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In Vitro and *In Vivo* biocompatibility evaluation of a left atrial appendage device: An ISO 10993-Based Safety Assessment

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Abstract

Background: The left atrial appendage (LAA) occluder is a durable medical device that will stay inside your body and come into contact with blood and heart tissue for a long period of time (i.e., greater than 30 days). Before patients can use the device, we must determine how well it works inside you for an extended period of time by completing an extensive evaluation of its biological safety under the ISO 10993 standard (International Standards Organization).

Objective of Study: This study will provide a thorough evaluation of the biocompatibility of the LAA occlusion device to meet the relevant requirements for long-term contact with blood under the ISO 10993 standards.

Methods: A complete biocompatibility test strategy was developed based on the amount of time (i.e., length of time) that the LAA occlusion device will be in contact with tissue (i.e., heart tissue) and blood. The studies will use both polar and non-polar solvents to prepare device extracts (i.e., imitate the way it would be used in the environment) to perform the evaluations described below. The evaluations included cytotoxicity (ISO 10993-5), cutaneous irritation (ISO 10993-23), sensitization (ISO 10993-10), hemocompatibility (ISO 10993-4), and pyrogenicity and systemic toxicity (ISO 10993-11).

Results: The LAA Occluder demonstrated no evidence of cytotoxic effects, did not induce any dermal irritation or sensitization in the patient's dermis. Hemocompatibility studies from *in vivo* blood tests proved that there would be no adverse reactions to the blood component(s) when interacting with the LAA occluder. The device extracts showed no signs of pyrogenicity and acute systemic toxicity.

Conclusion: Based on the LAA Occluder's extensive biocompatibility evaluation, it has been found that they comply with all applicable ISO 10993 standards, therefore providing strong evidence of the LAA occluders' clinically acceptable long-term safety for use in clinical practice.

Keywords: Left atrial appendage (LAA) occlusion device; Biocompatibility; Skin Irritation; Skin Sensitization; Cytotoxicity; Hemocompatibility; Pyrogenicity; Acute Systemic toxicity

1. Introduction

Atrial fibrillation (AF) increases the risk of death and morbidity due to cardiovascular disease (CVD) as a result of forming blood clots in the left atrial appendage (LAA). Most of the evidence showing that thrombus formation occurs in patients with nonvalvular AF has established that the LAA is the primary area where thrombus formation occurs [1,2].

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Thus, there has been a growing recognition that mechanical exclusion of the LAA can effectively reduce the risk of stroke in patients with contraindications or intolerance to long-term oral anticoagulation therapy (OAT), or patients who perceived themselves to be inadequately protected by long-term OAT [3]. In that regard, over the last decade, transcatheter devices designed for occluding the LAA have become progressively advanced and more commonly utilized in clinical practice [4].

However, no matter how advanced it may become, biological safety is one of the most important factors for the successful translation of any cardiovascular device into clinical use. [5]. Because long-term implantable LAA occluder devices continually contact circulating blood and myocardial tissue, they are classified as among the highest risk of all medical devices regarding their potential for biological interactions [6]. The long-term exposure of LAA occluder devices to blood and cardiac tissue increases the risk of adverse biological reactions, such as cytotoxicity, local tissue irritation, immunological sensitization, heamolysis, pyrogenic reactions, and systemic toxicity [7].

To assess these biological risks for implantable devices with permanent blood contact, ISO 10993 series International Standards use a risk-based approach to evaluate medical device biocompatibility and the selection of testing is based on the type of contact, duration of contact, and anatomical site of the contact [8]. For implantable cardiovascular devices that are in permanent contact with blood, ISO 10993-1 specifies the need for comprehensive biocompatibility testing, including: cytotoxicity testing, irritation and sensitization testing, hemocompatibility testing, pyrogenicity and acute Systemic toxicity testing [9].

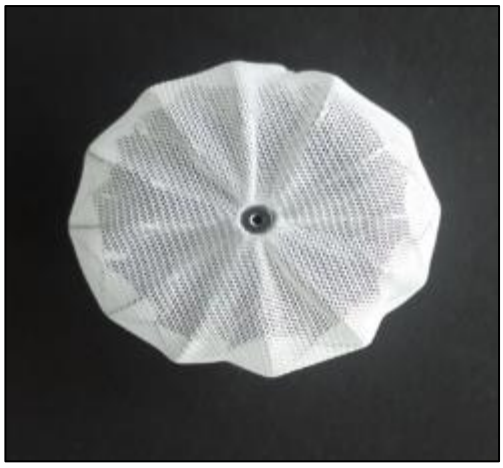
The device materials tested for biocompatibility did not produce cytotoxic effects and they induced and did not resulted into any dermal irritation or skin sensitization reactions. Hemolysis testing indicated that hemolysis occurred at acceptable levels, and therefore that the device materials are compatible with blood. There was no indication of a fever from the evaluation of pyrogenicity and there were no treatment-related death or other signs of toxicity at the endpoint in the evaluation of acute systemic toxicity. Based on all of these data, the device materials, at the doses tested, did not produce either local or systemic toxicity.

2. Materials And Methods

2.1. Device Description

The left atrial appendage occluder system is made of a self-expanding nitinol frame structure with fixation anchors and overlays made of permeable polyester (PET) fabric. LAA is comprised of a trunk or base (the left atrium) from which it protrudes. Using a LAA typically involves an endoleak (mechanical occlusion), which has been designed for efficient deployment and placement within complex LAA anatomies.

(a)



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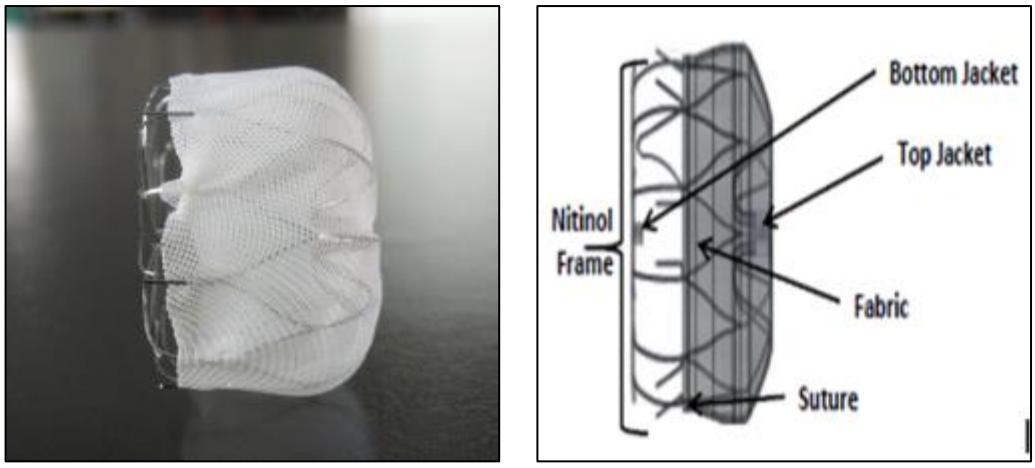


(c) (d)

(a). Front View of Left Atrial Appendage (LAA) Occluder Device,
(b). Top View of Left Atrial Appendage (LAA) Occluder Device,
(c). Side View of Left Atrial Appendage (LAA) Occluder Device,
(d). Schematic Diagram of Left Atrial Appendage (LAA) Occluder Device

Figure 1 Left Atrial Appendage (LAA) Occluder Device

The Left Atrial Appendage (LAA) Occluder System is a self-expanding nitinol frame structure with fixation anchors and permeable polyester (PET) fabric covering the Left Atrial Appendage (LAA) Occluder System. The nitinol frame structure is non-magnetic and is designed to be compatible with magnetic resonance imaging (MRI) environments under specified conditions.

2.2. Device Component Description

Table 1 Device components description

Sr. No.	Parameters	Specifications
Implant Details		
1	Frame	Nitinol
2	Fabric	Polyethylene terephthalate
3	Bottom Jacket	Stainless steel
4	Top Jacket	
5	Suture	Polyester
6	Device size; mm	18, 20, 24, 28, 32, 36, 38
7	Guidewire compability; inch	0.035″
8	Access Sheath; F	14
9	Delivery sheath; F	12
10	Y- Hub	Polypropylene
11	Delivery cable	Stainless steel

2.3. Extraction Procedure

Extracts were prepared from the test item (thickness <0.5 mm) by using a ratio of 6 cm^2/mL for the polar and non-polar solvent in accordance with the standards outlined in ISO 10993-12:2021 and BS EN ISO 10993-12:2021 in Part 12 for the "Biological evaluation of medical devices" [10, 15, 16]. The test item and solvent control extracts were produced independently and extracted for 72±2 hour at 50±2°C in an incubator shaker with stirring.

