BIO-C TEMP

***In vitro* Cytotoxicity Potential Testing**

(Cytotoxicity Test)

Final Report

DATA REQUIREMENTS:	ISO 10993-5:2009 - Annex C.
AUTHOR:	Mariana Aguilera Alencar da Silva
STUDY COMPLETION DATE:	24 May 2019
ENGLISH VERSION:	24 May 2019
PERFORMING LABORATORY:	TECAM Tecnologia Ambiental Ltda. Rua Fábia, 59 CEP: 05051-030 São Paulo/ SP - Brazil
LABORATORY PROJECT ID:	Report Number: **RL18464/2019CT-B** Study Number: **18464/2019CT**
SPONSOR:	ANGELUS INDÚSTRIA DE PRODUTOS ODONTOLÓGICOS S/A WALDIR LANDGRAF, 101 CEP: 86031-218 LONDRINA/PR - BRAZIL

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Study Title: BIO-C TEMP – *In vitro* Cytotoxicity Potential Testing (Cytotoxicity Test).

Study Number: 18464/2019CT

This study was conducted under my responsibility in accordance to NIT-DICLA-035 (INMETRO, Nov/18, Rev.03) and its complementary documents and the Good Laboratory Practice Principles as published by the OECD (N° 1 [ENV/MC/CHEM (98) 17]) which meet the United States Environmental Protection Agency Good Laboratory Practice Standards [40 CFR Part 160].

This study was conducted in accordance to the written study plan authorized by the Sponsor and TECAM Management and to TECAM standard operating procedures. This report represents a true and accurate record of the obtained results. There were no major known circumstances that may have affected the quality or integrity of the study.

All original raw data, including any storage medium for electronically recorded data, documentation, the signed study plan, the protocol amendments, the final report and a sample of the test item will be retained in the GLP Archives at TECAM Tecnologia Ambiental.

Mariana Aguilera Alencar da Silva (MSc)
Study Director

24 May 2019
Date

Performing Laboratory: TECAM Tecnologia Ambiental Ltda.
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STATEMENT OF QUALITY ASSURANCE

Study Title: BIO-C TEMP – *In vitro* Cytotoxicity Potential Testing (Cytotoxicity Test).

Study Number: 18464/2019CT

Based on a quality assurance review, it was concluded that the final report is a true reflection of the raw data.

The final report was examined with respect to the study plan, standard operating procedures and raw data. Proceedings of the present study were inspected by process-based inspections. The inspections were carried out according to the standard operating procedures of the Quality Assurance of TECAM Tecnologia Ambiental.

Dates of inspections and the dates on which the findings were reported to the Study Director and Management are given below. These reports are kept in the GLP Archives at TECAM Tecnologia Ambiental.

Inspection	Date of inspection	Reporting Dates	
		To Study Director	Test Facility Management
Study plan	07 May 2019	07 May 2019	07 May 2019
Experimental phase	20 February 2019	18 March 2019	18 March 2019
Raw data	24 May 2019	24 May 2019	24 May 2019
Final report	24 May 2019	24 May 2019	24 May 2019
English Version	24 May 2019	24 May 2019	24 May 2019

Carolina Satie Hayashida
Quality Assurance
TECAM Tecnologia Ambiental Ltda.

24 May 2019
Date

GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Mariana Aguilera Alencar da Silva (MSc)	Study Director.
Lilian Polakiewicz (MSc)	Test Facility Manager.
Lilian Mion (BSc)	Quality Assurance.
Carolina Satie Hayashida (BSc)	Quality Assurance.
Carmen Coelho Pita (BSc)	Technical Support.

Study Dates

Study Initiation Date:	08 May 2019.
Experimental Starting Date:	13 May 2019.
Experimental Termination date:	15 May 2019.
Study Completion Date:	24 May 2019.
English version:	24 May 2019.

Performing laboratory

The present study was conducted at TECAM Tecnologia Ambiental, located at Rua Fábia, 59 – CEP: 05051-030 - São Paulo – SP, Brazil.

