Final Report

Study Title	Test for *in vitro* cytotoxicity: Elution method
Test Item	Implants (Titanium)
Study Director	Ms. P. Pradeepa, MSc, M Phil
Sponsor	B. D. Surgical Industries 2082, Mie, Part-B Bahadurgarh, Jhajjar Haryana-124507
Study Monitor	Mr. Abhishek Sharma
Test Facility	GLR Laboratories Private Limited 444 Gokulam Street Mathur, Chennai - 600 068 Tamil Nadu, India
Study Number	304/001
Regulatory Guideline	Biological Evaluation of Medical Devices - Part 5, Tests for *in vitro* Cytotoxicity, ISO 10993-5:2009(E).
Report Issued	18 April 2017
Total Number of Pages	14

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CERTIFICATE

Implants (Titanium): Test for *in vitro* cytotoxicity: Elution method

This study was performed in accordance with the standard guideline, Biological Evaluation of Medical Devices - Part 5, Tests for *in vitro* Cytotoxicity, ISO 10993-5:2009(E), agreed study plan, one definitive study plan amendment and with GLR laboratories Pvt. Ltd's Standard Operating Procedures, unless otherwise stated, and the study objectives were achieved. The work and generated data are scientifically acceptable and valid; and this report provides a true and accurate record of the results obtained.

18 Apr 2017.

Ms. P. Pradeepa, MSc, M Phil
Study Director
GLR Laboratories Pvt Ltd

Date

18 Apr 2017

Dr. G. Velmani, M Pharm, PhD
Executive-Quality Assurance
GLR Laboratories Pvt Ltd

Date

18 Apr 2017

Dr. S. S. Murugan, PhD, ERT
Test Facility Management
GLR Laboratories Pvt Ltd

Date

SUMMARY

The test item, Implants (Titanium) was tested for its ability to induce cytotoxicity in Balb/c 3T3cells at *in vitro* condition using elution method.

Implants (Titanium) is a Metallic gold coloured rectangular shaped strips with 14.5 cm length, 1.5 cm breadth and 0.20 cm thickness. It is an implant device which comes in contact with tissue and bone. The duration of contact is greater than 30 days.

Test item was extracted in the ratio of 3 cm^2 per millilitre of serum supplemented 1x DMEM at 37 ºC for 24 h under sterile conditions. The test item measuring 22.16 cm^2 (4 no. Each measuring 5.54 cm^2) was extracted in 7.4 mL of serum supplemented 1x DMEM for 24 h at 37 °C. Negative control (High - Density Polyethylene) measuring 6 cm^2 (surface area of one side is 3 cm^2, both the sides were involved in extraction) was extracted in 2 mL of serum supplemented 1x DMEM at the ratio of 3 cm^2per mL at 37 ºC for 24 hours. Positive control (Sodium Lauryl Sulphate) of 0.0010 g in 5.0 mL of serum supplemented 1x DMEM in the final concentration of 0.2 mg / mL was freshly prepared before treating the cells. Extracts were used within 40 minutes of preparation and was considered stable during this time.

Exponentially growing Balb/c 3T3 cells were seeded in 96-well plate at a concentration of 1 x 10^4 cells/well. After 24 h, the culture medium was removed and cells were treated with controls (positive [SLS] and negative [High-Density Polyethylene]) and a series of eight different concentrations (30, 40, 50, 60, 70, 80, 90 and 100%) of the test item extract. Six replicate cultures were treated for each concentration and appropriate blanks were added. The plates were then incubated in a CO_2 incubator at 37 °C with 5% CO_2 for 24 h. After 24 h of incubation period, the cells were evaluated qualitatively (microscopic evaluation) and quantitatively (neutral red uptake) for cytotoxicity.

Under microscopic evaluation (qualitative evaluation), the cultures treated with the test item extract at different concentrations were found to be normal and no change in the morphology were observed. There were no qualitative changes in cells when compared with the negative control. Quantitative evaluation using neutral red uptake assay showed that cultures treated with test item extract in all eight different concentrations had a viability greater than 70% when compared with negative control.

The assay was considered valid as the confluency of cells before treatment was greater than 70%, mean absorbance in negative control wells was 0.601, positive

control induced a strong positive response and coefficient of variation (CV %) for the mean of replicate measurements were less than 15%.

Results of viability and cytotoxicity

	Negative Control	Viability in test item extract concentrations (%)								Positive Control
		30	40	50	60	70	80	90	100	
Mean OD	0.601	0.586	0.592	0.591	0.592	0.578	0.578	0.563	0.558	0.006
SD (±)	0.014	0.005	0.009	0.018	0.009	0.006	0.007	0.005	0.010	0.003
CV (%)	2.3	0.9	1.5	3.0	1.5	1.0	1.2	0.9	1.8	50.0
Viability (%)	--	97.50	98.50	98.34	98.50	96.17	96.17	93.68	92.85	1.00
Cytotoxicity (%)	--	2.50	1.50	1.66	1.50	3.83	3.83	6.32	7.15	99.00

Based upon the results obtained in this study and in line with ISO 10993-5:2009(E) it is concluded that, the given test item Implants (Titanium) supplied by B. D. Surgical Industries, is non-cytotoxic.

INTRODUCTION

Biocompatibility testing is a regulatory requirement for demonstrating the preclinical safety of medical devices. This is evaluated in line with the standard guideline, ISO 10993-1:2009/Cor 1:2010(E), Biological Evaluation of Medical Devices - Part 1, Evaluation and Testing within a Risk Management Process. This standard describes the test selection necessary to evaluate the biocompatibility.

Cytotoxicity assays are used to assess the effect of the device or its extract on cells grown *in vitro*. The elution method uses culture medium supplemented with serum as an extracting vehicle and are considered equivalent to the use of both polar and non-polar vehicles. The extracts are transferred onto a layer of cells and incubated for 24 hours. Following incubation, the cells are examined microscopically (qualitative) for their morphology, any malformation or degeneration, and cell lysis. In the quantitative assay, the neutral red (NR) uptake assay procedure is followed, which are based on the ability of viable cells to uptake neutral red dye. A reduction of > 30% viability in the test item treated cultures compared to concurrent control culture indicates cytotoxicity.

The test selection and methods used in this study were based upon the following standards:

1. Biological Evaluation of Medical Devices - Part 1, Evaluation and Testing within a Risk Management Process, ISO 10993-1:2009/Cor 1:2010(E).

2. Biological Evaluation of Medical Devices - Part 5, Tests for *in vitro* Cytotoxicity, ISO 10993-5:2009(E).

3. Biological Evaluation of Medical Devices - Part 12, Sample Preparation and Reference Materials, ISO 10993-12:2012(E).

OBJECTIVE

To evaluate the *in vitro* cytotoxicity potential of test item extract in Balb/c 3T3 cells using elution method.

STUDY DATES

Study Start Date	:	02 March 2017
Experiment Start Date (addition of test item extract to the cell system)	:	08 March 2017
Experiment Completion Date	:	09 March 2017

The study completion date is the date the final report is signed by the Study Director

This study was performed in line with agreed study plan and one amendment.

TEST ITEM DETAILS

The test item, Implants (Titanium) was received at GLR Laboratories Private Limited, on 21 February 2017 and stored at room temperature (23.2 to 25.7 °C) until used.

The following test item information provided by the Sponsor, are considered an adequate description of the characterisation and stability of the test item.

Test Item	Implants (Titanium)
Batch No.	TIT 001
Manufacture Date	15 February 2017
Expiry Date	Not Applicable
Appearance	Metallic gold coloured rectangular shaped strips with 14.5 cm length, 1.5 cm breadth and 0.20 cm thickness.
Ingredients	Not provided by the sponsor
Temperature Stability	Not provided by the sponsor
Sterility	Non-Sterile

No analysis was performed at GLR Laboratories Private Limited to confirm it. Determinations of stability and characteristics of the test item were the responsibility of the Sponsor. The test item and control items were handled with all necessary protective clothing and all recommended safety and sterile measures were followed.

Description of the test item

Implants (Titanium) is a Metallic gold coloured rectangular shaped strips with 14.5 cm length, 1.5 cm breadth and 0.20 cm thickness. It is an implant device which comes in contact with tissue and bone. The duration of contact is greater than 30 days.

DETAILS OF CONTROL ITEMS

Positive Control	Sodium Lauryl Sulphate (SLS) (0.2 mg/mL) in 1x DMEM; (Thermo Fisher Scientific, Batch no. 2433460215; Expiry date: January 2020). This material has been routinely tested in GLR Laboratories Private Limited gives consistently an excellent cytotoxic response with Balb/c 3T3 cells.
Negative Control	High-Density Polyethylene Film (RM-C) (Make: Hatano Research Institute, Food and Drug Safety Centre, Japan. Lot No.:C-141, Expiry Date: June 2021).

TEST SYSTEM

Cell line	Balb/c 3T3, supplied by National Centre for Cell Science, India.
Growth conditions	Dulbecco's Modified Eagle Medium with L-glutamine 1x DMEM (Thermo Fisher Scientific, Lot no.1789594; Expiry Date: April 2017) supplemented with 10% New Born Calf Serum (Thermo Fisher Scientific, Lot no.1418556; Expiry Date: July 2017), 1% Penicillin/Streptomycin solution (Himedia, Lot no. 0000241753, Expiry Date: August 2017) at 37 °C in CO_2 incubator with 5% CO_2. Antibiotics used does not adversely affect the assay.
Justification for use	Use of Balb/c 3T3 cells is recommended in ISO 10993, Part 5:2009 for assessing *in vitro* cytotoxicity.

TEST METHOD

Preparation of the test item extract

Test item was extracted in the ratio of 3 cm^2 per millilitre of serum supplemented 1x DMEM at 37 ºC for 24 h under sterile conditions. The test item measuring 22.16 cm^2 (4 no. Each measuring 5.54 cm^2) was extracted in 7.4 mL of serum supplemented 1x DMEM for 24 h at 37 °C (07 March 2017, 10:30 a.m. to 08 March 2017, 10:30 a.m.). Negative control (High - Density Polyethylene) measuring 6 cm^2 (surface area of one side is 3 cm^2, both the sides were involved in extraction) was extracted in 2 mL of serum supplemented 1x DMEM at the ratio of 3cm^2per mL at 37 ºC for 24 h. Positive control (Sodium Lauryl Sulphate) of 0.0010 g in 5.0 mL of serum supplemented 1x DMEM in the final concentration of 0.2 mg / mL was freshly prepared before treating the cells. This fulfils the requirements of ISO 10993-5:2009(E) and ISO 10993-12:2012(E).