In cytotoxicity testing, the positive control was 0.1% (Zinc Diethyldithiocarbamate) ZDEC polyurethane film, and the negative control was high density polyethylene film. In the hemocompatibility test, the positive control was sterile water for injection, the negative control was high density polyethylene film, and the blank control was the CMF-PBS (Calcium (Ca) and Magnesium (Mg) Free Phosphate Buffered Saline). There are no controls in the other tests.

The control samples were prepared in triplet and preserved in the incubator shaker agitated for 50 ± 2°C for 72 hours. After the incubated period, the extract was examined for the change in color, test item appearance, as well as the presence of particles.

The measured pH values of the polar solvent control (7.01) and the polar extract of the test item (7.06) were within the physiological range and did not meet the criteria of ≤ 2.0 or ≥ 11.5; therefore, an intracutaneous irritation study was conducted.

2.4. Skin Irritation Test

2.4.1. Dose Selection and Justification for Selection

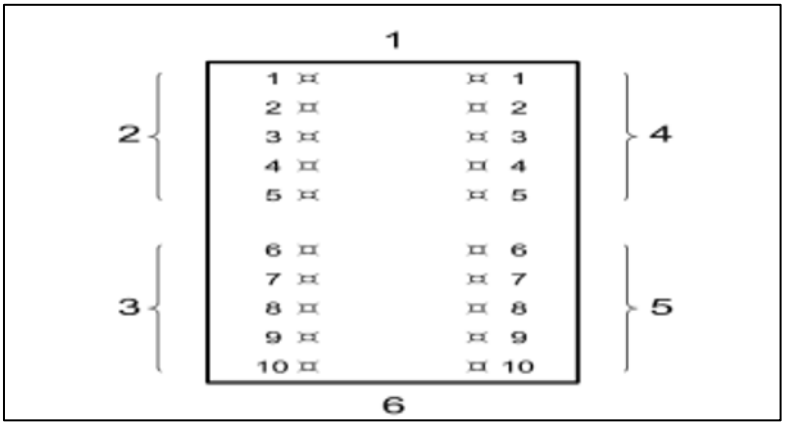
The testing was conducted using undiluted extracts (0.2 mL/site), as recommended by BS EN ISO 10993-23:2021 and ISO 10993, Part 23:2021(E) [10-14 17, 18].

2.4.2. Route of Administration and Justification for Selection

The polar, non-polar test item extracts and respective polar, non-polar solvent controls were given intradermally (by injection into the skin) to test how well they irritate the skin. This study was conducted according to the First Edition (2021-01) of the Standard (ISO 10993-23:2021 [E]) and BS EN ISO 10993-23:2021 standards for "Biological Evaluation of Medical Devices" [10-14 17, 18].

2.4.3. Administration of Test Item

Each rabbit was injected with 0.2 mL/site of polar extract using intracutaneous injection technique to the anterior aspect of its left trunk at five separate locations. Similarly, 0.2 mL/site of the respective polar solvent control was injected as an intracutaneous injection at five separate locations to the anterior portion of the right trunk of each rabbit. The non-polar test item extract and the non-polar solvent control were injected into each rabbit's posterior (contralateral) area in the same way. The injection locations were identified using a permanent marker pen. The observation will be terminated after 72 hour of administration of test item extracts.



Key: 1 - Cranial end (Anterior end); 2 - 0.2 mL injections of polar extract of test item; 3 - 0.2 mL injections of non-polar extract of test item; 4 - 0.2 mL injections of polar solvent control; 5 - 0.2 mL injections of non-polar solvent control; 6 - Caudal end (Posterior end)

Figure 2 Injection site in animal

2.4.4. Observations

The following observations were made during experimental period.

Clinical Signs of Toxicity and Mortality

For the duration of the study, animals were checked once a day for evidence of toxic reactions and twice a day for evidence of mortality. Systematic evaluations were performed to detect any changes in their skin/coat, eyes, respiratory system/circulatory system, autonomic system and central nervous system, as well as their somatomotor activity and behavior pattern.

Intracutaneous Reactions

Skin reactions were assessed at every injection site upon injection immediately and at intervals of 24 (±2 h), 48 (±2 h) and 72 (±2 h) hour after the injection occurred. Each tissue reaction was classified into grades based upon the grading scale supplied in the following table for all injections and all time intervals, with evaluations performed until 72 hour had elapsed after administering the test item extract. No adverse changes were observed at the injection sites.

Table 2 Grading System for Intracutaneous (Intradermal) Reactions

Intracutaneous 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 and extending beyond exposure area)	4
Maximum possible score for irritation	8

Body Weight

The individual animal body weight was recorded at the time of receipt, prior to initiation of the treatment (day 1) and at termination of the observation period.

Pathology

- Necropsy

All animals were euthanized via intravenous administration of sodium thiopentone at the end of the observation period and subsequently, the carcasses were disposed of Sodium thiopentone.

Interpretation of Results

All animals were monitored for numerical values that corresponded to their injection sites at each time interval during observations. All animals were given an erythema response (score) and an oedema response (score) for either 24 (±2 hour), 48 (±2 hour), or 72 (±2 hour) from each extract and control, respectively. Each animal's response scores were totaled for each extract and control. The total was divided by 15 (3 time points x 5 injection sites) to obtain the mean score for each test extract and each respective solvent control. Subtracting the mean score of the solvent control from the mean score of the test extract resulted in a final score for the test extract. For each test extract, all requirements of the experiment were satisfied, as the final test sample scores were zero.

2.5. Skin Sensitization Test

2.5.1. Dose Selection and Justification for Selection

The testing was conducted using the undiluted extracts as recommended by BS EN ISO 10993-10:2013 and ISO 10993, Part 10:2021 (E) [10-14, 19, 20].

2.5.2. Route of Administration and Justification for Selection

The insert test item was injected into the dermis during induction and applied to the skin during both the induction (boosting) and challenge phases of the study. The mode of administration was as defined in ISO 10993 Part 10:2021 (E) and BS EN ISO 10993-10:2013 [10-14, 19, 20].

2.5.3. Preparation of Intradermal Injections

Table 3 Intradermal Induction Injection Preparations for Control and Test Item Groups

Group	Injection Site	Composition	Total Volume (mL)
G1 (Polar Control)	Site 1	1:1 (v/v) mixture of Freunds Complete Adjuvant (FCA) in Polar Solvent Control Extract	2 mL+2 mL = 4 mL
	Site 2	Polar Solvent Control Extract	2 mL
	Site 3	50% (v/v) of Polar Solvent Control Extract with 1:1 mixture (v/v) of Freunds Complete Adjuvant (FCA) in Polar Solvent Control Extract	2 mL+1 mL +1 mL = 4 mL
G2 (Polar Test Item)	Site 1	1:1 (v/v) mixture of Freunds Complete Adjuvant (FCA) in Polar Solvent Control Extract	2 mL+2 mL = 4 mL
	Site 2	Undiluted Polar Test Item Extract	3 mL
	Site 3	50% (v/v) of Undiluted Polar Test Item Extract with 1:1 mixture (v/v) of Freunds Complete Adjuvant (FCA) in Polar Solvent Control Extract	2 mL+1 mL + 1 mL = 4 mL
G3 (Non Polar Control)	Site 1	1:1 (v/v) mixture of Freunds Complete Adjuvant (FCA) in Non-Polar Solvent Control Extract	2 mL+2 mL = 4 mL
	Site 2	Non-polar Solvent Control Extract	2 mL
	Site 3	50% (v/v) of Non-Polar Solvent Control Extract with 1:1 mixture (v/v) of Freunds Complete Adjuvant (FCA) in Non-Polar Solvent Control Extract	2 mL+1 mL +1 mL = 4 mL
G4 (Non Polar Test Item)	Site 1	1:1 (v/v) mixture of Freunds Complete Adjuvant (FCA) in Non-Polar Solvent Control Extract	2 mL+2 mL = 4 mL
	Site 2	Undiluted Non-Polar Test Item Extract	3 mL
	Site 3	50% (v/v) of Undiluted Non-polar Test Item Extract with 1:1 mixture (v/v) of Freunds Complete Adjuvant (FCA) in Non-Polar Solvent Control Extract	2 mL+1 mL + 1 mL = 4 mL

2.5.4. Preparation of Animals

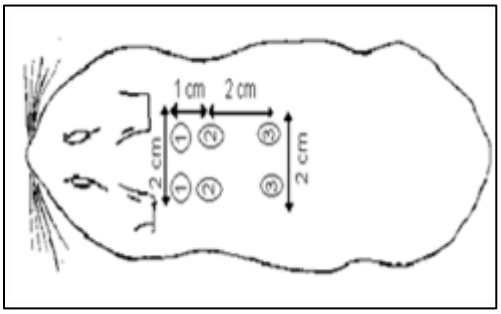
For both topical and intradermal injections, animal fur was cut back dramatically (i.e., approx. 24 hour) prior to administering the product on the shoulders, at an area of approximately 3 cm x 5 cm for topical only, and for both methods on the left side of the body, approximately 80 sq cm (8 cm x 10 cm). Prior to the challenge phase, all cuts must have been made prior to administering to ensure no skin irritation/abrasion.