Study plan adherence

No deviations or amendments were recorded from the study plan.

Archives

All the original raw data and records of this study are the property of the Sponsor. Data will be properly registered, signed and stored in TECAM´s archives for five years. Test item will be properly stored during the test and after that will be returned to the Sponsor. When possible a sample will be retained for two years or until the expiry date.

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1.0 EXECUTIVE SUMMARY

The study was carried out with the test item **BIO-C TEMP** (supplied by ANGELUS INDÚSTRIA DE PRODUTOS ODONTOLÓGICOS S/A) aiming to evaluate the cytotoxic potential of medical devices in an *in vitro* system using the V-79 cell line.

Fibroblasts from the V-79 cell line were cultured in 96-well cell culture plates and incubated for 24 hours for cell monolayer formation. After this period the culture medium was replaced by a new medium containing the treatments (n = 6) and the positive (n = 6), negative (n = 12) and extraction (n = 12) controls. The test item concentrations used for the treatment were 100%, 50%, 25% and 12.5% where they were incubated for 24 hours. At the end of the exposure period, the culture medium with the treatments was discarded and 50 µL of the MTT solution (1 mg / mL) was added to each well, the cells were incubated for 2 hours, then the MTT solution was discarded and 100 µL of Isopropanol was added to each well and the plate was kept shaking for 5 minutes. The absorbance was measured at a wavelength of 570 nm (reference wavelength 650 nm).

A reduction in the cell viability above 30% was observed in relation to the negative control at all concentrations of the test item **BIO-C TEMP** (12.5%, 25%, 50% and 100%). Therefore, the test item **BIO-C TEMP** was cytotoxic in this assay.

2.0 INTRODUCTION

2.1 Study Purpose

The aim of the study is to evaluate the cytotoxic potential of medical devices in an *in vitro* system using the V-79 cell line.

2.2 Study Guidelines

The study was performed in accordance with:

ISO 10993: 5 – Biological evaluation of medical devices Part 5: Tests for *in vitro* cytotoxicity (Annex C – MTT cytotoxicity test), 2009.

3.0 MATERIALS AND METHODS

3.1 Test Item

Identification:	BIO-C TEMP
Test item number:	1919107
Received on:	16 April 2019
Batch Nº:	48268
Register Nº:	10349450094
Study number:	18464/2019CT
Declared composition:	Tricalcium silicate, Dicalcium silicate, Tricalcium aluminate, Calcium oxide, Base Resin, Calcium Tungstate, Polyethylene Glycol and Titanium Oxide
Manufactured on:	11 March 2019
Expiry date:	11 March 2021
Product Purpose:	Intracanal dressing for endodontic treatment.
Indication of use of the product:	Root canal dressing for endodontic treatment of pulp necrosis, refractory lesions, retreatments, recurrent fistulas

	and excessive exudate. Root canal dressing in cases of perforations, internal and external resorptions, prior to the use of MTA ANGELUS OR MTA REPAIR HP. Incomplete root formation (rizogenesis): Apexification – the formation of the apex in cases of pulp necrosis. Apexigenesis – the formation of the apex in cases of vital pulp.
Which components of the product will have contact with the patient:	Compound paste of Tricalcium silicate, Dicalcium silicate, Tricalcium aluminate, Calcium oxide, Base Resin, Calcium Tungstate, Polyethylene Glycol and Titanium Oxide.
Product use:	Temporary
Period:	Up to 30 days
The product is absorbable?	No
Physical state:	Paste

3.2 Cell Line

The V-79 cell line is fibroblasts isolated from the lung tissue of young Chinese male hamsters, this strain was developed by Ford and Yerganian in 1958.

