxicity and composition [81]. It is estimated that adults can have up to four tooth surfaces restored with this alloy before exceeding the reference exposure level (REL) for silver [82].

Silver appears to be an inhibitor of the repair of DNA, but only at concentrations greater than 1mg/L on bacteria [83]. Some studies suggested that silver does not appear to induce gene mutations or primary lesions in bacteria and on mammalian cells [84-87]. As a resolution, it can be stated that scientific literature presents fragmented data on silver. Although certain studies are limited in their protocol, it is difficult to select available and reliable data that suggest a mutagenic and carcinogenic risk. However, in this context, relatively recent studies revealed that silver nanoparticles can produce genotoxic effects, observed especially for in vitro, but also for in vivo studies [88, 91]. A thorough Geno-toxicological assessment of silver nanoparticles is essential for informed decision-making.

Is there a Correlation Between Chemical Extraction and Migration of Cations in the Cell Medium (Cytotoxicity Test)?

The answer to this question is nuanced. Although there may be some correlation between the results of chemical extraction tests and those of biological cytotoxicity tests, several fundamental differences in the media and mechanisms involved limit the possibility of establishing a direct and systematic link. It is therefore

necessary to specify several key points:

a) Fundamental Differences Between the two Types of Environments

- Chemical extraction media: These media (NaCl, lactic acid, ultrapure water, or simulated fluids such as Ringer, Hank, Fusayama, artificial plasma, artificial sweat, etc.) are designed to evaluate the maximum possible release of cations under physico-controlled chemicals, often extreme, but devoid of biological compounds.
- Cell culture medium: It is much more complex and biologically active. It contains proteins (albumin, transferrin), amino acids, salts, and complexing agents such as bovine fetal serum (FBS), which interact directly with the released cations. These interactions strongly influence the speciation, solubility, and therefore the bioavailability of metal ions.

b) Factors Influencing the Migration of Cations in a Cellular Environment

- Complexation and Chelation
- oMetal ions can bind to proteins (albumin, metallothioneins) or organic ligands present in the medium, which modifies their biological activity.
- for example, Ag binds easily to protein thiols or precipitates as silver chloride, reducing its apparent toxicity.
- pH and Redox Conditions
- Chemical media can be acidified (e.g., with lactic acid) to promote cation solubilization.
- o Cellular media have a buffered pH (~7.4) and stable redox conditions that modify the chemical form of cations. Some ions, such as Zn+2 or Cu+2 can precipitate in the form of hydroxides, which limits their biological action.
- Membrane Adsorption and cell Transport
- Certain cations (Ni+2 , Co+2 , Cr+3) interact directly with membrane proteins or are internalized via specific transporters, which influences their cytotoxicity independently of their concentration in the extracellular medium.
- Solubility and Precipitation
- Insoluble precipitates can form (e.g. Zn(OH)2, Cu3(PO4)), limiting the effective concentration of available cations for cells, unlike chemical tests where this limitation is often absent.

c) Exposure Duration and Response Dynamics

- Chemical extraction tests evaluate cumulative ion release over long periods of time (often up to 7 days).
- Cytotoxicity tests are generally carried out over 24 to 72 hours, during which the cells react to a transient and biologically modulated exposure.
- The cellular response therefore depends not only on the quantity of ions released, but also on their chemical form, their release kinetics, and their biological interpretation by the cells.

d) Microstructural Structure and Phase Distribution of the Alloy

• In multiphase alloys, the heterogeneous distribution of elements such as Ag or Cu strongly influences corrosion kinetics and local ion distribution.

• The same total quantity of ions released can thus have different biological effects depending on their microstructural origin and chemical speciation.

In Conclusion, chemical extraction tests assess the potential of a material to release ions, while in vitro cytotoxicity tests analyze the actual biological effects of these ions on cells.

Although a partial correlation can be observed for some highly soluble and reactive ions (Ag , Cu+2 , Zn+2), it remains imperfect due to major differences between the two-milieu chemical and biologic.

Limitations and Perspectives

This study presents some methodological limitations. First of all, the limited number of silver-based dental alloys analyzed, as well as the limited number of tests performed, may reduce the statistical significance and representativeness of the results. Moreover, the use of an in vitro approach does not allow for a faithful reproduction of the complex physiological conditions of the oral cavity. The oral microenvironment is influenced by many factors such as saliva, fluctuating temperature, variable pH, microbial flora, mechanical forces of mastication, as well as interactions with living tissues. These dynamic parameters are difficult to simulate in a cell system in culture.

The cell lines used (L929 and THP-1) are well established standard models for cytotoxicity and inflammation tests, but they do not accurately represent oral epithelial cells or human gingival fibroblasts. Therefore, the observed biological responses only reflect an approximation of the actual behavior of materials in the oral environment.

Prospectively, it is conceivable that future scientific developments will not only allow the precise quantification of the release of metal cations (such as silver) in µg/cm 2/week, but also to correlate these values with the systemic concentrations observed in human blood or target tissues. This would allow for the establishment of biologically relevant exposure thresholds, thus facilitating toxicological risk assessment.

Silver, historically recognized for its antiseptic properties, remains today a reference inorganic antimicrobial agent. Many recent studies have highlighted the interest of silver-doped or silver-coated materials in preventing infections associated with dental implants [92-94]. These materials have demonstrated broad antibacterial efficacy without deleterious effect on human cells at the concentrations used [95, 96].

In conclusion, although the in vitro results of this study alone cannot predict with certainty the behavior of alloys under clinical conditions, they constitute a solid scientific basis justifying additional investigations to confirm their biocompatibility, their biofunctionality, and their long-term safety in the oral environment.

Conclusion

Within the limitations of this study, the following conclusions can be drawn:

1. The amounts of silver detected in the extraction tests were minimal.

2. Based on the results of the cytotoxicity assessment and TNF-α expression, we observed the following:

- No turbidity was observed in the cell cultures exposed to the dental alloys after 24 hours.

- L929 cell proliferation was only slightly inhibited in the presence of dental alloys, while in some cases, THP-1 cells exhibited a slight proliferative response. No significant cytotoxic effects were observed.
- Lipopolysaccharide (LPS) significantly induced TNF-α production in THP-1 cells, whereas none of the tested dental alloys triggered a similar response.

3. At present, we cannot draw definitive conclusions regarding the biocidal properties of silver-containing dental alloys. However, both the European Union and the European Chemicals Agency (ECHA) plan to introduce regulatory limits for silver and its alloys. These limits are expected to be expressed in µg/cm²/week. A forthcoming challenge will be to compare this biocidal threshold with toxicological reference values that have already been validated.

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