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RESEARCH ARTICLE

Comparative Cytotoxicity Assessment of Cobalt-Chromium-Molybdenum Casts Using Elution, Agar Diffusion, and Filter Diffusion Methods

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Abstract

Background/Objectives: Cobalt-Chromium-Molybdenum (CCM) alloys are widely used in dental abutments due to their mechanical strength, corrosion resistance, and durability in the harsh oral environment. However, despite these advantages, concerns remain regarding their potential cytotoxic effects. Therefore, we investigated the cytotoxic potential of CCM using three different cytotoxicity tests. **Methods**: In the current study, three different methods – Elution Method, Agar Diffusion Method, and Filter Diffusion Method - were used to evaluate the cytotoxic effects of CCM casts. **Findings**: CCM did not show cytotoxicity in any of the 3 assays tested. In the quantitative elution method, the viability was greater than 90%, indicating no cytotoxicity. Both in agar diffusion and filter diffusion methods, there was no qualitative evidence of cytotoxicity. **Novelty**: This study is the first to examine a detailed cytotoxic effect of CCM cast on cultured cells. The findings provide valuable insights into the safety profile of CCM implants.

Keywords: Cytotoxicity; Elution method; Agar diffusion; Filter diffusion; CobaltChromium Molybdenum alloy

1 Introduction

The increasing demand for dental implants has driven the development of advanced materials with enhanced biocompatibility, mechanical strength, and long-term durability. Among these, Cobalt-Chromium-Molybdenum (CCM) alloys have emerged as a popular choice for dental abutments due to their superior strength, corrosion resistance, and ability to endure the harsh oral environment. The abutment connect to the implant fixture and holds the crown—the artificial tooth—in place [(1)].

Despite their mechanical advantages, CCM alloys raise concerns regarding potential cytotoxic and genotoxic effects. As with all materials intended for medical applications, it is essential to evaluate their biocompatibility through cytotoxicity testing to assess the risk of leaching harmful substances [(2)]. Various standardized assays are available for this purpose, each with distinct advantages and limitations. Among those commonly used

for dental materials are the elution method, agar diffusion method, and filter diffusion method [3].

The elution method assesses the effect of medical device extracts on cell viability. One widely used assay is the Neutral Red Uptake (NRU) cytotoxicity test, which measures the ability of viable cells to incorporate and bind neutral red dye. This method is particularly relevant because it mimics the potential release of toxic substances into biological fluids over time [4]. It can also provide insights into possible long-term effects of material degradation on host tissues.

In the agar diffusion method, a layer of agarose separates the test material from the cultured cells. Any toxic compounds must diffuse through the agar to reach the cells. Toxicity is typically indicated by zones of inhibition, where cell growth is suppressed [5]. This method is useful for preliminary screening due to its simplicity and cost-effectiveness, although it may not accurately replicate the complexity of *in-vivo* interactions [6].

The filter diffusion method involves placing the medical device on one side of a filter membrane, with cells cultured on the opposite side. If toxic leachables are present, they diffuse through the membrane and affect the underlying cells. While similar in concept to the elution method, this approach allows for localized assessment of cytotoxicity without direct contact between the material and cells. It offers a different perspective on how materials might interact with surrounding tissues in a clinical context.

Given that each method has its own strengths and limitations, using multiple assays provides a more comprehensive evaluation of material biocompatibility especially for devices for dental use. In the present study, we aim to assess the cytotoxic potential of CCM casts by comparing the results obtained from the elution, agar diffusion, and filter diffusion methods.

2 Methodology

2.1. Test material

CCM casts measuring 2 x 1 cm, with thickness slightly less than 0.5 mm were used in this study. CCM casts were steam-sterilized at 132°C for 15 minutes. Additionally, sterile natural rubber latex and High-Density Polyethylene (HDPE) strips were used as positive and negative controls, respectively.

2.2 Preparation of extracts

CCM casts, positive and negative controls were extracted in 1x DMEM supplemented with 20 mM HEPES, 5% heat-inactivated newborn calf serum, 4 mM L-glutamine, and 1% penicillin/streptomycin solution at 37 $\pm$ 1 °C for 72 $\pm$ 2 hours. The extraction ratio used was 6 cm^2/mL for CCM casts and positive control, and 3 cm^2/mL for negative control [7]. At the end of the extraction, all extracts were clear, without any color change or particulates. There were no changes in the retrieved test item. No processing of extracts like filtration, centrifugation, or pH adjustments were made. Neat extracts were used in the three test systems. If necessary, extracts were diluted as required.