At the end of extraction period, the extract was filter sterilised prior to addition since the test item is non-sterile. Extracts were used within 40 minutes of preparation and was considered to be stable during this time. A series of eight different concentrations (30, 40, 50, 60, 70, 80, 90 and 100%) of the test item extract was prepared for the study.

Test procedure

Rationale for assay method

The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red dye.

Specified in ISO 10993, Part-5:2009 standard as an appropriate test to evaluate *in vitro* cytotoxicity for assessing the biocompatibility of medical devices.

Exponentially growing Balb/c 3T3 cells were trypsinised using trypsin-EDTA (Make: Sigma - Aldrich, Lot no. SLBN4359V, Expiry date: July 2017) and counted in a hemocytometer using 0.4% Trypan blue (Himedia, Lot no. 0000243749, Expiry Date: September 2017). Exactly 1 x 10^5 cells per mL was prepared (0.214 mL of cell suspension [32.75 x 10^5 cells per mL] was added to 6.786 mL of culture media to get 7 mL of cell suspension) and 100 µL was seeded in wells B2 to G11 of 96-well plates at a concentration of 1 x 10^4 cells per well. The plate was incubated in CO_2 incubator with 5% CO_2 at 37 °C for 24 h (07 March 2017, 10:50 a.m. to 08 March 2017, 10:50 a.m.).

The following day, the confluency and morphology of the cell was checked and found to be greater than 70 % confluent and normal. Then the medium was removed and six replicates of appropriate concentrations of the test item extract, positive, negative controls and appropriate blanks were added to the cultures as shown below:

96 - well plate template

	1	2	3	4	5	6	7	8	9	10	11	12
A	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media
B	Media	Negative	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	Positive	Media
C	Media	Negative	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	Positive	Media
D	Media	Negative	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	Positive	Media
E	Media	Negative	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	Positive	Media
F	Media	Negative	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	Positive	Media
G	Media	Negative	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	Positive	Media
H	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media

Media: Medium blank
Negative: Negative control
Positive: Positive control
Conc 1 to 8: Eight different concentrations of the test item extract - 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%, respectively
Alphabet A-H in the 96-Well Plate Layout represents each row of the plate.
Number 1-12 in the 96-Well Plate Layout represents each column of the plate.

The plate was then incubated in CO_2 incubator with 5% CO_2 at 37 °C for 24 h (08 March 2017, 11:10 a.m. to 09 March 2017, 11:10 a.m.). After 24 h of incubation, the cells were examined under inverted microscope for morphological evidence of cytotoxicity using a grading scheme according to ISO 10993-5:2009(E) (Table 1).

Immediately following the visual assessment, wells were washed with 150 µL of phosphate buffered saline (PBS) (Himedia, Lot no. 0000242278, Expiry Date: September 2017). This was removed, and 100 µL of neutral red medium was added. The plates were then incubated in CO_2 incubator with 5% CO_2 at 37 °C for exactly 3 h (09 March 2017, 11:30 a.m. to 02:30 p.m.). Following the incubation, the neutral red medium was removed and the cells were washed with 150 µL of PBS which was removed before adding 150 µL of neutral red desorb solution (ethanol: glacial acetic acid: distilled water, 10 mL:0.2 mL:9.8 mL). Plates were shaken periodically until all neutral red was removed from the cells, forming a homogenous solution. The resulting coloured solution was analysed using a microplate reader (Mindray MR-96A) at a wavelength setting of 546 nm. Neutral Red absorbance was expressed in terms of absolute optical density (OD_{546}; which was OD_{546} of the culture minus the mean OD_{546} of medium blanks). Cell viability was calculated as the percentage of culture OD_{546} divided by negative control OD_{546}.

DATA EVALUATION

Qualitative evaluation: Cultures treated with test item extract that induce cytotoxicity grades greater than 2 (see Table 1) will be considered cytotoxic.

Quantitative evaluation: Undiluted test item extract was considered non-cytotoxic if the viability measured by neutral red uptake was ≥ 70% than that of the negative control. Viability < 70% indicated cytotoxicity.

The results of the range finder experiment revealed negative response, no further main experiment was performed.

The coefficient of variation (CV %) was calculated using the following formula:

$$\text{CV \%} = \frac{\text{SD}}{\text{Mean } OD_{546}} \text{ X } 100$$

The assay was considered valid, as the positive control treated cultures gave a clear increase in cytotoxicity compared to that observed in negative control cultures.

Good scientific judgement was used in interpreting the data.

ACCEPTANCE CRITERIA

The assay was to be considered valid if all the following criteria were met:

1. Before treatment, cells should have a confluency of >70% and grown well.

2. Mean absorbance value of negative control should be ≥ 0.3.
3. The positive controls should show a strong positive cytotoxic response of >30%.
4. The coefficient of variation (CV %) for replicate measurements should be < 15%.

RESULTS

Before treatment, all wells had cells confluency of greater than 70%. The mean OD_{546} of negative treated cells were 0.601. Coefficient of variation for all test item extract replicate measurements were < 15%. Clear increase in cytotoxicity was observed in the positive control treated cultures. But, no such cell destruction was evident in the negative control. Hence, the test was considered valid.

Results of qualitative evaluation are given in Table 2. It is clear that the test item extract was non - cytotoxic.

Neutral red uptake assay's results reflected the results of qualitative analysis (Tables 3, 4, and 5). Implants (Titanium) extract at concentrations 30, 40, 50, 60, 70, 80, 90 and 100% showed viability greater than 70% when compared to negative control.

CONCLUSION

Based upon the results obtained in this study and in line with ISO 10993-5:2009(E) it is concluded that, the given test item Implants (Titanium) supplied by B. D. Surgical Industries, is non-cytotoxic.

REFERENCES

1. Biological Evaluation of Medical Devices - Part 1, Evaluation and Testing within a Risk Management Process, ISO 10993-1:2009/Cor 1:2010(E).

2. Biological Evaluation of Medical Devices - Part 5, Tests for *in vitro* Cytotoxicity, ISO 10993-5:2009(E).

3. Biological Evaluation of Medical Devices - Part 12, Sample Preparation and Reference Materials, ISO 10993-12:2012(E).

Table 1: Qualitative Morphological Grading of Cytotoxicity of Extracts

Grade	Reactivity	Conditions of all cultures
0	None	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth.
1	Slight	Not more than 20% of the cells are round, loosely attached and without intracytoplasmic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable.
2	Mild	Not more than 50% of the cells are round, devoid of intracytoplasmic granules, no extensive cell lysis; not more than 50% growth inhibition observable.
3	Moderate	Not more than 70% of the cell layers contain rounded cells or are lysed; cell layers not completely destroyed, but more than 50% growth inhibition observable.
4	Severe	Nearly complete or complete destruction of the cell layers.

Source: ISO 10993-5:2009(E).

Table 2: Results of qualitative scoring for cytotoxicity

	1	2	3	4	5	6	7	8	9	10	11	12
A	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells
B	No cells	0	0	0	0	0	0	0	0	0	4	No cells
C	No cells	0	0	0	0	0	0	0	0	0	4	No cells
D	No cells	0	0	0	0	0	0	0	0	0	4	No cells
E	No cells	0	0	0	0	0	0	0	0	0	4	No cells
F	No cells	0	0	0	0	0	0	0	0	0	4	No cells
G	No cells	0	0	0	0	0	0	0	0	0	4	No cells
H	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells

0, None; 1, Slight; 2, Mild; 3, Moderate; and 4, Severe cytotoxicity

Table 3: Results of optical density readings at 546 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.062	0.068	0.063	0.061	0.061	0.057	0.070	0.066	0.062	0.057	0.071	0.059
B	0.063	0.678	0.648	0.671	0.667	0.653	0.649	0.647	0.623	0.634	0.070	0.067
C	0.065	0.680	0.659	0.665	0.628	0.645	0.634	0.653	0.632	0.629	0.068	0.069
D	0.067	0.664	0.644	0.648	0.663	0.661	0.641	0.635	0.620	0.617	0.072	0.071
E	0.069	0.676	0.647	0.657	0.641	0.651	0.651	0.639	0.627	0.625	0.076	0.064
F	0.063	0.647	0.654	0.652	0.661	0.662	0.638	0.645	0.633	0.606	0.073	0.068
G	0.070	0.652	0.653	0.651	0.677	0.670	0.644	0.636	0.632	0.629	0.069	0.061
H	0.061	0.067	0.070	0.062	0.071	0.065	0.063	0.059	0.062	0.063	0.071	0.064

Mean of media blanks: 0.065

Table 4: ODs adjusted for media blank

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0.613	0.583	0.606	0.602	0.588	0.584	0.582	0.558	0.569	0.005	0
C	0	0.615	0.594	0.600	0.563	0.580	0.569	0.588	0.567	0.564	0.003	0
D	0	0.599	0.579	0.583	0.598	0.596	0.576	0.570	0.555	0.552	0.007	0
E	0	0.611	0.582	0.592	0.576	0.586	0.586	0.574	0.562	0.560	0.011	0
F	0	0.582	0.589	0.587	0.596	0.597	0.573	0.580	0.568	0.541	0.008	0
G	0	0.587	0.588	0.586	0.612	0.605	0.579	0.571	0.567	0.564	0.004	0
H	0	0	0	0	0	0	0	0	0	0	0	0

Table 5: Results of viability and cytotoxicity

	Negative Control	**Viability in test item extract concentrations (%)**								**Positive Control**
		30	**40**	**50**	**60**	**70**	**80**	**90**	**100**	
Mean OD	0.601	0.586	0.592	0.591	0.592	0.578	0.578	0.563	0.558	0.006
SD (±)	0.014	0.005	0.009	0.018	0.009	0.006	0.007	0.005	0.010	0.003
CV (%)	2.3	0.9	1.5	3.0	1.5	1.0	1.2	0.9	1.8	50.0
Viability (%)	--	97.50	98.50	98.34	98.50	96.17	96.17	93.68	92.85	1.00
Cytotoxicity (%)	--	2.50	1.50	1.66	1.50	3.83	3.83	6.32	7.15	99.00

RESPONSIBLE PERSONNEL

Ms. P. Pradeepa, MSc, M Phil	Study Director
Ms. G. Ashtalakshmi, MSc, M Phil	Study Scientist
Mr. S. Haribabu, B Tech (Biotech), MSc	Study Scientist

STUDY PLAN AMENDMENT

One definitive study plan amendment was made as per sponsor request to change the "Batch / Lot No. - BDS/2017/02/001" to "Batch No. - TIT 001" and "Manufacture Date - 10 February 2017" to "Manufacture Date - 15 February 2017".

