Istituto Zooprofilattico Sperimentale della Sicilia
Ente Sanitario di Diritto Pubblico
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Commissario Straordinario: **Dr. Salvatore Seminara**

DIPARTIMENTO ATTIVITÀ DIAGNOSTICHE
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Laboratorio Colture Cellulari
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REPORT OF CYTOTOXICITY TEST

TEST SUBSTANCE

Endodontic pins - *FiberSite*, made of Epoxy resin strengthened with fiberglass.

Diameter: 2 mm, 4 mm and 5 mm

CUSTOMER

MEGADENTAL ITALIA di Accardo Giovanni
Via Caprera, 16
91028 Partanna (TP)

TEST PERFORMED BY

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Identification number of samples:

30958-2mm

30958-4mm

30958-5mm

Receipt date: 03/11/2017

Samples description:

Endodontic pins - *FiberSite*, made of Epoxy resin strengthened with fiberglass, diameter of 2 mm, 4 mm and 5 mm

LOT: PK20004 (diameter 2 mm); PK40009 (diameter 4 mm); PK50006 (diameter 5 mm).

Sampling carried out by: Customer

Time schedule of analysis

The analysis was started on the 03.11.2017 and was completed on the 15.11.2017

Test Method:

In vitro cytotoxicity test on L929 cell line

References:

UNI EN ISO 10993-5 regulation "*Biological Evaluation of Medical Devices - In vitro Cytotoxicity Testing*"

UNI EN ISO 10993-12 regulation "*Biological Evaluation of Medical Devices - Preparation of samples and reference materials*"

Target cells:

L929 cell line (murine fibroblast), purchased by Cell Bank of National Reference Center for Alternative Methods, Welfare and Care of Laboratory Animals – Istituto Zooprofilattico Sperimentale of Lombardia and Emilia Romagna (IZS LER)

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Positive Control:

Phenol solution 0.5%, treated as samples

Negative Control:

High Density Polyethylene Film, purchased by *Food and Drug Safety Center, Hetano Research Institute*, treated as samples

Reagent Control:

Extraction medium without sample (MEM, supplemented with 10% FBS, 1% Antibiotic-antimitotic solution and 1% NEAA), treated as samples.

Apparatus:

Incubator at + 37°C ±1 and 5% CO2;
Microscope with inverted phase contrast optics;
Water Bath;
Incubator at + 37°C ±1;
Class II Biohazard laminar flow hood;
Chemical hood;
Plate shaker;
Fridge + 4°C ± 2;
Freezer - 20°C ± 3;
Liquid nitrogen tank;
Autoclave;
Centrifuge;
Sterile disposables;
Tissue culture flasks;
96-well culture plates;

Reagents:

Minimum Essential Medium with Earle's salt, L-Glutamine and Sodium Bicarbonate (MEM);

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Foetal Bovine Serum (FBS);
Antibiotic-Antimytotic 100X solution;
Non-Essential Amino Acid 100X solution (NEAA);
Cell Titer 96 AQueous One Solution Cell Proliferation 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) Assay – Promega.

Summary of method:

All manipulations of samples, cells and reagents were carried out in conformity with the Good Laboratory Practices standards, under a class II Biohazard laminar flow hood, using disposable gloves.

- **Samples preparation:**

Among the methods recommended by the UNI EN ISO 10993 regulation, the "Extraction dilution method" was adopted.
Before to perform the extraction procedure, each sample was sterilized at + 121 °C for 20 min and then placed into a sterile, chemically inert and closed flask, suitable for cell culture.
Extraction procedure was carried out in MEM, supplemented with 10% FBS, 1% Antibiotic-Antimytotic solution and 1% NEAA, at + 37°C ± 1 for 72 h, by continuous agitation.
Negative Control and Reagent Control were extracted as samples.

- **Cytotoxicity test on L929 cell line:**

L929 cell line were grown in culture flasks containing MEM supplemented with 10% FBS, 1% Antibiotic-Antimytotic solution and 1% NEAA, at + 37°C in a humidified atmosphere with 5% CO_2, to a near confluent monolayer.
Cells were seeded into 96-well culture plates. Three 96-well culture plate for each sample were prepared and incubated at + 37°C ± 1 in 5% CO2 for 24 h. Each 100% concentrated sample and a series of two-fold dilutions, were tested in triplicate with controls. All plates were incubated at + 37 ± 1 °C in a 5% CO_2 atmosphere and examined after 24 h, 48 h and 72 h of incubation.

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- **Microscope examination and MTS assay:**

After 24, 48 and 72 h of incubation period, general morphology of cells were microscopically assessed.

The MTS reagent was directly put into each well containing samples and controls and incubated at + 37 ± 1 °C in a 5% CO2 for 4 h. The absorbance was recordered at 490 nm with a 96-well plate reader.