2.3 Test system

Balb/c 3T3 cell lines, supplied by the National Centre for Cell Science, India were used in this study. These cells were cultured in Dulbecco's modified eagle medium (1x DMEM) supplemented with 20 mM HEPES, 10% heat-inactivated newborn calf serum, 4 mM L- glutamine, and 1% penicillin/streptomycin.

2.4 Elution method

Exponentially growing Balb/c 3T3 cells were seeded into wells B1–G12 of a 96-well plate at 1 x 10^4 cells/well and incubated at 37 $\pm$ 1 °C, 5% CO_2, and >90% humidity for 24 hours. Upon reaching ~80% confluency, the culture medium was replaced with six replicates of the test item extract (30–100%), positive control (neat extract), vehicle control, and negative control (neat extract), as illustrated in Figure 1. After 24 h incubation, cells were assessed qualitatively under an inverted microscope for morphological signs of cytotoxicity using ISO 10993-5:2009 grading criteria. For quantitative analysis, wells were washed with 150 μL PBS and incubated with 100 μL neutral red (NR) medium for 3 h. After incubation, NR medium was removed, wells were washed with PBS, and 150 μL of NR desorb solution (ethanol:glacial acetic acid:distilled water, 10:0.2:9.8 mL) was added. Absorbance was then measured at 540 nm using a microplate reader (Multiskan SkyHigh, Thermo Fisher, USA). NR uptake was expressed as OD_{540} (sample OD minus mean OD of blanks), and cell viability was calculated using the formula, Viability (%) = 100 $\times$ OD_{540e_5} / OD_{540v}, where OD_{540e_5} is the sample and OD_{540v} the vehicle control. Viability of less than 70% is considered cytotoxic.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	Negative	Vehicle	30%	40%	50%	60%	70%	80%	90%	100%	Vehicle	Positive
C	Negative	Vehicle	30%	40%	50%	60%	70%	80%	90%	100%	Vehicle	Positive
D	Negative	Vehicle	30%	40%	50%	60%	70%	80%	90%	100%	Vehicle	Positive
E	Negative	Vehicle	30%	40%	50%	60%	70%	80%	90%	100%	Vehicle	Positive
F	Negative	Vehicle	30%	40%	50%	60%	70%	80%	90%	100%	Vehicle	Positive
G	Negative	Vehicle	30%	40%	50%	60%	70%	80%	90%	100%	Vehicle	Positive
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Fig 1. 96-well plate layout for Elution Method

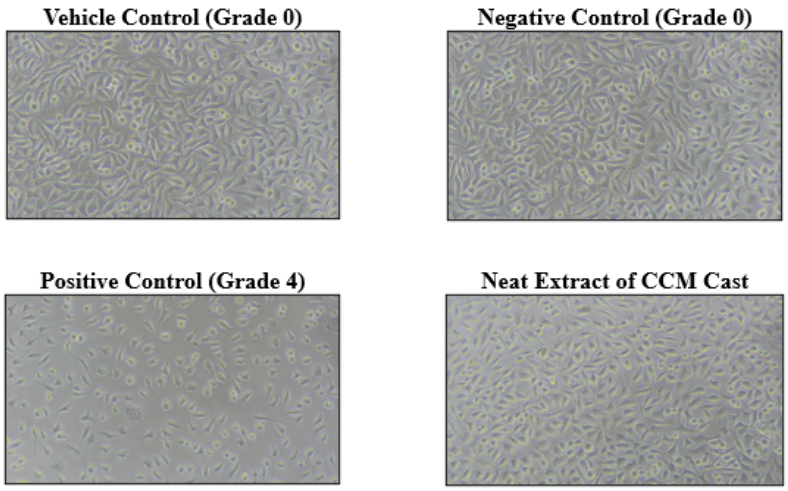

Fig 2. Representative photographs of cell cultures illustrating the qualitative outcomes observed in the elution assay *The images show cell confluency and morphology before and after treatment with Cobalt-Chromium-Molybdenum (CCM) cast extracts, as well as with positive and negative controls.*

2.5 Agar diffusion method

Exponentially growing Balb/c 3T3 cells were seeded into two 6-well plates at a concentration of 2.5 x 10^5 cells/mL and incubated at 37°C for 24 h in 5% CO_2. 70% confluency was confirmed post-incubation. The culture medium was removed, and a 3% agarose overlay—supplemented with culture medium containing 0.01% neutral red dye—was applied to the cell layer. Once the agarose solidified, 5 mm diameter filter paper discs soaked in the neat test, positive and negative control extracts were placed at the center of each well. Quadruplicates cultures were tested for each extract. After 24 h of incubation, the decolorization zone around the test and the control extracts were assessed using an inverted microscope. The decolorization index and lysis index were determined for each test and control items in accordance with the criteria specified in ISO 7405:2018.