3.3 Maintenance

From the defrosting of the cells, they were kept in bottles of 25 cm^2 or 75 cm^2 suitable for cell culture in complete culture medium (DMEM High Glucose plus 10% Bovine Fetal Serum and 1% Penicillin / Streptomycin Solution 10,000 Penicillin units / 10 mg / ml Streptomycin), the cells were maintained in culture medium at 37 °C and 5% CO_2 for about 48 hours to promote cell proliferation and to achieve the desired confluency (about 80%).

3.4 Controls

For the test, the complete culture medium (DMEM High Glucose plus 10% Fetal Bovine Serum and 1% Penicillin / Streptomycin Solution 10,000 penicillin units / 10 mg / mL

Streptomycin) was used as the negative control, as extraction control was (DMEM High Glucose plus 10% Fetal Bovine Serum and 1% Penicillin / Streptomycin Solution 10,000 Penicillin / 10 mg / mL Streptomycin) remained in a flask identical to that used to extract the test item and subjected to the same conditions as the test item) and positive control (Latex, subjected to the same extraction conditions as the test item and the extraction control) to ensure the test response.

3.5 Cell Plating

After reaching approximately 80% confluency, the cells were washed with Phosphate Buffer (0.05M / 0.15M NaCl, pH 7.4): 1X (Phosphate Buffered Saline (PBS) to remove excess Bovine Fetal Serum, they underwent a process of chemical detachment process using 0.25% Trypsin for 5 minutes at 37 °C and 5% CO_2. After this period Trypsin was inactivated with complete culture medium, the cells were then centrifuged for 3 minutes at 200G.

After centrifugation the supernatant was discarded and the cell pellet was resuspended in 1 mL of complete culture medium, an aliquot of 10 µL of cells was withdrawn and the Tripan Blue was added for counting. The counting was performed in the Neubauer chamber and then calculations were performed to adjust the correct concentration of cells per well ($1x10^4$ cells per well) and the volume of complete culture medium. The plating was performed in a 96 well plate where the cells remained for 24 hours to form the monolayer.

3.6 Test Item Preparation

The test item was manipulated in laminar flow, a portion of the test item was withdrawn, this portion was weighed and the volume of the complete culture medium proportional to the weight was added for performing the extraction process which occurred within a period of 24 ± 2 hours at 37 ± 1 °C in sterile Shott-type flask. After this period the test item extract remain directly in contact with the cells for 24 hours at 37 °C and 5% CO_2 at the following concentrations (12.5%, 25%, 50% and 100%).

3.7 Exposure to MTT

After the period of exposure of the cells to the test item extract, the cells were incubated with HBSS (Hanks' Balanced Salt solution) containing 1 mg/mL of MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-Diphenyltetrazolium Bromide) for 2 hours at 37 °C and 5% CO_2. After the exposure

period the remaining solution on the plate was discarded and Isopropanol was added for the solubilization of formed formazan crystals.

3.8 Evaluation of Results

The absorbance of the plate was read on the microplate reader using the wavelength of 570 nm as the main and a secondary wavelength of 650 nm (reference wavelength). To calculate viability, the optical density was measured to quantify the reduction in the number of viable cells relative to the negative control. The reduction of viability is a result of decreased metabolic activity (SCUDIERO et al., 1988), which in turn is proportional to the intensity of violet color observed in the wells (the less violet, the less viable the cells are). The final results are presented in percentage in relation to the negative control in the final report.

3.9 Evaluation Criteria

The mean optical density of untreated cells (negative control) and the cells treated with extraction control should be ≥ 0.2. The corrected mean of the columns designated to the negative control (columns 2 and 10) and extraction control (columns 3 and 9) shall not differ by more than 15% from the corrected mean of both. The lower the viability value (%), the greater the cytotoxic potential of the test item; if the viability is reduced to <70% in relation to the negative control, the test item has a cytotoxic potential.