STUDY PLAN DEVIATION

No deviations from the study plan were found during the conduct of the study.

DISTRIBUTION OF REPORTS

Two originals of the study report are prepared and distributed as mentioned below:

1. Sponsor.
2. GLR Laboratories Private Limited.

Final Report

Study Title	Intracutaneous reactivity test in New Zealand White rabbits
Test Item	Implants (Titanium)
Study Director	Ms. G. Ashtalakshmi, MSc, M Phil
Sponsor	B. D. Surgical Industries 2082, Mie, Part-B Bahadurgarh, Jhajjar Haryana-124507
Study Monitor	Mr. Abhishek Sharma
Test Facility	GLR Laboratories Private Limited 444 Gokulam Street Mathur, Chennai - 600 068 Tamil Nadu, India
Study Number	304/002
Regulatory Guideline	Biological Evaluation of Medical Devices - Part 10, Tests for Irritation and Skin Sensitization, ISO 10993-10:2010(E).
Report Issued	17 April 2017
Total Number of Pages	18

CONTENTS

CERTIFICATE

Implants (Titanium): Intracutaneous reactivity test in New Zealand White rabbits

This study was performed in accordance with the standard guideline, Biological Evaluation of Medical Devices - Part 10, Tests for Irritation and Skin Sensitization, ISO 10993-10:2010 (E), agreed study plan, one definitive study plan amendment and with GLR laboratories Pvt Ltd's Standard Operating Procedures, unless otherwise stated, and the study objectives were achieved. The work and generated data are scientifically acceptable and valid; and this report provides a true and accurate record of the results obtained.

17 Apr 2017

Ms. G. Ashtalakshmi, MSc, M Phil
Study Director
GLR Laboratories Pvt Ltd

Date

17 Apr 2017

Dr. G. Velmani, M Pharm, PhD
Executive-Quality Assurance
GLR Laboratories Pvt Ltd

Date

17 Apr 2017

Dr. S. S. Murugan, PhD, ERT
Test Facility Management
GLR Laboratories Pvt Ltd

Date

SUMMARY

Intracutaneous reactivity test of the test item Implants (Titanium), supplied by B. D. Surgical Industries, were conducted in male New Zealand White rabbits.

Implants (Titanium) is a metallic gold coloured rectangular shaped strips with 14.5 cm length, 1.5 cm breadth and 0.20 cm thickness. It is an implant device which comes in contact with tissue and bone. The duration of contact is greater than 30 days.

The test items measuring 16.62 cm^2 were extracted in 5.54 mL of polar (physiological saline) (3 nos. were used each measuring 5.54 cm^2) and similarly 16.62 cm^2 were extracted in 5.54 mL of non-polar (sesame oil) solvent prepared by ratio of 3 cm^2 of test item per millilitre of solvent at 50 ºC for 72 h under sterile conditions. Solvent controls were also subjected to same extraction conditions. At the end of extraction, the extracts and solvent controls were clear, there was no change in the colour and no particulates were found (pre- and post-extraction). Hence, no additional processing such as filtration, centrifugation, pH adjustments or any other processing were made. The extracts and solvent controls were transferred to sterile containers and stored at room temperature. All extracts and solvent controls were used within 2 h and 10 mins of preparation and were considered stable during this time. This fulfils the requirements of ISO 10993-12:2012(E).

Four hours prior to intracutaneous injections, all the rabbits were closely clipped off the fur on the backs, allowing sufficient distance on both the sides of the spine for injection of test item extracts.

Test item extracts and negative controls were injected as follows:

Animal No.	Sample	Injection site	Volume of each injection (mL)	No. of injections/ site
1	Test item extract in physiological saline Physiological saline (Negative control) Test item extract in sesame oil Sesame oil (Negative control)	Left cranial end Right cranial end Left caudal end Right caudal end	0.2	5
2	Test item extract in physiological saline Physiological saline (Negative control) Test item extract in sesame oil Sesame (Negative control)	Left cranial end Right cranial end Left caudal end Right caudal end	0.2	5
3	Test item extract in physiological saline Physiological saline (Negative control) Test item extract in sesame oil Sesame oil (Negative control)	Left cranial end Right cranial end Left caudal end Right caudal end	0.2	5

The skin reactions were visually scored according to ISO 10993-10:2010(E) at 24,48 and 72 h post injection.

The animals were observed for three consecutive days for morbidity, mortality and abnormal clinical signs and symptoms following injections.

Neither mortality nor morbidity was recorded, a gradual increase in body weight of test animals was reported and no signs of ill health or overt toxicity was observed.

No positive controls were included in this study. Positive control trials for irritation are carried out every three months in our laboratory to demonstrate the sensitivity of this strain of animals to 10% SLS in water. The last such positive control trial was completed on 08 December 2016 and gave a moderate irritant response. The current positive control trial was initiated and will be completed in March 2017.

Animals treated with the test item extracts did not show any skin reactions.

Solvent	Mean Reaction Score for test item extract	Mean Reaction Score for control	Overall difference (Test extract - control)
Physiological saline	0	0	0
Sesame oil	0	0	0

The difference of the mean skin reaction scores for the test item extracts and the control vehicle was zero.

Based upon the results obtained in this study and in line with ISO 10993-10:2010 (E) it is concluded that, the extract of the given test item Implants (Titanium) supplied by B. D. Surgical Industries, is non-reactive.

INTRODUCTION

Biocompatibility testing is a regulatory requirement for demonstrating the safety of medical devices. This is performed as per ISO 10993, Parts 1 to 20. The primary aim of this group of standards is the protection of humans from potential biological risks arising from the use of medical devices. The general guidance for biocompatibility testing is given in ISO 10993-1:2009/ Cor 1:2010 (E), Biological Evaluation of Medical Devices - Part 1, Evaluation and Testing within a Risk Management Process. This standard also describes the categorization of medical devices based on nature and duration of patient contact; and test selection necessary to evaluate biocompatibility. The technical guidance for the biocompatibility tests are given in other parts of ISO 10993.

Intracutaneous reactivity test is carried out according to ISO 10993 Part 10; Tests for irritation and skin sensitization. Types of irritation tests are listed below:

Irritation Tests	**Standard**
Animal Irritation Test Animal intracutaneous (intradermal) reactivity test	ISO 10993: Part 10
Special irritation tests	
Ocular irritation test Oral mucosa irritation test Penile irritation test Rectal irritation test Vaginal irritation test	ISO 10993: Part 10

In this study, intracutaneous reactivity test was carried out. The reactivity potential of a test device was assessed by injecting the extract of the test item intracutaneously in rabbits and the observed responses were graded as given in ISO 10993 Part 10.

The test selection and methods used in this study were based on the following standards:

1. Biological Evaluation of Medical Devices - Part 1, Evaluation and Testing within a Risk Management Process, ISO 10993-1:2009/Cor 1:2010(E).
2. Biological Evaluation of Medical Devices - Part 2, Animal Welfare Requirements, ISO 10993-2:2006(E).
3. Biological Evaluation of Medical Devices - Part 10, Tests for Irritation and Skin Sensitization, ISO 10993-10:2010(E).
4. Biological Evaluation of Medical Devices - Part 12, Sample Preparation and Reference Materials, ISO 10993-12:2012(E).

OBJECTIVE

To determine the reactivity potential of the test item extracts following intracutaneous injection into New Zealand White rabbits.

STUDY DATES

Study Start Date	02 March 2017
Experiment Start Date (Date of first dosing)	11 March 2017
Experiment Completion Date	14 March 2017

The study completion date is the date the final report is signed by the Study Director.

This study was performed in line with agreed study plan and one amendment.

TEST ITEMDETAILS

The test item, Implants (Titanium) was received at GLR Laboratories Private Limited on 21 February 2017 and stored at room temperature (23.2 to 25.7) °C until use. The following test item information provided by the sponsor were considered adequate.

Test Item	Implants (Titanium)
Batch / Lot No.	TIT 001
Manufacture Date	15 February 2017
Expiry Date	Not Applicable
Appearance	Metallic gold coloured rectangular shaped strips with 14.5 cm length, 1.5 cm breadth and 0.20 cm thickness.
Ingredients	Not provided by the sponsor
Temperature Stability	Not provided by the sponsor
Sterility	Non-Sterile

CONTROL ITEM DETAILS

Positive Control	Sodium lauryl sulphate-SLS No animals were used for positive control in this study. Positive control trials for irritation are conducted every three months in GLR laboratory. This strain of rabbits gives a clear positive response to 10% sodium lauryl sulphate (SLS) in water. The details of positive control trials are provided in Appendix 1.
Negative (Solvent) Control	Physiological saline and sesame oil

The test item was handled with all necessary protective clothing and all recommended safety and sterile measures were followed. The identity, composition stability and characteristics of the test item is the responsibility of the sponsor. No analysis was performed at GLR Laboratories Private Limited, to confirm it.

Description of the test item

Implants (Titanium) is a metallic gold coloured rectangular shaped strips with 14.5 cm length, 1.5 cm breadth and 0.20 cm thickness. It is an implant device which comes in contact with tissue and bone. The duration of contact is greater than 30 days.

TEST SYSTEM

Species	Rabbit (*Oryctolagus cuniculus*)
Strain	New Zealand White
Weight (g) (Start of the experiment)	2529.3 to 2619.4
Sex	Male
Source	NIN, Hyderabad, India. This supplier is approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India for breeding laboratory animals.
Number of animals used	3
Acclimation period	5 days
Justification for animal use	The intracutaneous injection test in rabbits are specified in the current ISO testing standards and has been used historically to evaluate biomaterial extracts.

The test system was approved by the GLR Laboratories Private Limited Institutional Animal Ethics Committee (IAEC).

ANIMAL HUSBANDRY

Test Room No.	07
Test room temperature (°C)	19.6 to 22.1
Relative humidity (%)	41 to 59
Housing	Animals were housed individually in stainless steel rabbit cages.