2.6 Filter diffusion method

Exponentially growing Balb/c 3T3 cells were seeded on cellulose acetate filters placed in the culture dish and were incubated for 24h. Freshly prepared agarose supplemented with DMEM and serum was allowed to solidify in petri plates at room temperature. Once the agarose was set, the filter paper with cells were placed over the agarose layer with the cell side down. Quadruplicates sterile CCM cast, positive, and negative controls were placed on the filter paper culture dish. Additionally, a filter containing cells and the test item, as well as a filter with the test item but without cells, were placed in separate culture media. The plates were incubated for 2 hours and 24 hours. After the respective incubation, the filters were removed from the cultures and incubated with succinate dehydrogenase for 3 hours at 37 $\pm$ 2°C. After incubation the filter papers were washed with distilled water and air-dried, the diameter of the zone formed was measured and graded based on the area of decolorization.

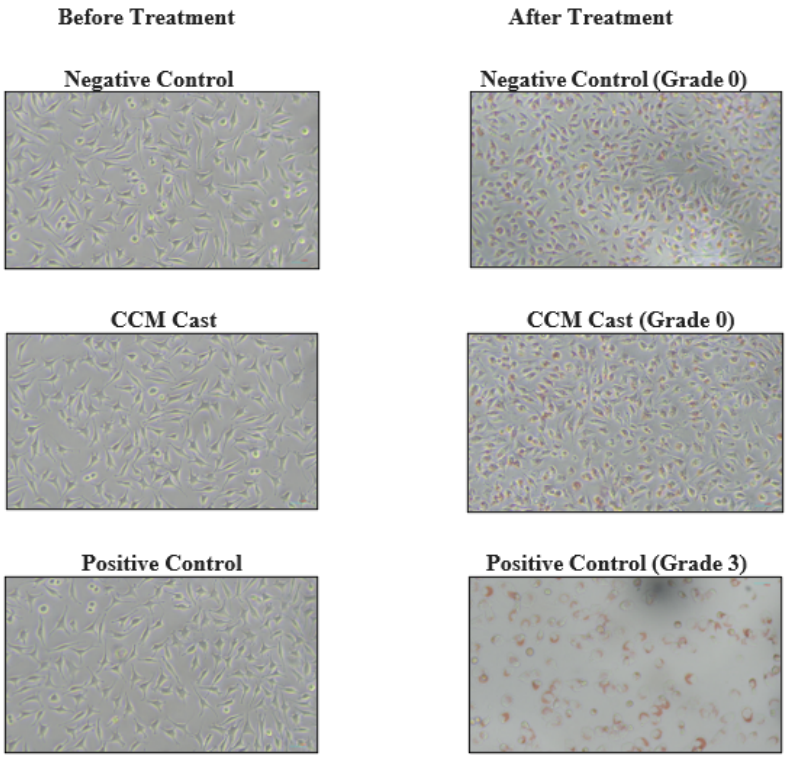

Fig 3. Representative photographs of cell cultures from the agar diffusion assay

3 Results and Discussion

3.1. Elution method

Qualitative analysis showed that cells treated with the negative control extracts did not show any morphological signs of cytotoxicity (Grade 0). Positive control extracts showed moderate cytotoxicity (Grade 3). Therefore, the assay was considered valid. CCM cast extract treated cells did not show any cytotoxicity (Grade 0). The cells were then taken through to the quantitative assay using NRU assay. The negative controls treated cultures showed a viability of 97% (indicating non-cytotoxic) and the positive control extract treated cultures showed viability of 47% (indicating cytotoxicity). These results showed that the quantitative assay worked. All concentrations including neat extract of CCM casts showed viability in the range of 88 to 96% and therefore was considered non-cytotoxic. (See Table 1)

Table 1. Quantitative analysis of cell viability and cytotoxicity in the elution assay.