2.5.5. Treatment

The test comprised of three phases, viz.,

- Induction :Intradermal injection
- Induction (Boosting) : Topical Application
- Challenge : Topical Application

Induction: Intradermal Injection



Site 1: 1st pair of injection; Site 2: 2nd pair of injection; Site 3: 3rd pair of injection.

Figure 3 Site of intradermal injections in the Guinea pig for maximization test of Magnusson and Kligman

- Day 1:

The animals received three pairs of intradermal injections (a total of 0.1 mL per injection) into their shoulders and were given two injections per pair (left and right side of the midline) approximately 2 cm from each other. Injections 1 and 2 were given near the head region at least 1 cm apart, and injection 3 was given 2 cm apart towards the caudal part of the body, as shown in figure 3. The injection sites were clearly identified by using the permanent marker pen to ensure accurate localization .

Induction: Topical Application

- Day 7:

Following intradermal administration of the test materials to the shoulder region of the test animals, the previously injected erythema areas were clipped (approximately 3 x 5 cm) from the shoulder region for both tests (solvent control and treatment groups). There was no irritancy seen when testing undiluted test item extracts for intradermal induction at site 2. To create local irritation prior to topical induction, 0.5 mL of 10% sodium lauryl sulphate and Vaseline were pre-applied before the 24±2 hour topical induction period.

- Day 8:

After applying intradermal injections, 2 x 4 cm filter paper was fully submerged into both control solvent and test item extraction. After making sure the filter paper was saturated with control solvent and test material extract, the soaked filter paper was then placed at the same location as the intradermal injections and secured in place using non-irritating adhesive tape, followed by the use of crepe bandage with an occlusive dressing for 48±2 hour. Upon completion of the contact period (48±2 hour), the test patches were removed and the test sites were thoroughly cleaned using normal saline swabs and dried with sterile cotton.

Challenge: Topical Application

- Day 21:

The fur from the right flank region, which was not treated during the induction phase, was clipped for all the animals.

- Day 22:

The filter paper (2 cm x 4 cm) was saturated by soaking in the respective solvent control and test item extract. The soaked filter paper was applied to the respective groups over the pre-clipped area. The test item extract was applied on the posterior part of the right flank region, and solvent control was applied on the anterior part of the right flank region in the G1, G2, G3 and G4 group animals. The patches were held in place with non-irritating adhesive tape and further wrapped with crepe bandage by an occlusive dressing.

The test patch was held in its position for 24±2 hour. After 24±2 hour of contact period, the test patches were removed, and the area was cleaned with normal saline swabs and dried with cotton.

Re-challenge: Topical Application

Since there were no unclear scores from the challenge phase treatment, re-challenge was not carried out.

2.5.6. Observations

During the course of the experiment, the following observations were made.

Clinical Signs of Toxicity and Mortality

Every animal was checked twice a day for mortality and once a day for clinical symptoms of toxicity.

Skin Reactions Scoring

Induction via Intradermal Injection: The skin reaction result for the intradermal injection (induction) was recorded at 24 ± 2 hour and 48 ± 2 hour.

Induction via Topical Application: The skin reaction for topical application (induction) was recorded at approximately 1 hr & 24 hour after the test patch was removed.

Topical Challenge Response: The skin reaction in the challenge phase was recorded at 24 ± 2 hour and 48 ± 2 hour after the removal of the test patch.

Table 4 Magnusson and Kligman Grading Scale for the Evaluation of Challenge Patch Test Reactions

Patch Test Reaction	**Grading Scale**
No visible change	0
Discrete or patchy erythema	1
Moderate and confluent erythema	2
Erythema and/or swelling	3

Body Weight

The individual animal body weights were recorded at the time of receipt, prior to initiation of the treatment (Day 1) and at termination.

2.5.7. Evaluation of Results

Both the treatment groups and the solvent control groups received a Magnusson and Kligman grade of "0". As a result, the extracted test item is regarded as a non-sensitizer to the animals' skin.

2.6. Cytotoxicity Test

2.6.1. Extraction Procedure for Cytotoxicity Test

An extraction ratio of 6 cm^2/mL of surface area to volume was utilized in order to extract the material. This extraction ratio of 6 cm^2/mL of surface area multiplied by the volume was determined following the guidelines outlined in ISO 10993-12:2021 (E) “Sample Preparation and Reference Materials"[10,15, 16].

The sample was received as a complete unit and utilized in the extraction process in accordance with the extraction ratio based upon the surface area of the sample and the volume of MEM culture media to be added. Before beginning the study, the negative and positive controls were autoclaved at 121ºC and 15 pounds for 15 minute to guarantee the sterility of the two controls. Both controls were cut into small pieces prior to being placed in the sterile working area for extraction purposes. The medium used as the control was prepared with no prior treatment to serve as a blank.

Post extraction showed clearly that there were no particles and no color change in the test item extraction medium, and there were no physical changes to the test item before and after extraction. The extracted test items were treated identically to the materials used during the extraction process. Test item extract was treated without modification. Extracts were kept at room temperature until the time of use, which was no more than 54 minute after the extraction process had been completed.

2.6.2. Test Procedure

The test item extract, Positive control(0.1% ZDEC polyurethane film (RM-A)), Negative control(High density polyethylene film (RM-C)) and Blank was made in an incubator shaker at 37±1ºC for 71 hour and 45 minute using an extraction ratio of 6 cm^2/mL surface area/volume [10-14,21, 22].

Table 5 Treatment groups and corresponding extraction mixtures used for cell exposure

Treatment	Details
Blank wells	1 mL of cell culture media without any treatment maintained similar to extraction procedure.
Test item	1 mL of test item extraction mixture.
Negative control	1 mL of high density polyethylene (HDPE) sheet extraction mixture.
Positive control	1 mL of polyurethane sheet extraction mixture.

Using L-929 mouse fibroblast cells and the Elution Method, the study evaluated the cytotoxic potential of the extract of the test item, the Left Atrial Appendage (LAA) Occluder System. The subconfluent cell monolayer-containing culture wells were chosen. Every treatment was kept in duplicate. The extraction combination of the test item, positive control, and negative control for each well was substituted for 1 mL of growth media to maintain the wells. Similarly, 1 mL of untreated extraction growth medium was used to maintain blank wells. The trial number, replicate number, matching treatment, and treatment date were labeled on the wells [10-14, 21, 22].

For 24 hour and 36 minute, cells were incubated at 37±1°C with 5±1%CO_2.

To confirm any alterations in cell morphology following treatment with the test item, the cultures were inspected under a microscope after incubation.

Table 6 Scoring for cytotoxicity was done based on the following criteria

Grade	Reactivity	Description of Reactivity Condition
0	None	Discrete intracytoplasmic granules, no cell lysis.
1	Slight	Less than or equal to 20% of the cells are round, loosely attached and without intracytoplasmic granules; occasional lysed cells are present.
2	Mild	Greater than 20% to less than or equal to 50% of the cells are round and devoid of intracytoplasmic granules; no extensive cell lysis and empty areas between cells.
3	Moderate	Greater than 50% to less than 70% of the cell layers contain rounded cells or are lysed.
4	Severe	Nearly complete destruction of the cell layers.

2.6.3. Acceptance Criteria

Due to the results of the test items with a numerical grade of zero and the test items not producing any reaction, a result is determined as follows. As there was no variation between replicates in this study, the results of the study are deemed valid. The study is considered as accepted based on both the negative and positive control throughout the testing, as the negative control met the criteria for a numerical grade of zero and the positive control met the criteria for a numerical grade of 4 based on the testing system.

2.7. Hemocompatibility

The aim of this study was to assess the hemolytic effect of the Left Atrial Appendage (LAA) Occluder System through direct contact and indirect contact methods when it comes into contact with rabbit blood [23-26].

2.7.1. Pre-Test Procedure For Heamolysis Test

Collection and Preparation of Blood Substrates

Whole blood samples were collected from healthy New Zealand White rabbits under controlled experimental conditions. The collected blood was in 3.8% (w/v) sodium citrate anticoagulant. About 5 mL of blood was collected from each rabbit. All collected blood was pooled equally from all three rabbits.