Method of identification	Animals were identified using cage cards indicating cage no., study no., species, strain, animal no.,sex, age/bodyweight, dose, signature and individual earmarking.
Diet	Rabbit pellet feed (Amrut feeds)
Water	Purified drinking water was provided *ad libitum*
Bedding material	No bedding materials were used as rabbits were housed in stainless steel cages with mesh floors. Absorbent paper paddings used to collect the excreta and urine was changed routinely.
Photoperiod	12: 12 h light and dark cycle
Contaminants	Contaminants, reasonably expected in feed and/or water supplied were not believed to influence the outcome of the study.
Personnel	Associates involved in this study were appropriately qualified and trained.
Selection of animals	Previously unused and healthy young adults were selected for this study.

TEST METHOD

Preparation of the test item extracts

The test items measuring 16.62 cm^2 were extracted in 5.54 mL of polar (physiological saline) (3 nos. were used each measuring 5.54 cm^2) and similarly 16.62 cm^2 were extracted in 5.54 mL of non-polar (sesame oil) solvent prepared by ratio of 3 cm^2 of test item per millilitre of solvent at 50 ºC for 72 h under sterile conditions. Solvent controls were also subjected to same extraction conditions. At the end of extraction, the extracts and solvent controls were clear, there was no change in the colour and no particulates were found (pre-and post-extraction). Hence, no additional processing such as filtration, centrifugation, pH adjustments or any other processing were made. The extracts and solvent controls were transferred to sterile containers and stored at room temperature. All extracts and solvent controls were used within 2 h and 10 mins of preparation and were considered stable during this time. This fulfils the requirements of ISO 10993-12:2012(E).

The details of extract preparation are given below,

Extract	Extraction vehicle	Surface area of the test item taken (cm^2)	Volume of vehicle (mL)	Extract preparation start time	Extract preparation end time	Appearance of extracts*
Polar Extract	Physiological saline	16.62	5.54	10:20 a.m. on 08 Mar 2017	10:20 a.m. on 11 Mar 2017	Colourless clear solution, no particulates
Polar Vehicle Negative Control	Physiological saline	NA	10.0			Colourless clear solution, no particulates
Non-polar Extract	sesame oil	16.62	5.54			Light brown viscous liquid; no particulates
Non-polar Vehicle Negative Control	Sesame oil	NA	10.0			Light brown viscous liquid; no particulates

*extraction vehicles did not undergo any colour changes during the extraction process; NA-Not applicable

The pH of the polar extract was 7.03. Therefore, the extract was found suitable to conduct intracutaneous reactivity study in rabbits. The pH of the oil extract cannot be measured, but it is assumed acceptable for intracutaneous injections.

The details of the solvents were as follows:

Physiological saline (0.9% w/v sodium chloride solution)

Manufacturer	Baxter (India) Pvt. Limited
Batch No.	10150892B
Expiry Date	August 2018
Appearance	Colourless clear solution

Sesame oil

Manufacturer	Sigma-Aldrich
Lot No.	MKBT8141V
Expiry Date	December 2021
Appearance	Light brown viscous liquid

Dosing Procedure

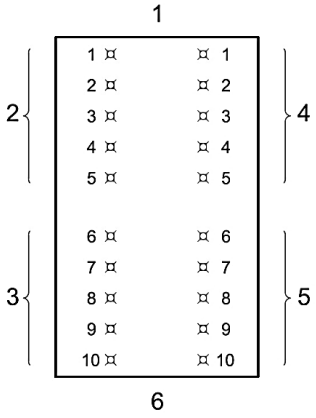
Justification	Recommended in ISO 10993, Part-10: 2010 (E), intracutaneous injection of test item extracts to rabbit as a suitable route of administration and the dose volume was 0.2 mL per injection without any dilution, to determine biocompatibility of materials used in medical devices.

Test procedure

Four hours prior to intracutaneous injections, all the rabbits were closely clipped off the fur on the backs, allowing sufficient distance on both the sides of the spine for injection of test item extracts (see diagram below). Intracutaneous injections of polar

and non-polar extracts and corresponding controls were given using, sterile syringes and needles (Hindustan Syringes & Medical Devices Ltd.; Batch No.:444016G32; Expiry date: October 2019) as given in the table and figure:

Animal No.	Sample	Injection site	Volume of each injection (mL)	No. of injections/ site
1	Test item extract in physiological saline Physiological saline (Negative control) Test item extract in sesame oil Sesame oil (Negative control)	Left cranial end Right cranial end Left caudal end Right caudal end	0.2	5
2	Test item extract in physiological saline Physiological saline (Negative control) Test item extract in sesame oil Sesame oil (Negative control)	Left cranial end Right cranial end Left caudal end Right caudal end	0.2	5
3	Test item extract in physiological saline Physiological saline (Negative control) Test item extract in sesame oil Sesame oil (Negative control)	Left cranial end Right cranial end Left caudal end Right caudal end	0.2	5



1. Cranial end; 2. 0.2 ml injections of polar extract; 3. 0.2 ml injections of non-polar extract; 4. 0.2 ml injections of polar solvent control; 5. 0.2 ml injections of non-polar solvent control; 6. Caudal end

OBSERVATIONS

Mortality & Morbidity

All the animals were observed daily for mortality and morbidity throughout the experiment.

Body Weight

Body weight of each animal was recorded at the start and at the end of the experiment.

Clinical Observation

All animals were observed for clinical signs of toxicity immediately after intracutaneous injection, and at 24 h, 48 h, and 72 h.

Scoring of Skin Reaction

Observations and scoring of skin reactions viz., oedema, erythema and eschar formation were performed visually with naked eyes as per ISO 10993-10:2010(E) at 24 h, 48 h and 72 h following the intracutaneous injection. Observations were graded on a numerical scale for both the test item extracts and vehicle controls.

Grading system for intracutaneous reactions are shown in the following table:

Reaction	Numerical grading
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet-redness) to eschar formation preventing grading of erythema	4
Oedema formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Well-defined oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm extending beyond exposure area)	4
Maximal possible score for irritation	8

Source: ISO 10993- Part 10: 2010 (E)

Necropsy

No animals were found dead or in moribund condition, hence gross pathology was not performed. All animals were euthanized by ketamine + xylazine injection at the end of the experiment.

EVALUATION CRITERIA

After 72 h grading, all erythema and oedema grades at 24 h, 48 h and 72 h were totalled for each test item extract or control for each individual animal. For calculating the score of a test item and control on each individual animal, the derived value was divided each of the totals by 15 (3 scoring periods x 5 test or control sample injection sites). To determine the overall mean score for each test item and each corresponding control, the scores for the 3 animals were added and divided by three. The final test item score was obtained by subtracting the score of the control from the test item score.

Solvent	Mean Reaction Score for test item extract	Mean Reaction Score for negative control	Overall difference (Test extract - control)
Physiological saline	A	B	(A-B)
Sesame oil	C	D	(C-D)

The requirements of the test were met, the difference (final score) of the mean reaction grades (erythema/ oedema) for the test item and the control was less than 1.0.

RESULTS

Mortality & Morbidity
No animal was observed for mortality and morbidity throughout the experiment

Body Weight
Body weight of each animal increased after test item administration, was recorded at the start and at the end of the experiment are presented in Table 1.

Clinical Observation
No signs of ill health or overt toxicity were observed in any of the test animals.

Scoring of Skin Reaction
Injection sites appeared normal immediately after the injections. The results of grading of skin reactions for individual animals are given in Table 2. The difference of the mean skin reaction scores for the test item extracts and the vehicle control was zero (see Table 3).

Positive control trial
Positive control trial conducted within the test facility gave clear positive results (Appendix 1).

CONCLUSION

Based upon the results obtained in this study and in line with ISO 10993-10:2010 (E) it is concluded that, the extract of the given test item Implants (Titanium) supplied by B. D. Surgical Industries, is non-reactive.

REFERENCES

1. Biological Evaluation of Medical Devices - Part 1, Evaluation and Testing within a Risk Management Process, ISO 10993-1:2009/Cor 1:2010(E).

2. Biological Evaluation of Medical Devices - Part 2, Animal Welfare Requirements, ISO 10993-2:2006(E).

3. Biological Evaluation of Medical Devices - Part 10, Tests for Irritation and Skin Sensitization, ISO 10993-10:2010(E).

4. Biological Evaluation of Medical Devices - Part 12, Sample Preparation and Reference Materials, ISO 10993-12:2012(E).

Table 1: Individual body weights of New Zealand White rabbits

Animal No.	Sex	Bodyweight (g)	
		Initial	Final
1	M	2571.7	2577.0
2	M	2619.4	2624.3
3	M	2529.3	2534.4

M - Male

Table 2: Grading of skin reactions for individual New Zealand White rabbits

Animal No.	Sex	Solvent	24 h				48 h				72 h			
			Test item extract		Negative Control		Test item extract		Negative Control		Test item extract		Negative Control	
			E	O	E	O	E	O	E	O	E	O	E	O
1	M	Physiological saline	0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
1	M	Sesame oil	0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
2	M	Physiological saline	0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
2	M	Sesame oil	0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
3	M	Physiological saline	0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
3	M	Sesame oil	0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0

M, Male; E, Erythema; O, Oedema

Table 3: Mean reaction scores

Solvent	Test item extract	Negative Control	Overall difference
	E+O	E+O	E+O
Physiological saline	0	0	0
Sesame oil	0	0	0

E, Erythema; O, Oedema

APPENDIX 1

Summary of Positive Control Trial (GLR Study number 000/016)

Study number	Study start date	Experiment start date	Experiment completion date	Study completion date	Agent used	Result
000/016	15 November 2016	24 November 2016	01 December 2016	08 December 2016	10% sodium lauryl sulphate	Moderate irritant

The current positive control trial was initiated and will be completed in March 2017.

RESPONSIBLE PERSONNEL

Ms. G. Ashtalakshmi, MSc, M Phil	Study Director
Mr. K. Sakthivel, MSc	Animal House In-charge
Dr. B. Rajan, MSc, PhD	Study Scientist
Dr. J.S.I. Rajkumar, MSc, M Phil, PhD	Study Scientist

STUDY PLAN AMENDMENT

Based on sponsor request, study plan amendment was made to modify the batch no. and manufacturing date of the test item.

STUDY PLAN DEVIATION

No deviations from the study plan were found during the conduct of the study.