		Negative Control	Vehicle Control	Cobalt-Chromium-Molybdenum (CCM) extract concentrations (%)								Positive Control
				30	40	50	60	70	80	90	100	
Optical Density	Mean	0.367	0.376	0.365	0.358	0.340	0.344	0.333	0.337	0.342	0.322	0.177
	(±) SD	0.011	0.013	0.021	0.011	0.012	0.006	0.020	0.008	0.008	0.008	0.022
Viability (%)		97.35	NA	96.82	94.96	90.19	91.25	88.33	89.39	90.72	85.41	46.95
Cytotoxicity (%)		2.65	NA	3.18	5.04	9.81	8.75	11.67	10.61	9.28	14.59	53.05

3.2 Agar diffusion method

Qualitative evaluations revealed that the cells treated with the negative control showed a non-cytotoxic response (Scale 0), and cells treated with the positive control showed a severe cytotoxic response (Scale 3), hence validating the assay. A non-cytotoxic response (Scale 0) was observed in CCM extract-treated cultures. Results are summarized in Table 2.

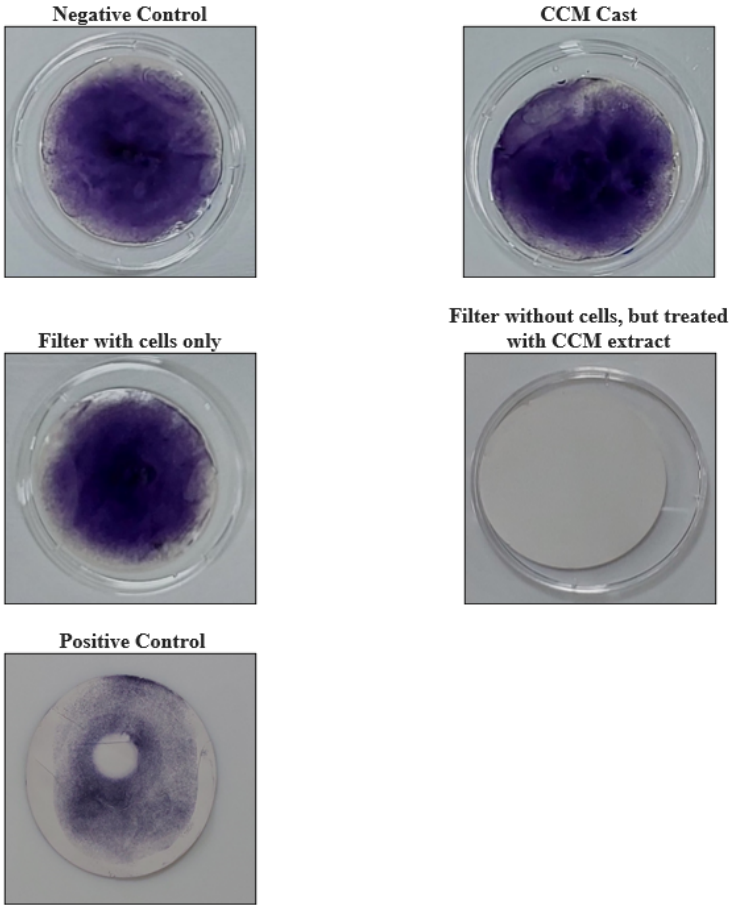

Fig 4. Representative photographs of stained cell cultureson filter membranes from the filter diffusion assay at 24 hour time point

Table 2. Results from Agar Diffusion Assay

Sample	Culture	Decolorization index	Median	Cell response*	Lysis index	Median	Cell response*	Interpretation
Negative control	1	0	0	0	0	0	0	Non-cytotoxic
	2	0			0			
	3	0			0			
	4	0			0			
CCM Cast	1	0	0	0	0	0	0	Non-cytotoxic
	2	0			0			
	3	0			0			
	4	0			0			
Positive control	1	4	4	4	4	4	4	Severely cytotoxic
	2	4			4			
	3	4			4			
	4	4			4			

*Cell response is graded between 0 (no cytotoxicity) to 4 (severe cytotoxicity)

Filter diffusion method

Dark blue staining, indicative of intact, viable cells, was observed in wells treated with the negative control extract and in wells containing cells without exposure to test items. In contrast, the positive control showed clear cytotoxicity, with unstained zones measuring 7 mm at 2 h and 10 mm at 24 h. The corresponding cell damage indices were assessed as 2 and 3, respectively, confirming a cytotoxic response. These results validated the assay, as the positive control produced well-defined zones of cell damage compared to the negative control. Filters without cells showed no staining, suggesting that the test item had no visible effect on the filter material itself. Filters treated with CCM casts exhibited uniform dark blue staining, indicating the absence of cytotoxicity. Based on these observations, the test item was graded as non-cytotoxic, with a cell damage index of 0. The results are summarized in Table 3 .