Establishment of Standard Curve for Hemoglobin (Hb) Determination

In this study six dilutions of hemoglobin standard were prepared from the stock solution, Sigma Hemoglobin reference standard, which contained 2 mg/mL of hemoglobin, and a purchase of Drabkin's Solution as the solvent for diluting. The concentrations prepared, ranging from 0.03 mg/mL to 0.70 mg/mL

Table 7 Preparation of Hemoglobin Calibration Standards Using Drabkin's Solution

Standard	Volume Taken	Drabkin's Solution (mL)	Concentration (mg/ mL)
Std 6	1.75 mL from Stock	3.25	0.70
Std 5	2.5 mL from Std 6	2.5	0.35
Std 4	3.6 mL from Std 5	1.4	0.25
Std 3	2.6 mL from Std 4	2.4	0.13
Std 2	2.3 mL from Std 3	2.7	0.06
Std 1	2.5 mL from Std 2	2.5	0.03

The same 200 µL concentrations from tube samples were placed on 96-well microplates with duplicate samples for each concentration with blank samples containing Drabkin's solution, to measure the OD of the hemoglobin standard, where the hemoglobin standard concentrations were plotted on the y-axis and the ODs noted in nm at 540 nm were plotted on the x-axis to generate a calibration curve. The calibration coefficient (F) is represented by the slope of the calibration curve. The standard calibration coefficient (F), calculated from the slope of the calibration curve, is used to calculate the concentration of hemoglobin (mg/mL) in the tube samples from the duplicate absorbance readings at 540 nm.

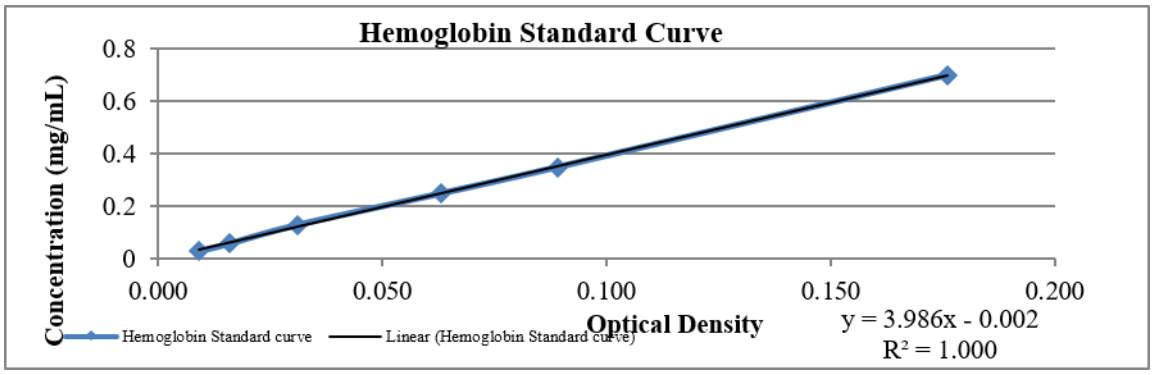


Figure 4 Hemoglobin Standard Curve Showing the Relationship between Optical Density and Concentration

Determination of Plasma Free Hemoglobin (PFH)

Centrifugation of pooled blood containing 3 mL at 800 xg was performed at 4°C to separate the plasma, which was collected. A graphical representation of signal results can be created by using the Drabkin dye technique. After an incubation time of 15 minute at room temperature, 200 µL of each test sample was transferred to the microplate wells and was tested by means of 540 nm. To be able to determine the full concentration of plasma-free hemoglobin (PFH), the formula of PFH should be used.

$$\text{Plasma Free Hemoglobin (PFH) (mg/mL)} = \text{APFH} \times F \times 2$$

APFH = Average Sample OD at 540 nm, F = Calibration Coefficient (Slope), 2 = Dilution Factor [obtained by dividing the total reaction mixture volume (1 mL) with sample volume (0.5 mL)]

As the value of the PFH was less than 2 mg/mL, hence blood was used for study.

Determination of Total Blood Hemoglobin Concentration (TBH)

To prepare for analysis, a 20 µL volume of a pooled and mixed blood sample (whole blood) was added to 5.0 mL of Drabkin's reagent and allowed to incubate (room temperature) for 15 minute (2 sets of incubated pure blood/Drabkin's reagent were prepared). Following incubation, 200 µL portions of each pure blood/Drabkin's reagent were placed into wells of a 96-well microtiter plate. The Drabkin's reagent was used in this experiment as the blanking reference substance. The microtiter plates were read on a microplate reader (measures based upon OD at the 540 nm wavelength), and the hemoglobin concentration in the total volume of blood used for the experiment was determined using the following equation:

$$\text{Total blood hemoglobin concentration (C)} = \text{AC} \times F \times 251$$

AC= Average Sample OD at 540 nm

F = Calibration Coefficient (Slope)

251 = Dilution factor [obtained by dividing the total reaction mixture volume (5.02 mL) with sample volume (0.02 mL)]

To establish the final concentration of hemoglobin, the calculated level of hemoglobin in the blood was established as being 09.95 mg/mL. This figure was obtained by dilution with CMF-PBS (the amount diluted was 2.31 mL of blood to 37.69 mL of CMF-PBS). The hemoglobin concentration was determined by performing the hemoglobin test using Drabkin's solution. A sample was added to Drabkin's solution at a volume of 300 µL into a total volume of 4.5 mL. This sample solution was allowed to incubate for approximately 15 minute at room temperature. The reader used for measuring the optical density (OD) of the sample solution was based on the above-mentioned protocol. The samples were tested in triplicate. The OD was obtained at 540 nm using a microplate reader. The OD value obtained from Drabkin's solution blank served as the baseline OD value. To calculate the hemoglobin concentration of the total diluted blood, the following equation was used.

$$\text{Total diluted blood hemoglobin concentration (T)} = \text{AT} \times F \times 16$$

AT = Average Sample OD at 540 nm, F = Calibration Coefficient (Slope), 16 = Dilution factor [obtained by dividing the total reaction volume (4.8 mL) with sample volume (0.3 mL)]

2.7.2. Test Procedure

Sample Preparation For Direct Contact Procedure

The test item is a solid device, and according to ISO 10993-12 and ASTM F619-20, the test item was considered as a whole device, and the extraction was done as a 6 cm^2/mL extraction ratio in CMF-PBS. The test item, negative control, positive control, and sample blank were prepared in triplicate. As per ASTM F756-17 for surface area to volume ratio, approximately 12 cm^2 HDPE sheets (> 0.5 mm thick) have been cut into small pieces and placed into a tube containing 4 mL of CMF-PBS. Sterile water for injection (SWFI) was used for positive control and CMF-PBS for blank.

Table 8 Preparation of Test Item and Control Extracts Including Thickness, Extraction Ratio, and Extraction Volume

Samples	Thickness	Extraction	Quantity	Volume of CMF-PBS(mL)
Blank	-	-	-	8
			-	8
			-	8
Test Item	<0.5	6 cm^2/mL	54.3792	9.063
			54.3792	9.063
			54.3792	9.063
Negative Control	>0.5	3 cm^2/mL	12 cm^2	4
			12 cm^2	4
			12 cm^2	4
Positive Control	-	-	8 mL (SWFI)	-
			8 mL (SWFI)	-
			8mL(SWFI)	-

Indirect Contact Method

The extraction was performed following these steps: The test item, blank, negative control, and positive controls were prepared in triplicate and incubated with constant stirring at a temperature of 50 ± 2°C for 72 hour. After the incubation period, the test item extracts were evaluated for any changes in colour, physical properties, and any remaining particle material. The extracts were stored at room temperature. The extracts were to be used within 1 hour and 8 minute after preparing the samples. The supernatants of all tubes were pooled into pre-labelled tubes containing the following extracts: Both test item and negative control, as well as both positive controls. The pre-labelled tubes of the test item and negative control were analysed to determine any background absorbance present when measuring the optical density at an absorption wavelength of 540 nm using a 96-well plate (200 µL). A CMF-PBS buffer was used as the blank. If any absorbance was detected, the average optical density of the extracts was subtracted from the average optical density of the test item and the average optical density of the negative control.