DISTRIBUTION OF REPORTS

Two originals of the study report are prepared and distributed as mentioned below:
1. Sponsor.
2. GLR Laboratories Private Limited.

Final Report

Study Title	Test for *in vitro* cytotoxicity: Elution method
Test Item	Implants (SS316L)
Study Director	Ms. P. Pradeepa, MSc, M Phil
Sponsor	B. D. Surgical Industries 2082, Mie, Part-B Bahadurgarh, Jhajjar Haryana-124507
Study Monitor	Mr. Abhishek Sharma
Test Facility	GLR Laboratories Private Limited 444 Gokulam Street Mathur, Chennai - 600 068 Tamil Nadu, India
Study Number	304/003
Regulatory Guideline	Biological Evaluation of Medical Devices - Part 5, Tests for *in vitro* Cytotoxicity, ISO 10993-5:2009(E).
Report Issued	18 April 2017
Total Number of Pages	15

CONTENTS

CERTIFICATE

Implants (SS316L): Test for *in vitro* cytotoxicity: Elution method

This study was performed in accordance with the standard guideline, Biological Evaluation of Medical Devices - Part 5, Tests for *in vitro* Cytotoxicity, ISO 10993-5:2009(E), agreed study plan, One definitive study plan amendment and with GLR laboratories Pvt Ltd's Standard Operating Procedures, unless otherwise stated, and the study objectives were achieved. The work and generated data are scientifically acceptable and valid; and this report provides a true and accurate record of the results obtained.

18 Apr 2017

Ms. P. Pradeepa, MSc, M Phil
Study Director
GLR Laboratories Pvt Ltd

Date

18 Apr 2017

Dr. G. Velmani, M Pharm, PhD
Executive-Quality Assurance
GLR Laboratories Pvt Ltd

Date

18 Apr 2017

Dr. S. S. Murugan, PhD, ERT
Test Facility Management
GLR Laboratories Pvt Ltd

Date

SUMMARY

The test item, Implants (SS316L) were tested for its ability to induce cytotoxicity in Balb/c 3T3cells at *in vitro* condition using elution method.

Implants (SS316L) is a metallic silver coloured rectangular shaped strips with 14.5 cm length, 1.1 cm breadth and 0.18 cm thickness. It is an implant device which comes in contact with tissue and bone. The duration of contact is greater than 30 days.

Test item was extracted in the ratio of 3 cm^2 per millilitre of serum supplemented 1x DMEM at 37 ºC for 24 h under sterile conditions. The test item measuring 20.5 cm^2 (5 no. each measuring 4.1 cm^2) was extracted in 6.83 mL of serum supplemented 1X DMEM for 24 h at 37 °C in an incubator. Negative control (High - Density Polyethylene) measuring 6 cm^2 (surface area of one side is 3 cm^2, both the sides were involved in extraction) was extracted in 2 mL of serum supplemented 1x DMEM at the ratio of 3 cm^2 per mL at 37 ºC for 24 hours. Positive control (Sodium Lauryl Sulphate) of 0.0011 g in 5.5 mL of serum supplemented 1x DMEM in the final concentration of 0.2 mg / mL was freshly prepared before treating the cells. Extracts were used within 40 minutes of preparation and was considered stable during this time.

Exponentially growing Balb/c 3T3 cells were seeded in 96-well plate at a concentration of1 x 10^4 cells/well. After 24 h, the culture medium was removed and cells were treated with controls (positive [SLS] and negative [High-Density Polyethylene]) and a series of eight different concentrations (30, 40, 50, 60, 70, 80, 90 and 100%) of the test item extract. Six replicate cultures were treated for each concentration and appropriate blanks were added. The plates were then incubated in a CO_2 incubator at 37 °C with 5% CO_2 for 24 h. After 24 h of incubation period, the cells were evaluated qualitatively (microscopic evaluation) and quantitatively (neutral red uptake) for cytotoxicity.

Under microscopic evaluation (qualitative evaluation), the cultures treated with the test item extract at different concentrations were found to be normal and no change in the morphology were observed. There were no qualitative changes in cells when compared with the negative control. Quantitative evaluation using neutral red uptake assay showed that cultures treated with test item extract in all eight different concentrations had a viability greater than 70% when compared with negative control.

The assay was considered valid as the confluency of cells before treatment was greater than 70%, mean absorbance in negative control wells was 0.593, positive

control induced a strong positive response and coefficient of variation (CV %) for the mean of replicate measurements were less than 15%.

Results of viability and cytotoxicity

	Negative Control	Viability in test item extract concentrations (%)								Positive Control
		30	40	50	60	70	80	90	100	
Mean OD	0.593	0.572	0.565	0.559	0.562	0.555	0.552	0.548	0.541	0.007
SD (±)	0.012	0.012	0.008	0.009	0.007	0.018	0.005	0.012	0.010	0.003
CV (%)	2.0	2.1	1.4	1.6	1.2	3.2	0.9	2.2	1.8	42.9
Viability (%)	--	96.46	95.28	94.27	94.77	93.59	93.09	92.41	91.23	1.18
Cytotoxicity (%)	--	3.54	4.72	5.73	5.23	6.41	6.91	7.59	8.77	98.82

Based upon the results obtained in this study and in line with ISO 10993-5:2009(E) it is concluded that, the extracts of the given test item, Implants (SS316L) supplied by B. D. Surgical Industries, is non-cytotoxic.

INTRODUCTION

Biocompatibility testing is a regulatory requirement for demonstrating the preclinical safety of medical devices. This is evaluated in line with the standard guideline, ISO 10993-1:2009/Cor 1:2010(E), Biological Evaluation of Medical Devices - Part 1, Evaluation and Testing within a Risk Management Process. This standard describes the test selection necessary to evaluate the biocompatibility.

Cytotoxicity assays are used to assess the effect of the device or its extract on cells grown *in vitro*. The elution method uses culture medium supplemented with serum as an extracting vehicle and are considered equivalent to the use of both polar and non-polar vehicles. The extracts are transferred onto a layer of cells and incubated for 24 hours. Following incubation, the cells are examined microscopically (qualitative) for their morphology, any malformation or degeneration, and cell lysis. In the quantitative assay, the neutral red (NR) uptake assay procedure is followed, which are based on the ability of viable cells to uptake neutral red dye. A reduction of > 30% viability in the test item treated cultures compared to concurrent control culture indicates cytotoxicity.

The test selection and methods used in this study were based upon the following standards:

1. Biological Evaluation of Medical Devices - Part 1, Evaluation and Testing within a Risk Management Process, ISO 10993-1:2009/Cor 1:2010(E).

2. Biological Evaluation of Medical Devices - Part 5, Tests for *in vitro* Cytotoxicity, ISO 10993-5:2009(E).

3. Biological Evaluation of Medical Devices - Part 12, Sample Preparation and Reference Materials, ISO 10993-12:2012(E).

OBJECTIVE

To evaluate the *in vitro* cytotoxicity potential of test item extract in Balb/c 3T3 cells using elution method.

STUDY DATES

Study Start Date	:	02 March 2017
Experiment Start Date (addition of test item extract to the cell system)	:	14 March 2017
Experiment Completion Date	:	15 March 2017

The study completion date is the date the final report is signed by the Study Director

This study was performed in line with agreed study plan and one amendment.

TEST ITEM DETAILS

The test item, Implants (SS316L) was received at GLR Laboratories Private Limited, on 21 February 2017 and stored at room temperature (23.4 to 25.4 °C) until used.

The following test item information provided by the Sponsor, are considered an adequate description of the characterisation and stability of the test item.

Test Item	Implants (SS316L)
Batch No.	SS 001
Manufacture Date	10 February 2017
Expiry Date	Not Applicable
Appearance	Metallic silver coloured rectangular shaped strips with 14.5 cm length, 1.1 cm breadth and 0.18 cm thickness.
Ingredients	Not provided by the sponsor
Temperature Stability	Not provided by the sponsor
Sterility	Non-Sterile

No analysis was performed at GLR Laboratories Private Limited to confirm it. Determinations of stability and characteristics of the test item were the responsibility of the Sponsor. The test item and control items were handled with all necessary protective clothing and all recommended safety and sterile measures were followed.

Description of the test item

Implants (SS316L) is a metallic silver coloured rectangular shaped strips with 14.5 cm length, 1.1 cm breadth and 0.18 cm thickness. It is an implant device which comes in contact with tissue and bone. The duration of contact is greater than 30 days.

DETAILS OF CONTROL ITEMS

Positive Control	Sodium Lauryl Sulphate (SLS) (0.2 mg/mL) in 1X DMEM; (Thermo Fisher Scientific, Batch no. 2433460215; Expiry date: January 2020). This material has been routinely tested in GLR Laboratories Private Limited gives consistently an excellent cytotoxic response with Balb/c 3T3 cells.
Negative Control	High-Density Polyethylene Film (RM-C) (Make: Hatano Research Institute, Food and Drug Safety Centre, Japan. Lot No.:C-141, Expiry Date: June 2021).

TEST SYSTEM

Cell line	Balb/c 3T3, supplied by National Centre for Cell Science, India.
Growth conditions	Dulbecco's Modified Eagle Medium with L-glutamine 1X DMEM (Thermo Fisher Scientific, Lot no.1789594; Expiry Date: April 2017) supplemented with 10 % New Born Calf Serum (Thermo Fisher Scientific, Lot no.1418556; Expiry Date: July 2017), 1% Penicillin/Streptomycin solution (Himedia, Lot no. 0000241753, Expiry Date: August 2017) at 37 °C in CO_2incubator with 5% CO_2. Antibiotics used does not adversely affect the assay.
Justification for use	Use of Balb/c 3T3 cells is recommended in ISO 10993, Part 5:2009 for assessing *in vitro* cytotoxicity.

TEST METHOD

Preparation of the test item extract

Test item was extracted in the ratio of 3 cm^2 per millilitre of serum supplemented 1x DMEM at 37 ºC for 24 h under sterile conditions. The test item measuring 20.5 cm^2 (5 no. each measuring 4.1 cm^2) was extracted in 6.83 mL of serum supplemented 1x DMEM for 24 h at 37 °C (13 March 2017, 09:50 a.m. to 14 March 2017, 09:50 a.m.). Negative control (High - Density Polyethylene)

measuring 6 cm^2 (surface area of one side is 3 cm^2, both the sides were involved in extraction) was extracted in 2 mL of serum supplemented 1x DMEM at the ratio of 3cm^2per mL at 37 ºC for 24 hours. Positive control (Sodium Lauryl Sulphate) of 0.0011 g in 5.5 mL of serum supplemented 1x DMEM in the final concentration of 0.2 mg / mL was freshly prepared before treating the cells. This fulfils the requirements of ISO 10993-5:2009(E) and ISO 10993-12:2012(E).