Interpretation of Results:

The determination of cytotoxicity is performed examinating the cells under the microscope to assess general morphology, vacuolation, detachment, cell lysis, membrane integrity. Any change from the normal morphology is rated on a reactivity grade from 0 to 4 (see Grading System).

Grading system

Grade	Reactivity	Reactivity description
0	None	Discrete intracytoplasmatic granules; no cell lysis
1	Slight	Not more than 20% of the cells are round, loosely attached and without intracytoplasmatic granules; occasional lysed cells are present
2	Mild	Not more than 50% of the cells are round and devoid of intracytoplasmic granules; no extensive cell lysis and empty areas between cells
3	Moderate	Not more than 70% of the cell layers contain rounded cells or are lysed
4	Severe	Nearly complete destruction of the cell layers

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The percentage of vital cells is assessed through MTS assay:

- A sample is considered ***Cytotoxic*** if the percentage vitality value compared to the Reagent Control is < 70%.
- A sample is considered ***Not cytotoxic*** if the percentage vitality value, compared to the Reagent Control is > 70%.

Results

See table 1, 2 and 3

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Table 1: results after 24 h

Samples/Controls	Concentration	Score	Cell vitality %	Result
30958-2mm	100%	0	100	Not Cytotoxic
	1 : 2	0	100	
	1 : 4	0	100	
	1 : 8	0	100	
	1: 16	0	100	
	1: 32	0	100	
30958-4mm	100%	0	100	Not Cytotoxic
	1 : 2	0	100	
	1 : 4	0	100	
	1 : 8	0	100	
	1: 16	0	100	
	1: 32	0	100	
30958-5mm	100%	0	100	Not Cytotoxic
	1 : 2	0	100	
	1 : 4	0	100	
	1 : 8	0	100	
	1: 16	0	100	
	1: 32	0	100	
Positive control	100%	4	15,5	Cytotoxic to 1: 2
	1 : 2	3	58.65	
	1 : 4	0	100	
	1 : 8	0	100	
	1: 16	0	100	
	1: 32	0	100	
Negative control	/	0	100	Not Cytotoxic
Reagent control	/	0	100	Not Cytotoxic

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Table 2: results after 48 h

Samples/Controls	Concentration	Score	Cell vitality %	Result
30958-2mm	100 %	0	100	Not Cytotoxic
	1 : 2	0	100	
	1 : 4	0	100	
	1 : 8	0	100	
	1: 16	0	100	
	1: 32	0	100	
30958-4mm	100	0	100	Not Cytotoxic
	1 : 2	0	100	
	1 : 4	0	100	
	1 : 8	0	100	
	1: 16	0	100	
	1: 32	0	100	
30958-5mm	100%	0	100	Not Cytotoxic
	1 : 2	0	100	
	1 : 4	0	100	
	1 : 8	0	100	
	1: 16	0	100	
	1: 32	0	100	
Positive control	100%	4	8.97	Cytotoxic to 1: 2
	1 : 2	3	31.3	
	1 : 4	0	100	
	1 : 8	0	100	
	1: 16	0	100	
	1: 32	0	100	
Negative control	/	0	100	Not Cytotoxic
Reagent control	/	0	100	Not Cytotoxic

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Table 3: results after 72 h

Samples/Controls	Concentration	Score	Cell vitality %	Result
30958-2mm	100%	0	100	Not Cytotoxic
	1 : 2	0	100	
	1 : 4	0	100	
	1 : 8	0	100	
	1: 16	0	100	
	1: 32	0	100	
30958-4mm	100%	0	100	Not Cytotoxic
	1 : 2	0	100	
	1 : 4	0	100	
	1 : 8	0	100	
	1: 16	0	100	
	1: 32	0	100	
30958-5mm	100%	0	100	Not Cytotoxic
	1 : 2	0	100	
	1 : 4	0	100	
	1 : 8	0	100	
	1: 16	0	100	
	1: 32	0	100	
Positive control	100%	4	6.3	Cytotoxic to 1: 2
	1 : 2	3	13.5	
	1 : 4	0	100	
	1 : 8	0	100	
	1: 16	0	100	
	1: 32	0	100	
Negative control	/	0	100	Not Cytotoxic
Reagent control	/	0	100	Not Cytotoxic

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Conclusions:

The cells treated with the extracts of the samples 100% concentrated and their dilutions, tested after 24, 48 and 72 hours of incubation did not showed any morphological changes from normal morphology compared to the Reagent Control and showed a percentage vitality value > 70%.

Since Negative and Positive Controls gave the expected results, the test is considered valid.

Il Responsabile del Laboratorio
Dott.ssa Vincenza Cannella

Il Direttore dell'Area
Dott.ssa Annalisa Guercio