Determination of Supernatant Hemoglobin

Table 9 Each tube holding solutions received the prepared reconstituted diluted blood as follows

Sample	Replicates	Direct Contact Method			Indirect Contact Method	
		Quantity	Total Volume of CMF-PBS (mL)	Volume of Diluted Blood (mL)	Volume of Extracts (mL)	Volume of Diluted Blood (mL)
Test Item	R1	54.3792	9.063	1.295	2	0.286
	R2	54.3792	9.063	1.295	2	0.286
	R3	54.3792	9.063	1.295	2	0.286
Blank	R1	-	7	1	2	0.286
	R2	-	7	1	2	0.286
	R3	-	7	1	2	0.286
	R1	-	7(SWFI)	1	2	0.286

Positive Control (SWFI)	R2	-	7(SWFI)	1	2	0.286
	R3	-	7(SWFI)	1	2	0.286
Negative Control	R1	12 cm^2	4	0.571	2	0.286
	R2	12 cm^2	4	0.571	2	0.286
	R3	12 cm^2	4	0.571	2	0.286

The diluted blood added to both contact and indirect contact methods has been diluted 1 part to 7 parts of either CMF-PBS or source extract. A single sample was placed in each of three replicates of direct and indirect contact method tubes. The tubes have been capped and placed in a water bath at 37±1ºC for 3 hour and inverted gently every 30 minute to ensure good mixing of the blood with the tube's interior wall. Following the 3-hour incubation time, both direct contact method tubes (with test items, negative controls and positive controls) and indirect contact method tubes (containing the same solutions) were removed from the water bath, labelled, and placed in a centrifuge for 15 minute at 800 xg and 4ºC. The supernatant from each tube was carefully removed, ensuring disruption of the settled erythrocytes at the bottom of the tube did not occur during this process. The removed supernatants were combined into a new set of labelled screw-cap tubes for colour comparison and clarity. As for supernatants of each of the test items, sample extracts, negative controls, negative control extracts, blank controls and blank control extracts, they were colourless, clear and free from visible particulates. However, supernatant from both the positive control and positive control extract was visibly red. The test items (direct and indirect contact), negative control, positive control and blank supernatant samples (1.0 mL each) were added onto newly labelled tubes that contained Drabkin's solution (1.0 mL each), and all samples were incubated for 15 minute (room temperature). Post the 15-minute incubation, 200 µL of reaction mixture each was added per sample to 96 wells of a microplate. The Drabkin's solution was used as the blank. A microplate reader was used to measure the optical density (OD) of the reaction mixture at 540 nm. Hemoglobin concentrations were calculated from the OD values of the blank, test item, negative control and positive control supernatants using the following formula:

$$\text{Supernatant hemoglobin concentration (D)} = AS \times F \times 2$$

AS = Average Sample OD at 540 nm, F = Calibration Coefficient (Slope), 2 = Dilution factor [obtained by dividing the total reaction volume (2.0 mL) with supernatant volume (1 mL)]
Blank adjusted % Heamolysis or Hemolytic index was determined using the below formula:
Blank corrected hemolytic index = ((A^S-A^B))/((A^T-A^B))×100
AS = Average OD of negative control or positive control or test item
AB = Average OD of blank
AT= Average OD of diluted blood

2.7.3. Evaluation Criteria

The absorbance measurements were used to determine the concentration of hemoglobin in the supernatant. After subtracting the negative control values from the blank corrected percentage of Heamolysis, the Hemolytic index above the test item's negative control was computed. The outcome of the test sample was compared to the results of the negative control.

Table 10 Hemolysis Grading Criteria Based on Hemolytic Index Relative to Negative Control

Hemolytic Index Above the Negative Control	**Hemolytic Grade**
0-2	Non hemolytic
2-5	Slightly hemolytic
>5	hemolytic

The test is judged valid, as the blank-corrected % Heamolysis of the negative control was within the range of 0 to 2.

2.8. Pyrogenicity

2.8.1. Route of Administration and Justification for Selection

The samples (test item extract or blank extract) were administered through the intravenous route as per guideline USP 43-NF 38, General Chapters: <151> Pyrogen test and 21 CFR Part 610, Section 610.13(b) Test for Pyrogenic Substances [13, 14, 27-32].

2.8.2. Dose Selection and Justification for Selection

In accordance with USP 43-NF 38, General Chapters: In the pyrogen test, the samples (test item extract or blank extract (preheated and kept 37±2°C throughout administration)) were injected at a dosage volume of 10 mL/kg body weight [13, 14, 27-32].

2.8.3. Preliminary Test

Before the test, a week before the test, 3 rabbits were selected to be used in the study based on these criteria (all animals were injected before the test); Rabbits were not allowed to eat or drink during the testing period but had the same diet as the rest of the rabbits used for this study. Rabbits were maintained on a 16 hr fast prior to removing all food and water during the tests. In addition to the restrainer, the animals were to be restrained for a minimum of 1 hr prior to measuring the rectal temperature of the animals. The temperature probe will be inserted into the rectum of each rabbit and will remain there for the same time as it takes to stabilise the rabbits. Control temperatures will be taken after stabilising at least once in all cases that were tested. The following temperature measurements were taken at an interval of 30±2 min after the time of the previous temperature test (from 1 to 3 hour after completion of the previous temperature test). The animals selected for testing must have similar (i.e., <1°C difference between) control temperature ranges (<39.23°C maximum) as the animals used in the control group (i.e., within the control group).

2.8.4. Pyrogen Test: Study Design and Procedure

Six rabbits were used in the first main test (three for blank extract and three for test item extract). Since none of the six animal individual responses exceeded 0.5°C, main test was not carried out.

- Procedure:

Prior to the administration of samples, all animals used in this study underwent a fast for approximately 15 hour. During fasting, the animals had access to water but both feed and water were restricted during the course of the test. The body weights of the animals were recorded and the animals were placed into their restraining devices for at least one hour before the animals temperatures were recorded; temperature probes were inserted into the rectum of each rabbit to a depth of not less than 7.5 cm and allowed to remain for several minute so as to allow temperature probes to stabilize before recording the control temperature of each rabbit. The injection of the samples occurred within 30 minute of the control temperature recording. The samples were pre-heated to 37±2°C prior to administration and were maintained at this temperature throughout the administration procedure. The samples were injected into the marginal ear veins of the animals at a dose of 10 mL per kilogram of body weight and the administration process was completed within 10 minute of the initiation of administration. Body temperatures were measured at 30±2 minute intervals for a period of one to three hour after sample injection.

2.8.5. Observations

The following observations were made throughout the experiment.

Clinical Signs of Toxicity and Morbidity/Mortality

Rabbits were observed once daily for clinical signs of toxicity and twice daily for morbidity/mortality throughout the experimental period.

Body weight

The body weight of animals was taken on the day of receipt and on the day of the main test before restraining the animals.

Determination of Rectal Temperature

The highest temperature that was recorded for each rabbit was the maximum temperature observed in the first three hour after administration of the samples (with a collection time ranging from 30±2 minute). Each rabbit's response to the treatment was determined by subtracting its controlled temperature from its maximum temperature.

2.8.6. Procedure For Interpretation of Results

A decrease in temperature is recorded as 0. If the temperature of all three subjects in Test I increased by 0.5°C or less than their respective control temperature(s), then this indicates that the sample does not contain any pyrogenic substance. In the case wherein one subject in Test I demonstrates an increase in temperature of 0.5°C, or higher, from their respective control temperature(s) and the combined maximum increase in temperature of all three subjects does not exceed 3.3°C, Test II must be conducted with five additional subjects. If three out of the eight subjects from Tests I and II have each exhibited a rise in temperature of 0.5°C or more, and the total of their combined maximum temperatures does not exceed 3.3°C, then the tested item demonstrates absence of pyrogenic activity.

2.9. Acute Systemic Toxicity

2.9.1. Study Design

Table 11 The selected animals were assigned to solvent control and different test groups as shown below

Group	Group Description	Dose Volume (mL/kg Body Weight)	Route of Administration	No. of animals/ group
G1	Polar Solvent Control	50	Intravenous	5
G2	Polar Test Item Extract	50	Intravenous	5
G3	Non-polar Solvent Control	50	Intraperitoneal	5
G4	Non-polar Test Item Extract	50	Intraperitoneal	5

2.9.2. Dose Selection and Justification for Selection

The test item and the respective solvent controls were extracted at an extraction ratio of volume 6 cm^2/mL using both non-polar and polar solvents. The undiluted extracts were administered to the mice as a single bolus dose of 50 mL/kg body weight. 50 mL/kg is a recommended amount for intraperitoneal (IP) and intravenous (IV) administration for laboratory testing as outlined in the ISO 10993 - Part 11 "Tests for Systemic Toxicity" and BS EN ISO 10993 - Part 11 "Tests for Systemic Toxicity" [31, 32].

2.9.3. Route of Administration and Justification for Selection

The ISO 10993 (Part 11)-"Systemic Toxicity test methods" and BS EN ISO (Part 11)-"Systemic Toxicity test methods" guided both methods of administration for polar and non-polar extracts/solvents. The polar substance and solvent were administered by intravenous route, and the non-polar extracts/solvents were administered by intraperitoneal route.