At the end of extraction period, the extract was filter sterilised prior to addition since the test item is non-sterile. Extracts were used within 40 minutes of preparation and was considered to be stable during this time. A series of eight different concentrations (30, 40, 50, 60, 70, 80, 90 and 100%) of the test item extract was prepared for the study.

Test procedure

Rationale for assay method	The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red dye. Specified in ISO 10993, Part-5:2009 standard as an appropriate test to evaluate *in vitro* cytotoxicity for assessing the biocompatibility of medical devices.

Exponentially growing Balb/c 3T3 cells were trypsinised using trypsin-EDTA (Make: Sigma - Aldrich, Lot no. SLBN4359V, Expiry date: July 2017) and counted in a hemocytometer using 0.4% Trypan blue (Himedia, Lot no. 0000243749, Expiry Date: September 2017). Exactly 1 x 10^5 cells per mL was prepared (0.160 mL of cell suspension [44.50 x 10^5 cells per mL] was added to 6.840 mL of culture media to get 7 mL of cell suspension) and 100 µL was seeded in wells B2 to G11 of 96-well plates at a concentration of 1 x 10^4 cells per well. The plate was incubated in CO_2 incubator with 5% CO_2 at 37 °C for 24 h (13 March 2017, 10:10 a.m. to 14 March 2017, 10:10 a.m.).

The following day, the confluency and morphology of the cell was checked and found to be greater than 70 % confluent and normal. Then the medium was removed and six replicates of appropriate concentrations of the test item extract, positive, negative controls and appropriate blanks were added to the cultures as shown below:

96 - well plate template

	1	2	3	4	5	6	7	8	9	10	11	12
A	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media
B	Media	Negative	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	Positive	Media
C	Media	Negative	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	Positive	Media
D	Media	Negative	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	Positive	Media
E	Media	Negative	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	Positive	Media
F	Media	Negative	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	Positive	Media
G	Media	Negative	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	Positive	Media
H	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media

Media: Medium blank
Negative: Negative control
Positive: Positive control
Conc 1 to 8: Eight different concentrations of the test item extract - 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%, respectively
Alphabet A-H in the 96-Well Plate Layout represents each row of the plate.
Number 1-12 in the 96-Well Plate Layout represents each column of the plate.

The plate was then incubated in CO_2 incubator with 5% CO_2 at 37 °C for 24 h (14 March 2017, 10:30 a.m. to 15 March 2017, 10:30 a.m.). After 24 h of incubation, the cells were examined under inverted microscope for morphological evidence of cytotoxicity using a grading scheme according to ISO 10993-5:2009(E) (Table 1). Immediately following the visual assessment, wells were washed with 150 µL of phosphate buffered saline (PBS) (Himedia, Lot no. 0000242278, Expiry Date: September 2017). This was removed, and 100 µL of neutral red medium was added. The plates were then incubated in CO_2 incubator with 5% CO_2 at 37 °C for exactly 3 h (15 March 2017, 10:40 a.m. to 01:40 p.m.).

Following the incubation, the neutral red medium was removed and the cells were washed with 150 µL of PBS which was removed before adding 150 µL of neutral red desorb solution (ethanol: glacial acetic acid: distilled water, 10 mL:0.2 mL:9.8 mL). Plates were shaken periodically until all neutral red was removed from the cells, forming a homogenous solution. The resulting coloured solution was analysed using a microplate reader (Mindray MR-96A) at a wavelength setting of 546 nm. Neutral Red absorbance was expressed in terms of absolute optical density (OD_{546}; which was OD_{546} of the culture minus the mean OD_{546} of medium blanks). Cell viability was calculated as the percentage of culture OD_{546} divided by negative control OD_{546}.

DATA EVALUATION

Qualitative evaluation: Cultures treated with test item extract that induce cytotoxicity grades greater than 2 (see Table 1) were considered cytotoxic.

Quantitative evaluation: Undiluted test item extract was considered non-cytotoxic if the viability measured by neutral red uptake was ≥ 70% than that of the negative control. Viability < 70% indicated cytotoxicity.

The results of the range finder experiment revealed negative response, no further main experiment was performed.

The coefficient of variation (CV %) was calculated using the following formula:

$$CV\ \% = \frac{SD}{\text{Mean } OD_{546}} \times 100$$

The assay was considered valid, as the positive control treated cultures gave a clear increase in cytotoxicity compared to that observed in negative control cultures.

Good scientific judgement was used in interpreting the data.

ACCEPTANCE CRITERIA

The assay was to be considered valid if all the following criteria were met:

1. Before treatment, cells should have a confluency of >70% and grown well.
2. Mean absorbance value of negative control should be ≥0.3.
3. The positive controls should show a strong positive cytotoxic response of >30%.
4. The coefficient of variation (CV %) for replicate measurements should be < 15%.

RESULTS

Before treatment, all wells had cells confluency of greater than 70%. The mean OD_{546} of negative treated cells were 0.593. Coefficient of variation for all test item extract replicate measurements were < 15%. Clear increase in cytotoxicity was observed in the positive control treated cultures. But, no such cell destruction was evident in the negative control. Hence, the test was considered valid.

Results of qualitative evaluation are given in Table 2. It is clear that the test item extract was non - cytotoxic.

Neutral red uptake assay's results reflected the results of qualitative analysis (Tables 3, 4, and 5). Implants (SS316L) extract at concentrations 30, 40, 50, 60, 70, 80, 90 and 100% showed viability greater than 70% when compared to negative control.

CONCLUSION

Based upon the results obtained in this study and in line with ISO 10993-5:2009(E) it is concluded that, the extracts of the given test item, Implants (SS316L) supplied by B. D. Surgical Industries, is non-cytotoxic

REFERENCES

1. Biological Evaluation of Medical Devices - Part 1, Evaluation and Testing within a Risk Management Process, ISO 10993-1:2009/Cor 1:2010(E).

2. Biological Evaluation of Medical Devices - Part 5, Tests for *in vitro* Cytotoxicity, ISO 10993-5:2009(E).

3. Biological Evaluation of Medical Devices - Part 12, Sample Preparation and Reference Materials, ISO 10993-12:2012(E).

Table 1: Qualitative Morphological Grading of Cytotoxicity of Extracts

Grade	Reactivity	Conditions of all cultures
0	None	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth.
1	Slight	Not more than 20% of the cells are round, loosely attached and without intracytoplasmic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable.
2	Mild	Not more than 50% of the cells are round, devoid of intracytoplasmic granules, no extensive cell lysis; not more than 50% growth inhibition observable.
3	Moderate	Not more than 70% of the cell layers contain rounded cells or are lysed; cell layers not completely destroyed, but more than 50% growth inhibition observable.
4	Severe	Nearly complete or complete destruction of the cell layers.

Source: ISO 10993-5:2009(E).

Table 2: Results of qualitative scoring for cytotoxicity

	1	2	3	4	5	6	7	8	9	10	11	12
A	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells
B	No cells	0	0	0	0	0	0	0	0	0	4	No cells
C	No cells	0	0	0	0	0	0	0	0	0	4	No cells
D	No cells	0	0	0	0	0	0	0	0	0	4	No cells
E	No cells	0	0	0	0	0	0	0	0	0	4	No cells
F	No cells	0	0	0	0	0	0	0	0	0	4	No cells
G	No cells	0	0	0	0	0	0	0	0	0	4	No cells
H	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells	No cells

0, None; 1, Slight; 2, Mild; 3, Moderate; and 4, Severe cytotoxicity

Table 3: Results of optical density readings at 546 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.071	0.062	0.064	0.07	0.067	0.071	0.068	0.069	0.069	0.066	0.066	0.071
B	0.066	0.660	0.648	0.623	0.624	0.619	0.614	0.616	0.601	0.594	0.075	0.067
C	0.069	0.654	0.633	0.637	0.638	0.630	0.624	0.611	0.627	0.619	0.074	0.060
D	0.067	0.668	0.641	0.623	0.629	0.632	0.592	0.618	0.625	0.607	0.076	0.071
E	0.070	0.650	0.627	0.641	0.626	0.620	0.644	0.621	0.619	0.605	0.069	0.069
F	0.068	0.643	0.623	0.626	0.619	0.629	0.614	0.626	0.598	0.616	0.073	0.064
G	0.070	0.676	0.653	0.636	0.613	0.635	0.636	0.613	0.612	0.599	0.070	0.066
H	0.066	0.057	0.062	0.058	0.061	0.061	0.063	0.058	0.062	0.063	0.071	0.063

Mean of media blanks: 0.066

Table 4: ODs adjusted for media blank

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0.594	0.582	0.557	0.558	0.553	0.548	0.550	0.535	0.528	0.009	0
C	0	0.588	0.567	0.571	0.572	0.564	0.558	0.545	0.561	0.553	0.008	0
D	0	0.602	0.575	0.557	0.563	0.566	0.526	0.552	0.559	0.541	0.010	0
E	0	0.584	0.561	0.575	0.560	0.554	0.578	0.555	0.553	0.539	0.003	0
F	0	0.577	0.557	0.560	0.553	0.563	0.548	0.560	0.532	0.550	0.007	0
G	0	0.610	0.587	0.570	0.547	0.569	0.570	0.547	0.546	0.533	0.004	0
H	0	0	0	0	0	0	0	0	0	0	0	0

Table 5: Results of viability and cytotoxicity

	Negative Control	Viability in test item extract concentrations (%)								Positive Control
		30	40	50	60	70	80	90	100	
Mean OD	0.593	0.572	0.565	0.559	0.562	0.555	0.552	0.548	0.541	0.007
SD (±)	0.012	0.012	0.008	0.009	0.007	0.018	0.005	0.012	0.010	0.003
CV (%)	2.0	2.1	1.4	1.6	1.2	3.2	0.9	2.2	1.8	42.9
Viability (%)	--	96.46	95.28	94.27	94.77	93.59	93.09	92.41	91.23	1.18
Cytotoxicity (%	--	3.54	4.72	5.73	5.23	6.41	6.91	7.59	8.77	98.82

RESPONSIBLE PERSONNEL

Ms. P. Pradeepa, MSc, M Phil	Study Director
Ms. G. Ashtalakshmi, MSc, M Phil	Study Scientist
Mr. S. Haribabu, B Tech (Biotech), MSc	Study Scientist

STUDY PLAN AMENDMENT

One definitive study plan amendment was made as per sponsor request to change the "Batch / Lot No. - BDS/2017/02/001" to "Batch no. - SS 001".