4.0 RESULTS AND DISCUSSION

4.1 Negative and Positive Controls

The results of the negative control, extraction control and positive control used in the tests were adequate. The mean optical density of the negative control was 1.346 and the extraction control was 1.294. The corrected mean of the columns designated for the negative control showed a difference of 5.1% and the extraction control presented a difference of 2.8% between the columns. The negative control as well as the extraction control presented optical density ≥ 0.2 and the corrected mean of the columns designated for each one, negative control (columns 2 and 10) and extraction control (columns 3 and 9) maintained the difference ≤ 15%. Therefore, the experiments were considered valid for the analysis.

The positive control clearly induced a decrease in cell viability, presenting a reduction >70% in relation to the negative control.

4.2 Test item

The final test results obtained after 24 hours of incubation of the V-79 cell line exposed to the extract of the test item **BIO-C TEMP** at concentrations of 12.5%, 25%, 50% and 100% are set out in Table 1 and Figure 1.

A cell viability reduction of 47% was observed for the concentration of 12.5%, 72% for the 25% concentration, 98% for the 50% concentration and 99% for the 100% concentration. All concentrations had a reduction in cell viability higher than 30% in relation to the negative control.

5.0 CONCLUSION

Under the conditions of this study, the test item **BIO-C TEMP** promote reduction of cell viability higher than 30%. Therefore, the test item **BIO-C TEMP** was cytotoxic in this assay.

6.0 REFERENCES

INMETRO: NIT-DICLA-035 - Principles of Good Laboratory Practice – GLP, Rev. 03, November/2018 and its complementary documents.

ISO 10993: 5 – Biological evaluation of medical devices Part 5: Tests for *in vitro* cytotoxicity (Annex C – MTT cytotoxicity test), 2009.

ISO 10993-12: 2012 (Sample preparation and reference materials).

Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Research, v. 48, n. 17, p. 4827-4833, 1988.

TABLES SECTION

TABLE 1: Cell viability (%) of mean replicate percentages of negative and positive controls and test item concentrations **BIO-C TEMP.**

Substance	Cell viability (%)	Reduction of cell viability (%)
Negative Control – C-	100	-
Extraction Control – CE	96	-
Extract Concentration – 12.5%	53	47
Extract Concentration – 25%	28	72
Extract Concentration – 50%	2	98
Extract Concentration – 100%	1	99
Positive Control – Latex	2	98

FIGURES

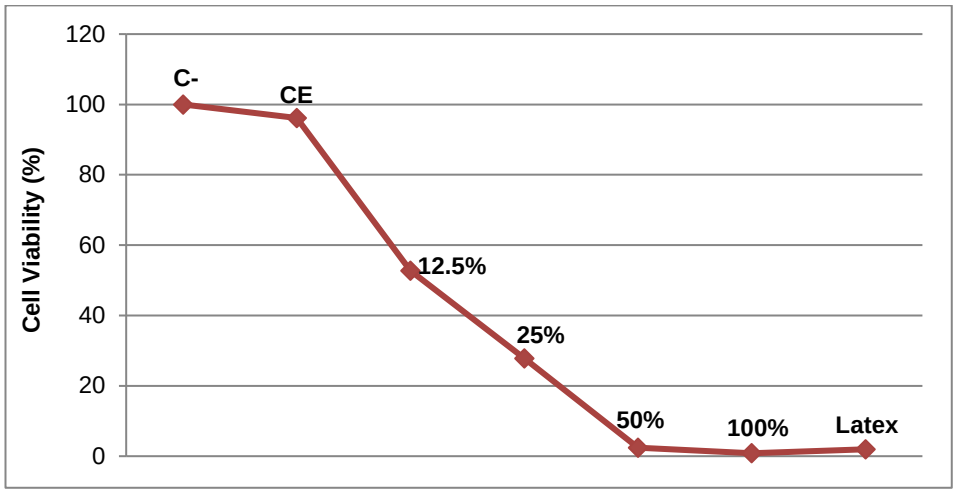

FIGURE 1: Reduction of cell viability of test item **BIO-C TEMP** at concentrations of 12.5%, 25%, 50% and 100% after exposure of 24 hours.