2.9.4. Administration of Test Item

In accordance with the standards established within ISO 10993 Part 11–"Test for Systemic Toxicity" and BS EN ISO 10993 Part 11–"Test for Systemic Toxicity", the test items and respective solvents were administered via intravenous (IV) or intraperitoneal (IP) routes, respectively, in a dosing scheme of 50 mL/kg body weight.

2.9.5. Observations

The following findings were noted throughout the experiment.

- Clinical Signs Observation

Observations of all the animals were carried out for signs of clinical signs of toxicity at 4-hourly intervals }of thirty to 40 minute following the first dose and 1 hour (plus or minus ten minute), 2 hour (±10 minute) and 4 hour (±10 minute). All animals were also observed the next three days for signs of toxicity, once a day from day two to day four, and twice

a day for mortalities. There were no signs of toxicity noted for any of the animals during the fourth day; therefore, the study was ended.

Body Weight

During the observation period, body weight was measured at the time of receipt, on the day the test item was administered before dosing, and every day for the first three days following dosing (days 2, 3, and 4).

Pathology

- Necropsy

All of the animals underwent necropsy and gross pathological evaluation after being mercifully sacrificed by carbon dioxide asphyxiation at the conclusion of the observation period.

- Histopathology

Since none of the animals had any obvious lesions, a histopathological study was not carried out.

2.9.6. Interpretation of Results

All tested animal groups compared favourably to their solvent control groups on the basis of toxicity and manifested no clinical signs of toxicity; no treated animal exhibited any form of death, seizure, or prostration, and no treated animals experienced a loss of body weight attributed to treatment during the observation period.

3. Results And Discussion

3.1. Skin Irritation

3.1.1. Clinical Signs of Toxicity and Mortality

None of the animals showed any clinical evidence of toxicity or mortality due to the therapy.

Table 12 Clinical Signs of Toxicity and Mortality

Animal No.	Dose (mL/site)	Sex	Clinical Signs of Toxicity on Days			
			1	2	3	4
Nc4307	0.2	Male	N	N	N	N
Nc4308	0.2	Male	N	N	N	N
Nc4309	0.2	Male	N	N	N	N

Note: N-Normal

3.1.2. Intracutaneous Reactions

No treatment related intracutaneous reactions were observed in any of the animals.

Table 13 Skin Reaction Scoring Record

Animal No.	Sex	Dose (mL/site)	Test Articles Evaluated	Observation Periods	Skin Reactions (Erythema / Oedema)	Total Irritation Score	Result
Nc4307	Male	0.2	Polar Extract, Polar Solvent, Non-polar Extract, Non-polar Solvent	24, 48, 72 h	No erythema or oedema observed at any site	0.0	Non-irritant

Nc4308	Male	0.2	Polar Extract, Polar Solvent, Non-polar Extract, Non-polar Solvent	24, 48, 72 h	No erythema or oedema observed at any site	0.0	Non-irritant
Nc4309	Male	0.2	Polar Extract, Polar Solvent, Non-polar Extract, Non-polar Solvent	24, 48, 72 h	No erythema or oedema observed at any site	0.0	Non-irritant
Mean Score (Blank control) = total score of 3 animals = 0/3 = 0							
Mean Score (Test Item sample) = total score of 3 animals = 0/3 = 0							
Final test sample score = Mean score of the test item sample - Mean score of the blank control = 0-0 =0							

Mean Score (Blank control) = total score of 3 animals = 0/3 = 0

Mean Score (Test Item sample) = total score of 3 animals = 0/3 = 0

Final test sample score = Mean score of the test item sample - Mean score of the blank control = 0-0 =0

3.1.3. Body Weight

No treatment related changes in body weight and percent change in body weight with respect to day 1 were observed in any of the animals.

Table 14 Body Weight (Kg) and Percent Change in Body Weight with Respect to Day 1

Animal No.	**Dose (mL/site)**	**Sex**	**Body Weight (kg) on Day**		**Percent Change in Body Weight with Respect to Day 1**
			1	**4**	
Nc4307	0.2	Male	2.30219	2.33537	1.44124
Nc4308	0.2	Male	2.25708	2.28914	1.42042
Nc4309	0.2	Male	2.31249	2.34801	1.53601
		Mean	2.29059	2.32417	1.46589
		±SD	0.02947	0.03099	0.06161
		n	3	3	3

n: Number of animals; SD: Standard Deviation

3.2. Skin Sensitization

3.2.1. Clinical Sign of Toxicity and Mortality

No clinical sign of toxicity and mortality were observed in any of the animal in all the groups.

Table 15 Clinical Signs of Toxicity and Mortality Record

Group, Sex & Treatment	**Animal No.**	**Clinical Signs of Toxicity and Mortality on Days**												
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
G1, Female &	Gf3867	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3868	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3869	N	N	N	N	N	N	N	N	N	N	N	N	N

Polar Solvent Control	Gf3870	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3871	N	N	N	N	N	N	N	N	N	N	N	N	N
G2, Female & Polar Test Item Extract	Gf3872	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3873	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3874	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3875	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3876	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3877	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3878	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3879	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3880	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3881	N	N	N	N	N	N	N	N	N	N	N	N	N
G3, Female & Non-polar Solvent Control	Gf3882	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3883	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3884	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3885	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3886	N	N	N	N	N	N	N	N	N	N	N	N	N
G4, Female & Non-polar Test Item Extract	Gf3887	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3888	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3889	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3890	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3891	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3892	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3893	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3894	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3895	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3896	N	N	N	N	N	N	N	N	N	N	N	N	N

N: Normal

Table 16 Clinical Signs of Toxicity and Mortality Record

Group, Sex & Treatment	**Animal No.**	**Clinical Signs of Toxicity and Mortality on Days**												
		14	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**
G1, Female & Polar Solvent Control	Gf3867	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3868	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3869	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3870	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3871	N	N	N	N	N	N	N	N	N	N	N	N	N
G2,	Gf3872	N	N	N	N	N	N	N	N	N	N	N	N	N

Female & Polar Test Item Extract	Gf3873	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3874	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3875	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3876	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3877	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3878	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3879	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3880	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3881	N	N	N	N	N	N	N	N	N	N	N	N	N
G3, Female & Non-polar Solvent Control	Gf3882	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3883	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3884	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3885	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3886	N	N	N	N	N	N	N	N	N	N	N	N	N
G4, Female & Non-polar Test Item Extract	Gf3887	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3888	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3889	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3890	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3891	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3892	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3893	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3894	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3895	N	N	N	N	N	N	N	N	N	N	N	N	N
	Gf3896	N	N	N	N	N	N	N	N	N	N	N	N	N

3.2.2. Skin Reactions Scoring

Intradermal Induction (Day 1)

No treatment-related skin reactions were observed at injection site 2 after intradermal injection at 24±2 and 48±2 hour of observation in the G1, G2, G3 and G4 group animals. However, at 24±2 and 48±2 hour of observation after intradermal injection, skin reactions like moderate and confluent erythema to discrete or patchy erythema were noted at injection site 1 in the G1, G2, G3 and G4 group animals. At 24±2 and 48±2 hour' skin reactions like discrete or patchy erythema were noted after intradermal injection at injection site 3 in all the group animals.

Topical Induction (Day 8)

No treatment related skin reactions were observed approximately at 1 hour and 24 hour after removal of the test patch in any of the animals of all the groups tested.

Challenge (Day 22)

No treatment related skin reactions were observed at (24±2) and (48±2) hour after removal of the test patch in any of the animals of all the groups tested.

Table 17 Skin Reactions Scoring Record

Group	Sex	Treatment	No. of Animals	Induction Reactions	Challenge Reactions (24 & 48 h)	Sensitization Result
G1	Female	Polar Solvent Control	5	Mild erythema (scores 1–2) at some induction sites	No reactions observed (score 0)	Negative
G2	Female	Polar Test Item Extract	10	Mild erythema (scores 1–2) at some induction sites	No reactions observed (score 0)	Negative
G3	Female	Non-Polar Solvent Control	5	Mild erythema (scores 1–2) observed at some induction sites	No reactions observed (score 0)	Negative
G4	Female	Non-Polar Test Item Extract	10	Mild erythema (scores 1–2) observed at some induction sites	No reactions observed (score 0)	Negative

3.2.3. Body Weight

There were no treatment-related changes in body weight or percentage changes in body weight compared to day 1. Every animal displayed a typical physiological rise in body weight.