STUDY PLAN DEVIATION

No deviations from the study plan were found during the conduct of the study.

DISTRIBUTION OF REPORTS

Two originals of the study report are prepared and distributed as mentioned below:

1. Sponsor.
2. GLR Laboratories Private Limited.

Final Report

Study Title	Intracutaneous reactivity test in New Zealand White rabbits
Test Item	Implants (SS316L)
Study Director	Ms. G. Ashtalakshmi, MSc, M Phil
Sponsor	B. D. Surgical Industries 2082, Mie, Part-B Bahadurgarh, Jhajjar Haryana-124507
Study Monitor	Mr. Abhishek Sharma
Test Facility	GLR Laboratories Private Limited 444 Gokulam Street Mathur, Chennai - 600 068 Tamil Nadu, India
Study Number	304/004
Regulatory Guideline	Biological Evaluation of Medical Devices - Part 10, Tests for Irritation and Skin Sensitization, ISO 10993-10:2010(E).
Report Issued	17 April 2017
Total Number of Pages	18

CONTENTS

CERTIFICATE

Implants (SS316L): Intracutaneous reactivity test in New Zealand White rabbits

This study was performed in accordance with the standard guideline, Biological Evaluation of Medical Devices - Part 10, Tests for Irritation and Skin Sensitization, ISO 10993-10:2010 (E), agreed study plan, one definitive study plan amendment and with GLR laboratories Pvt Ltd's Standard Operating Procedures, unless otherwise stated, and the study objectives were achieved. The work and generated data are scientifically acceptable and valid; and this report provides a true and accurate record of the results obtained.

17 Apr 2017

Ms. G. Ashtalakshmi, MSc, M Phil
Study Director
GLR Laboratories Pvt Ltd

Date

17 Apr 2017

Dr. G. Velmani, M Pharm, PhD
Executive-Quality Assurance
GLR Laboratories Pvt Ltd

Date

17 Apr 2017

Dr. S. S. Murugan, PhD, ERT
Test Facility Management
GLR Laboratories Pvt Ltd

Date

SUMMARY

Intracutaneous reactivity test of the test item Implants (SS316L), supplied by B. D. Surgical Industries, were conducted in male New Zealand White rabbits.

Implants (SS316L) is a metallic silver coloured rectangular shaped strips with 14.5 cm length, 1.1 cm breadth and 0.18 cm thickness. It is an implant device which comes in contact with tissue and bone. The duration of contact is greater than 30 days.

The test items measuring 16.4 cm^2 were extracted in 5.5 mL of polar (physiological saline) (4 nos. were used each measuring 4.1 cm^2) and similarly 16.4 cm^2 were extracted in 5.5 mL of non-polar (sesame oil) solvent prepared by ratio of 3 cm^2 of test item per millilitre of solvent at 50 ºC for 72 h under sterile conditions. Solvent controls were also subjected to same extraction conditions. At the end of extraction, the extracts and solvent controls were clear, there was no change in the colour and no particulates were found (pre- and post-extraction). Hence, no additional processing such as filtration, centrifugation, pH adjustments or any other processing were made. The extracts and solvent controls were transferred to sterile containers and stored at room temperature. All extracts and solvent controls were used within 2 h of preparation and were considered stable during this time. This fulfils the requirements of ISO 10993-12:2012(E)

Five hours prior to intracutaneous injections, all the rabbits were closely clipped off the fur on the backs, allowing sufficient distance on both the sides of the spine for injection of test item extracts.

Test item extracts and negative controls were injected as follows:

Animal No.	Sample	Injection site	Volume of each injection (mL)	No. of injections/ site
1	Test item extract in physiological saline Physiological saline (Negative control) Test item extract in sesame oil Sesame oil (Negative control)	Left cranial end Right cranial end Left caudal end Right caudal end	0.2	5
2	Test item extract in physiological saline Physiological saline (Negative control) Test item extract in sesame oil Sesame (Negative control)	Left cranial end Right cranial end Left caudal end Right caudal end	0.2	5
3	Test item extract in physiological saline Physiological saline (Negative control) Test item extract in sesame oil Sesame oil (Negative control)	Left cranial end Right cranial end Left caudal end Right caudal end	0.2	5

The skin reactions were visually scored according to ISO 10993-10:2010(E) at 24,48 and 72 h post injection.

The animals were observed for three consecutive days for morbidity, mortality and abnormal clinical signs and symptoms following injections.

Neither mortality nor morbidity was recorded, a gradual increase in body weight of test animals was reported and no signs of ill health or overt toxicity was observed.

No positive controls were included in this study. Positive control trials for irritation are carried out every three months in our laboratory to demonstrate the sensitivity of this strain of animals to 10% SLS in water. The last such positive control trial was completed on 08 December 2016 and gave a moderate irritant response. The current positive control trial was initiated and will be completed in March 2017.

Animals treated with the test item extracts did not show any skin reactions.

Solvent	Mean Reaction Score for test item extract	Mean Reaction Score for control	Overall difference (Test extract - control)
Physiological saline	0	0	0
Sesame oil	0	0	0

The difference of the mean skin reaction scores for the test item extracts and the control vehicle was zero.

Based upon the results obtained in this study and in line with ISO 10993-10:2010 (E) it is concluded that, the extract of the given test item Implants (SS316L) supplied by B. D. Surgical Industries, is non-reactive.

INTRODUCTION

Biocompatibility testing is a regulatory requirement for demonstrating the safety of medical devices. This is performed as per ISO 10993, Parts 1 to 20. The primary aim of this group of standards is the protection of humans from potential biological risks arising from the use of medical devices. The general guidance for biocompatibility testing is given in ISO 10993-1:2009/ Cor 1:2010 (E), Biological Evaluation of Medical Devices - Part 1, Evaluation and Testing within a Risk Management Process. This standard also describes the categorization of medical devices based on nature and duration of patient contact; and test selection necessary to evaluate biocompatibility. The technical guidance for the biocompatibility tests are given in other parts of ISO 10993.

Intracutaneous reactivity test is carried out according to ISO 10993 Part 10; Tests for irritation and skin sensitization. Types of irritation tests are listed below:

Irritation Tests	**Standard**
Animal Irritation Test Animal intracutaneous (intradermal) reactivity test	ISO 10993: Part 10
Special irritation tests	
Ocular irritation test Oral mucosa irritation test Penile irritation test Rectal irritation test Vaginal irritation test	ISO 10993: Part 10

In this study, intracutaneous reactivity test was carried out. The reactivity potential of a test device was assessed by injecting the extract of the test item intracutaneously in rabbits and the observed responses were graded as given in ISO 10993 Part 10.

The test selection and methods used in this study were based on the following standards:

1. Biological Evaluation of Medical Devices - Part 1, Evaluation and Testing within a Risk Management Process, ISO 10993-1:2009/Cor 1:2010(E).
2. Biological Evaluation of Medical Devices - Part 2, Animal Welfare Requirements, ISO 10993-2:2006(E).
3. Biological Evaluation of Medical Devices - Part 10, Tests for Irritation and Skin Sensitization, ISO 10993-10:2010(E).
4. Biological Evaluation of Medical Devices - Part 12, Sample Preparation and Reference Materials, ISO 10993-12:2012(E).

OBJECTIVE

To determine the reactivity potential of the test item extracts following intracutaneous injection into New Zealand White rabbits.

STUDY DATES

Study Start Date	02 March 2017
Experiment Start Date (Date of first dosing)	20 March 2017
Experiment Completion Date	23 March 2017

The study completion date is the date the final report is signed by the Study Director.

This study was performed in line with agreed study plan and one amendment.

TEST ITEMDETAILS

The test item, Implants (SS316L) was received at GLR Laboratories Private Limited on 21 February 2017 and stored at room temperature (23.2 to 25.7) °C until use. The following test item information provided by the sponsor were considered adequate.

Test Item	Implants (SS316L)
Batch No.	SS 001
Manufacture Date	10 February 2017
Expiry Date	Not Applicable
Appearance	Metallic silver coloured rectangular shaped strips with 14.5 cm length, 1.1 cm breadth and 0.18 cm thickness.
Ingredients	Not provided by the sponsor
Temperature Stability	Not provided by the sponsor
Sterility	Non-Sterile

CONTROL ITEM DETAILS

Positive Control	Sodium lauryl sulphate-SLS No animals were used for positive control in this study. Positive control trials for irritation are conducted every three months in GLR laboratory. This strain of rabbits gives a clear positive response to 10% sodium lauryl sulphate (SLS) in water. The details of positive control trials are provided in Appendix 1.
Negative (Solvent) Control	Physiological saline and sesame oil

The test item was handled with all necessary protective clothing and all recommended safety and sterile measures were followed. The identity, composition stability and characteristics of the test item is the responsibility of the sponsor. No analysis was performed at GLR Laboratories Private Limited, to confirm it.

Description of the test item

Implants (SS316L) is a metallic silver coloured rectangular shaped strips with 14.5 cm length, 1.1 cm breadth and 0.18 cm thickness. It is an implant device which comes in contact with tissue and bone. The duration of contact is greater than 30 days.

TEST SYSTEM

Species	Rabbit (*Oryctolagus cuniculus*)
Strain	New Zealand White
Weight (g) (Start of the experiment)	2464.7 to 2611.4
Sex	Male
Source	NIN, Hyderabad, India. This supplier is approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India for breeding laboratory animals.
Number of animals used	3
Acclimation period	5 days
Justification for animal use	The intracutaneous injection test in rabbits are specified in the current ISO testing standards and has been used historically to evaluate biomaterial extracts.

The test system was approved by the GLR Laboratories Private Limited Institutional Animal Ethics Committee (IAEC).

ANIMAL HUSBANDRY

Test Room No.	03
Test room temperature (°C)	19.2 to 22.3
Relative humidity (%)	44 to 60
Housing	Animals were housed individually in stainless steel rabbit cages.