Table 3. Results from Filter Diffusion Assay

Sample	Culture	Cell damage index		Grade
		2 hours treatment	24 hours treatment	
Negative Control	1	0	0	Non-cytotoxic
	2	0	0	
	3	0	0	
	4	0	0	
CCM Cast	1	0	0	Non-cytotoxic
	2	0	0	
	3	0	0	
	4	0	0	
Positive control	1	2	3	Severely cytotoxic
	2	2	3	
	3	2	3	
	4	2	3	
Filter with cell monolayer but without test item	1	0	0	Non-cytotoxic
Filter without cells but with test item	1	-	-	Not applicable

Balb/c 3T3 cells share genetic, immunological, and physiological similarities with human cells, making them widely used in cytotoxicity testing [8]. Toxic effects observed in Balb/c 3T3 cells can often be correlated with human cellular responses. Studies have shown that cobalt is both cytotoxic and genotoxic to mammalian cells [9]. Soluble and particulate forms of cobalt have been reported to be lethal to urothelial cells, human fibroblasts, and lung epithelial cells [10].

Chromium also exhibits cytotoxic potential, with Cr (VI) being significantly more toxic than Cr(III) due to its higher solubility and mobility [11]. While Cr(III) is an essential trace element involved in glucose, lipid, and protein metabolism, it can exert harmful effects at elevated concentrations. Previous studies have demonstrated that chromium exposure can damage the kidneys, liver, heart, and reproductive organs [12]. Cr (VI) readily crosses cell membranes and can cause damage to DNA, RNA, proteins, and mitochondria. It has also been associated with cardiovascular, developmental, immunological, and endocrine dysfunctions [13].

Molybdenum is another essential trace element, playing a key role in various enzymatic functions. However, at high concentrations, it may disrupt copper metabolism [14]. Among its oxidation states, hexavalent molybdenum (Mo^{6+}) is the most toxic due to its high solubility and bioavailability, compared to Mo^{55+}, Mo^{4+}, and Mo^{3+} [15]. In a recent MTT assay, molybdenum was found to be cytotoxic to human bronchial cells [16].

Despite the known cytotoxic potential of cobalt, chromium, and molybdenum in their free ionic forms, their incorporation into the CCM alloy significantly reduces this risk. In the alloyed state, these metals are tightly bound within a stable crystalline matrix, which greatly limits their bioavailability. The strong metallurgical bonding and corrosion resistance of the CCM alloy minimize the release of free metal ions under physiological conditions. As a result, the likelihood of toxic exposure due to leaching is negligible, which explains the absence of cytotoxic effects observed in this study.

To our knowledge, this is the first study evaluating the cytotoxicity of CCM alloy using elution (NRU), agar diffusion, and filter diffusion methods. As outlined above, Co, Cr, and Mo can be cytotoxic above specific threshold levels. However, all three

assays yielded negative results, suggesting that the test item is stable and did not release metallic ions beyond permissible limits—both under extraction and direct contact conditions.

4 Conclusion

In summary, whereas cobalt, chromium, and molybdenum demonstrate cytotoxicity in their free ionic states, their integration into a stable CCM alloy matrix considerably reduces this hazard. The lack of cytotoxicity in all three assessment techniques corroborates the alloy's biocompatibility under simulated physiological settings. The findings offer compelling early evidence that the CCM alloy is safe for dental applications, as it does not leach large quantities of metal ions when in contact with biological systems.

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Contributory statement

Ms. Mariya George: Conceptualized the study problem and conducted data generation. Ms. P. Pavithra: Assisted with data generation. Dr. V.N. Sangeetha and Dr. S. S. Murugan: Contributed to data analysis, and facilitating research. Dr. T.N. Sathya: Designed the experimental framework, and interpretation of results. Provided overall research guidance. Dr. T. S. Kumaravel: Facilitating research activities, reviewed and edited the manuscript, ensuring accuracy and coherence. Provided overall research guidance.

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