Table 18 Body Weight (G) and Percent Change in Body Weight with Respect to Day 1

Group	Sex	Treatment	No. of Animals	Body Weight Day 1 (g) Mean ± SD	Body Weight Day 26 (g) Mean ± SD	% Change from Day 1 Mean ± SD
G1	Female	Polar Solvent Control	5	369.05 ± 6.32	448.05 ± 5.83	21.41 ± 0.83
G2	Female	Polar Test Item Extract	10	371.39 ± 6.32	449.91 ± 7.04	21.15 ± 1.49
G3	Female	Non-Polar Solvent Control	5	371.35 ± 5.73	450.77 ± 6.65	21.39 ± 1.08
G4	Female	Non-Polar Test Item Extract	10	372.38 ± 6.00	450.90 ± 5.36	21.10 ± 1.34

3.2.4. Cytotoxicity Toxicity

The test item was deemed "non-cytotoxic" since its numerical grading was "0" and "none" response was noted. The test was deemed legitimate since there was no indication that the test results for replicates differed. The study is approved since the positive control in the test system has a numerical grade of 4 and the negative control had a numerical grade of 0

Table 19 Individual Results of Cytotoxicity Test

Treatment	Microscopic Observation		
	Replicate	Grade	Reactivity
Blank	1	0	None
	2	0	
	3	0	
Test Item (Left Artial Appandage(LAA) Occluder System)	1	0	None
	2	0	
	3	0	
Negative Control	1	0	None

(HDPE)	2	0	
	3	0	
Positive Control (Polyurethane)	1	4	None
	2	4	
	3	4	

3.3. Hemocompatibility

3.3.1. Plasma Free Hemoglobin Concentration

The concentration of free hemoglobin in plasma was 0.423 mg/mL.

3.3.2. Total Blood Hemoglobin

Concentration Total Blood Hemoglobin Concentration (C) was 173.09 mg/mL.

3.3.3. Total Diluted Blood Hemoglobin

Focus The concentration of total diluted blood hemoglobin (T) was 9.95 mg/mL.

Table 20 Plasma Free Hemoglobin, Total Blood Hemoglobin and Total Diluted Blood Hemoglobin Concentration

Sample	Absorbance Replicate 1	Absorbance Replicate 2	Absorbance Replicate 3	Average Absorbance	Standard Deviation	Hemoglobin Concentration mg/mL
Plasma Free Hemoglobin (PFH)	0.049	0.056	-	0.053	0.005	0.423
Total Blood Hemoglobin (C)	0.173	0.173	-	0.173	0.000	173.09
Total Diluted Blood Hemoglobin (T)	0.156	0.156	0.156	0.156	0.000	9.95

Plasma free hemoglobin concentration (mg/mL) = A^{PFH}× F × 2; Total blood hemoglobin concentration (mg/mL) = A^{C}× F × 251; Total diluted blood hemoglobin concentration (mg/mL) = A^{T}× F ×16; A^{PFH} – Average absorbance of plasma free hemoglobin; A^{C} – Average absorbance of total blood hemoglobin; A^{T} – Average absorbance of total diluted blood hemoglobin; Calibration coefficient (F) = 3.986

3.3.4. Supernatant Hemoglobin Concentration

Blank Control: Hemoglobin Concentration from the Blank Control With the Direct Method (By Direct Contact) = 0.016 mg/ml Hemoglobin Concentration from the Blank Control with the Indirect Method (By Indirect Contact) = 0.013 mg/ml.

Accounts for the negative control assessment of hemoglobin levels for both the direct and indirect contact methods: a) the level at which no hemoglobin is present (-0.027 mg/mL for the direct contact method) and blank corrected percent Heamolysis (-3.463%), and b) the level at which hemoglobin is in contact with the indirect contact method (0.008 mg/mL for the indirect contact method) and blank corrected percent Heamolysis (1.948%). In the analysis of direct contact method, the positive control Hemoglobin concentration is 1.390 mg/mL, the percentage of Heamolysis, corrected for the blank, is 111.905%, and the Hemolytic Index of the positive control was 115.368% higher than that of the negative control. The positive control's hemoglobin concentration for the indirect contact method was 1.318 mg/mL, its hemolytic index was 108.009% greater than the negative control's, and the percentage of heamolysis adjusted for the blank was 106.061%.

For the test item, hemoglobin concentration measured by direct contact was determined to be -0.021 mg/mL, and -3.030% was obtained for % Heamolysis, indicating no detection of Hemolytic activity. An index of Heamolysis for this item obtained would thus be 0.433%, compared to the negative control. When using the direct contact method to assess hemoglobin concentration from the test item, 0.000 mg/mL was determined and -1.299% for % Heamolysis, also indicating the absence of Hemolytic activity. An index of

Heamolysis of 0.649% was determined for this item above the negative control.

Table 21 Blank Corrected Percent Heamolysis and Hemolytic Index - Direct Contact Method

Replicates	Absorbance at 540 nm				Hemoglobin Concentration (mg/mL)				Blank Corrected Heamolysis (%) *			Hemolytic Index≠%	
	Blank Control	Negative Control	Positive Control	Test Item	Blank Control	Negative Control	Positive Control	Test Item	Negative Control	Positive Control	Test Item	Positive Control	Test Item
Replicate 1	0.001	-0.004	0.180	-0.003	0.008	-0.032	1.435	-0.024	-3.896	115.584	-3.247	119.481	0.649
Replicate 2	0.001	-0.003	0.170	-0.002	0.008	-0.024	1.355	-0.016	-3.247	109.091	-2.597	112.338	0.649
Replicate 3	0.004	-0.003	0.173	-0.003	0.032	-0.024	1.379	-0.024	-3.247	111.039	-3.247	114.286	0.000
Mean	0.002	-0.003	0.174	-0.003	0.016	-0.027	1.390	-0.021	-3.463	111.905	-3.030	115.368	0.433
±SD	0.002	0.001	0.005	0.001	0.014	0.005	0.041	0.005	0.375	3.332	0.375	3.692	0.375

D= Average absorbance × F ×2; F = Calibration Coefficient (Slope); *Blank corrected % heamolysis = [(AS - AB)/(AT - AB)] ×100

AS = OD of negative control, positive control or test item; AB = Average OD of blank; AT = Average OD of total diluted blood

Hemolytic index above the negative control = Blank corrected % Heamolysis of test item/positive control – Blank corrected % Heamolysis of negative control

Table 22 Hemolytic Evaluation Based on Absorbance, Hemoglobin Concentration, and Hemolytic Index- Indirect Contact Method

Replicates	Absorbance at 540 nm				Hemoglobin Concentration (mg/mL)				Blank Corrected Heamolysis (%) *			Hemolytic index (%) ≠	
	Blank Control	Negative Control	Positive Control	Test Item	Blank Control	Negative Control	Positive Control	Test Item	Negative Control	Positive Control	Test Item	Positive Control	Test Item
Replicate 1	0.001	-0.002	0.165	-0.002	0.008	-0.016	1.315	-0.016	-2.597	105.844	-2.597	108.442	0.000
Replicate 2	0.002	-0.002	0.166	0.000	0.016	-0.016	1.323	0.000	-2.597	106.494	-1.299	109.091	1.299
Replicate 3	0.002	0.001	0.165	0.002	0.016	0.008	1.315	0.016	-0.649	105.844	0.000	106.494	0.649
Mean	0.002	-0.001	0.165	0.000	0.013	-0.008	1.318	0.000	-1.948	106.061	-1.299	108.009	0.649
±SD	0.001	0.002	0.001	0.002	0.005	0.014	0.005	0.016	1.125	0.375	1.299	1.352	0.649

D= Average absorbance × F × 2
F = Calibration Coefficient (Slope)
*Blank corrected % hemolysis = [(AS - AB)/(AT - AB)] ×100
AS = OD of negative control, positive control or test item
AB = Average OD of blank
AT = Average OD of total diluted blood
# Hemolytic index above the negative control = Blank corrected % hemolysis of test item/positive control – Blank corrected % hemolysis of negative control

3.4. Pyrogenicity Test

3.4.1. Clinical Signs of Toxicity and Morbidity/Mortality

No treatment related clinical signs of toxicity and mortality were observed in the animals.

Table 23 Individual Animal Clinical Signs of Toxicity and Mortality Record

Treatment	Sex	Animal No.	Clinical Signs on Day 1	Mortality (No. of Mortality/ No. of Animals Dosed)
Blank Extract	M	Nc4861	N	0/3
	M	Nc4863	N	
	M	Nc4864	N	
Test Item Extract	M	Nc4865	N	0/3
	M	Nc4866	N	
	M	Nc4868	N	

N: Normal, M: Male

3.4.2. Determination of Rectal Temperature

Preliminary Test

The Maximum control temperature variation observed in the selected animals was 0.52°C and the temperature of the rabbits did not exceed 39.23°C (Table 22).