Method of identification	Animals were identified using cage cards indicating cage no., study no., species, strain, animal no., sex, age/bodyweight, dose, signature and individual earmarking.
Diet	Rabbit pellet feed (Amrut feeds)
Water	Purified drinking water was provided *ad libitum*
Bedding material	No bedding materials were used as rabbits were housed in stainless steel cages with mesh floors. Absorbent paper paddings used to collect the excreta and urine was changed routinely.
Photoperiod	12: 12 h light and dark cycle
Contaminants	Contaminants, reasonably expected in feed and/or water supplied were not believed to influence the outcome of the study.
Personnel	Associates involved in this study were appropriately qualified and trained.
Selection of animals	Previously unused and healthy young adults were selected for this study.

TEST METHOD

Preparation of the test item extracts

The test items measuring 16.4 cm^2 were extracted in 5.5 mL of polar (physiological saline) (4 nos. were used each measuring 4.1 cm^2) and similarly 16.4 cm^2 were extracted in 5.5 mL of non-polar (sesame oil) solvent prepared by ratio of 3 cm^2 of test item per millilitre of solvent at 50 ºC for 72 h under sterile conditions. Solvent controls were also subjected to same extraction conditions. At the end of extraction, the extracts and solvent controls were clear, there was no change in the colour and no particulates were found (pre-and post-extraction). Hence, no additional processing such as filtration, centrifugation, pH adjustments or any other processing were made. The extracts and solvent controls were transferred to sterile containers and stored at room temperature. All extracts and solvent controls were used within 2 h of preparation and were considered stable during this time. This fulfils the requirements of ISO 10993-12:2012(E).

The details of extract preparation are given below,

Extract	Extraction vehicle	Surface area of the test item taken (cm^2)	Volume of vehicle (mL)	Extract preparation start time	Extract preparation end time	Appearance of extracts*
Polar Extract	Physiological saline	16.4	5.5	10:40 a.m. on 17 Mar 2017	10:40 a.m. on 20 Mar 2017	Colourless clear solution, no particulates
Polar Vehicle Negative Control	Physiological saline	NA	10.0			Colourless clear solution, no particulates
Non-polar Extract	sesame oil	16.4	5.5			Light brown viscous liquid; no particulates
Non-polar Vehicle Negative Control	Sesame oil	NA	10.0			Light brown viscous liquid; no particulates

*extraction vehicles did not undergo any colour changes during the extraction process; NA-Not applicable

The pH of the polar extract was 7.04. Therefore, the extract was found suitable to conduct intracutaneous reactivity study in rabbits. The pH of the oil extract cannot be measured, but it is assumed acceptable for intracutaneous injections.

The details of the solvents were as follows:

Physiological saline (0.9% w/v sodium chloride solution)

Manufacturer	Baxter (India) Pvt. Limited
Batch No.	10150892B
Expiry Date	August 2018
Appearance	Colourless clear solution

Sesame oil

Manufacturer	Sigma-Aldrich
Lot No.	MKBT8141V
Expiry Date	December 2021
Appearance	Light brown viscous liquid

Dosing Procedure

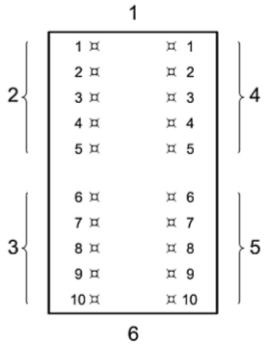
Justification	Recommended in ISO 10993, Part-10: 2010 (E), intracutaneous injection of test item extracts to rabbit as a suitable route of administration and the dose volume was 0.2 mL per injection without any dilution, to determine biocompatibility of materials used in medical devices.

Test procedure

Five hours prior to intracutaneous injections, all the rabbits were closely clipped off the fur on the backs, allowing sufficient distance on both the sides of the spine for injection of test item extracts (see diagram). Intracutaneous injections of polar and

non-polar extracts and corresponding controls were given using, sterile syringes and needles (Hindustan Syringes & Medical Devices Ltd.; Batch No.:444016G32; Expiry date: October 2019) as given in the table and figure:

Animal No.	Sample	Injection site	Volume of each injection (mL)	No. of injections/ site
1	Test item extract in physiological saline Physiological saline (Negative control) Test item extract in sesame oil Sesame oil (Negative control)	Left cranial end Right cranial end Left caudal end Right caudal end	0.2	5
2	Test item extract in physiological saline Physiological saline (Negative control) Test item extract in sesame oil Sesame oil (Negative control)	Left cranial end Right cranial end Left caudal end Right caudal end	0.2	5
3	Test item extract in physiological saline Physiological saline (Negative control) Test item extract in sesame oil Sesame oil (Negative control)	Left cranial end Right cranial end Left caudal end Right caudal end	0.2	5



1. Cranial end; 2. 0.2 ml injections of polar extract; 3. 0.2 ml injections of non-polar extract; 4. 0.2 ml injections of polar solvent control; 5. 0.2 ml injections of non-polar solvent control; 6. Caudal end

OBSERVATIONS

Mortality & Morbidity

All the animals were observed daily for mortality and morbidity throughout the experiment.

Body Weight

Body weight of each animal was recorded at the start and at the end of the experiment.

Clinical Observation

All animals were observed for clinical signs of toxicity immediately after intracutaneous injection, and at 24 h, 48 h, and 72 h.

Scoring of Skin Reaction

Observations and scoring of skin reactions viz., oedema, erythema and eschar formation were performed visually with naked eyes as per ISO 10993-10:2010(E) at 24 h, 48 h and 72 h following the intracutaneous injection. Observations were graded on a numerical scale for both the test item extracts and vehicle controls.

Grading system for intracutaneous reactions are shown in the following table:

Reaction	Numerical grading
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet-redness) to eschar formation preventing grading of erythema	4
Oedema formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Well-defined oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm extending beyond exposure area)	4
Maximal possible score for irritation	8

Source: ISO 10993- Part 10: 2010 (E)

Necropsy

No animals were found dead or in moribund condition, hence gross pathology was not performed. All animals were euthanized by ketamine + xylazine injection at the end of the experiment.

EVALUATION CRITERIA

After 72h grading, all erythema and oedema grades at 24 h, 48 h and 72 h were totalled for each test item extract or control for each individual animal. For calculating the score of a test item and control on each individual animal, the derived value was divided each of the totals by 15 (3 scoring periods x 5 test or control sample injection sites). To determine the overall mean score for each test item and each corresponding control, the scores for the 3 animals were added and divided by three. The final test item score was obtained by subtracting the score of the control from the test item score.

Solvent	Mean Reaction Score for test item extract	Mean Reaction Score for negative control	Overall difference (Test extract - control)
Physiological saline	A	B	(A-B)
Sesame oil	C	D	(C-D)

The requirements of the test were met, the difference (final score) of the mean reaction grades (erythema/ oedema) for the test item and the control was less than 1.0.

RESULTS

Mortality & Morbidity
No animal was observed for mortality and morbidity throughout the experiment

Body Weight
Body weight of each animal increased after test item administration, was recorded at the start and at the end of the experiment are presented in Table 1.

Clinical Observation
No signs of ill health or overt toxicity were observed in any of the test animals.

Scoring of Skin Reaction
Injection sites appeared normal immediately after the injections. The results of grading of skin reactions for individual animals are given in Table 2. The difference of the mean skin reaction scores for the test item extracts and the vehicle control was zero (see Table 3).

Positive control trial
Positive control trial conducted within the test facility gave clear positive results (Appendix 1).

CONCLUSION

Based upon the results obtained in this study and in line with ISO 10993-10:2010 (E) it is concluded that, the extract of the given test item Implants (SS316L) supplied by B. D. Surgical Industries, is non-reactive.

REFERENCES

1. Biological Evaluation of Medical Devices - Part 1, Evaluation and Testing within a Risk Management Process, ISO 10993-1:2009/Cor 1:2010(E).

2. Biological Evaluation of Medical Devices - Part 2, Animal Welfare Requirements, ISO 10993-2:2006(E).

3. Biological Evaluation of Medical Devices - Part 10, Tests for Irritation and Skin Sensitization, ISO 10993-10:2010(E).

4. Biological Evaluation of Medical Devices - Part 12, Sample Preparation and Reference Materials, ISO 10993-12:2012(E).

Table 1: Individual body weights of New Zealand White rabbits

Animal No.	Sex	Bodyweight (g)	
		Initial	Final
1	M	2464.7	2469.6
2	M	2539.4	2544.3
3	M	2611.4	2616.7

M - Male

Table 2: Grading of skin reactions for individual New Zealand White rabbits

Animal No.	Sex	Solvent	24 h				48 h				72 h			
			Test item extract		Negative Control		Test item extract		Negative Control		Test item extract		Negative Control	
			E	O	E	O	E	O	E	O	E	O	E	O
1	M	Physiological saline	0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
1	M	Sesame oil	0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
2	M	Physiological saline	0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
2	M	Sesame oil	0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
3	M	Physiological saline	0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
3	M	Sesame oil	0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0

M, Male; E, Erythema; O, Oedema

Table 3: Mean reaction scores

Solvent	Test item extract	Negative Control	Overall difference
	E+O	E+O	E+O
Physiological saline	0	0	0
Sesame oil	0	0	0

E, Erythema; O, Oedema

APPENDIX 1

Summary of Positive Control Trial (GLR Study number 000/016)

Study number	Study start date	Experiment start date	Experiment completion date	Study completion date	Agent used	Result
000/016	15 November 2016	24 November 2016	01 December 2016	08 December 2016	10% sodium lauryl sulphate	Moderate irritant

The current positive control trial was initiated and will be completed in March 2017.

RESPONSIBLE PERSONNEL

Ms. G. Ashtalakshmi, MSc, M Phil	Study Director
Mr. K. Sakthivel, MSc	Animal House In-charge
Dr. B. Rajan, MSc, PhD	Study Scientist
Dr. J.S.I. Rajkumar, MSc, M Phil, PhD	Study Scientist

STUDY PLAN AMENDMENT

Based on sponsor request, study plan amendment was made to modify the batch no. of the test item.

STUDY PLAN DEVIATION

No deviations from the study plan were found during the conduct of the study.

DISTRIBUTION OF REPORTS

Two originals of the study report are prepared and distributed as mentioned below:
1. Sponsor.
2. GLR Laboratories Private Limited.