Main Test (for Blank Extract)

The individual rise in temperature of animals was 0.19°C, 0.19°C and 0.00°C respectively when compared to respective pre-dose control temperature (Table 23).

Main Test (for Test Item Extract)

The individual rise in temperature of animals was 0.14°C, 0.00°C and 0.00°C respectively when compared to respective pre-dose control temperature (Table 23).

3.4.3. Interpretation of Results

No rabbit showed an increase in individual temperature of 0.5°C or more above the respective control temperature in main test. Hence the test item meets the requirements of the pyrogen test and is considered to be non-pyrogenic.

Table 24 Temperature Record for Preliminary Test

Animal No.	Sex	Temperature (°C)					
		Control Temperature	1 hour	1½ hr	2 hr	2½ hr	3 hr
*Nc4861	M	38.72	39.06	39.17	39.10	38.89	38.91
Nc4862	M	39.41	39.52	39.61	39.56	39.49	39.49
*Nc4863	M	38.64	38.56	38.32	38.21	38.33	38.26
*Nc4864	M	38.73	38.61	38.71	38.71	38.69	38.70
*Nc4865	M	38.71	38.78	38.89	38.85	38.94	38.99
*Nc4866	M	39.16	39.13	39.17	39.15	39.23	39.23
Nc4867	M	39.36	38.99	39.03	39.02	39.01	38.90

*Nc4868	M	39.01	38.93	38.86	38.76	38.76	38.76

*: Represents the animals selected for the main study; hr: hour, M: Male

Table 25 Temperature Record - Main Test

Animal No.	Sex	Body Weight (kg)	Dose Volume Per Animal (mL)	Duration of Administration (Minute)	Temperature (°C)						Response Temperature (°C)		
					Control Temperature	After Administration of Blank Extract							
						1 hour	1½ hr	2 hr	2½ hr	3 hr	A	B	C
For Blank Extract													
Nc4861	M	1.87261	18.7	02	38.69	38.84	38.85	38.88	38.82	38.87	38.69	38.88	0.19
Nc4863	M	1.79221	17.9	03	38.80	38.95	38.99	38.95	38.93	38.92	38.80	38.99	0.19
Nc4864	M	1.72996	17.3	01	39.20	38.92	38.94	38.95	38.93	38.92	39.20	38.95	-0.25
For Test Item Extract													
Nc4865	M	1.76219	17.6	02	38.98	39.12	38.69	38.70	38.93	38.80	38.98	39.12	0.14
Nc4866	M	1.74342	17.4	03	39.06	39.06	38.91	38.81	38.76	38.80	38.06	39.06	0.00
Nc4868	M	1.75499	17.5	02	38.89	38.78	38.72	38.72	38.61	38.61	38.89	38.89	-0.11

A: Control Temperature, B: Maximum Temperature, C: Individual Animal Response, Hr: hour, M: Male

Individual animal temperature variation (Response) = Maximum temperature - Control temperature

3.5. For Acute Systemic Toxicity

3.5.1. Clinical Signs of Toxicity and Mortality

None of the animals showed any clinical evidence of toxicity or mortality due to the therapy.

Table 26 Clinical Signs of Toxicity and Mortality Record

Group, Sex & Group Description	Animal No.	Clinical Signs of Toxicity/Mortality						
		Observation on Day 1				Observation on days		
		30 - 40 min	1 hr (±10 min)	2 hr (±10 min)	4 hr (±10 min)	2	3	4
G1, Female & Polar Solvent Control Extract	Md8705	N	N	N	N	N	N	N
	Md8706	N	N	N	N	N	N	N
	Md8707	N	N	N	N	N	N	N
	Md8708	N	N	N	N	N	N	N

	Md8709	N	N	N	N	N	N	N
G2, Female & Polar Test Item Extract	Md8710	N	N	N	N	N	N	N
	Md8711	N	N	N	N	N	N	N
	Md8712	N	N	N	N	N	N	N
	Md8713	N	N	N	N	N	N	N
	Md8714	N	N	N	N	N	N	N
G3, Female & Non-Polar Solvent Control Extract	Md8715	N	N	N	N	N	N	N
	Md8716	N	N	N	N	N	N	N
	Md8717	N	N	N	N	N	N	N
	Md8718	N	N	N	N	N	N	N
	Md8719	N	N	N	N	N	N	N
G4, Female & Non-Polar Test Item Extract	Md8720	N	N	N	N	N	N	N
	Md8721	N	N	N	N	N	N	N
	Md8722	N	N	N	N	N	N	N
	Md8723	N	N	N	N	N	N	N
	Md8724	N	N	N	N	N	N	N

3.5.2. Body Weight

There were no treatment-related changes observed in body weight or the percentage change in body weight compared to day 1. Throughout the monitoring period, every animal showed a typical increase in body weight.

Table 27 Dose Volume Administered (Ml), Body Weight (G) and Percent Change In Body Weight with Respect to Day 1

Group, Sex & Group Description	**Animal No.**	**Dose Volume Administered**	**Body Weight (g) on Days**				**Percent Change in Body Weight With Respect to Day**		
			1	**2**	**3**	**4**	**1-2**	**1-3**	**1-4**
G1, Female &Polar Solvent Control Extract	Md8705	1.1	21.26	21.42	21.56	21.72	0.75	1.41	2.16
	Md8706	1.1	21.46	22.60	22.75	22.90	0.62	1.29	1.96
	Md8707	1.2	23.85	24.03	24.17	24.33	0.75	1.34	2.01
	Md8708	1.2	24.83	25.00	25.16	25.32	0.68	1.33	1.97
	Md8709	1.3	25.11	25.28	25.44	25.61	0.68	1.31	1.99
	Mean	-	23.50	23.67	23.82	23.98	0.70	1.34	2.02
	±SD	-	1.63	1.64	1.64	1.65	0.06	0.05	0.08
	n	-	5	5	5	5	5	5	5
G2, Female &Polar Test Item Extract	Md8710	1.1	21.81	21.96	22.09	22.25	0.69	1.28	2.02
	Md8711	1.1	22.90	23.04	23.19	23.19	0.61	1.27	2.01
	Md87012	1.2	23.99	24.12	24.25	24.25	0.54	1.08	1.75
	Md8713	1.2	24.47	24.65	24.79	24.94	0.74	1.31	1.92
	Md8714	1.3	25.12	25.26	25.41	25.57	0.56	1.15	1.79
	Mean	-	23.66	23.81	23.95	24.11	0.63	1.22	1.90

	±SD	-	1.63	1.32	1.32	1.32	0.08	0.10	0.12
	n	-	5	5	5	5	5	5	5
G3, Female & Non-Polar Solvent Control Extract	Md8715	1.1	21.91	22.09	22.24	22.40	0.82	1.51	2.24
	Md8716	1.1	22.98	23.12	23.26	23.42	0.61	1.22	1.91
	Md8717	1.2	23.14	23.32	23.47	23.62	0.78	1.43	2.07
	Md8718	1.2	24.63	24.80	24.93	25.09	0.69	1.22	1.87
	Md8719	1.3	25.53	25.69	25.84	26.01	0.63	1.21	1.88
	Mean	-	23.64	23.80	23.95	24.11	0.71	1.32	1.99
	±SD	-	1.43	1.43	1.43	1.43	0.09	0.14	0.16
	n	-	5	5	5	5	5	5	5
G4, Female & Non-Polar Test Item Extract	Md8720	1.1	22.14	22.30	22.44	22.59	0.72	1.36	2.03
	Md8721	1.1	22.17	22.31	22.45	22.61	0.63	1.26	1.98
	Md8722	1.2	23.55	23.70	23.83	23.99	0.64	1.19	1.87
	Md8723	1.2	24.70	24.86	24.99	25.16	0.65	1.17	1.86
	Md8724	1.3	25.86	26.02	26.17	26.32	0.62	1.20	1.78
	Mean	-	23.68	23.84	23.98	24.13	0.65	1.24	1.91
	±SD	-	1.62	1.62	1.62	1.62	0.04	0.07	0.10
	n	-	5	5	5	5	5	5	5

SD: Standard Deviation, n: No. of animals

3.5.3. Pathology

None of the animals showed any obvious pathogenic alterations.

Table 28 Gross Pathology Findings

Group, Sex & Group Description	**Animal No.**	**Fate**	**Gross Pathology Findings**	
			External	**Internal**
G1, Female & Polar Solvent Control Extract	Md8705	TS	NAD	NAD
	Md8706	TS	NAD	NAD
	Md8707	TS	NAD	NAD
	Md8708	TS	NAD	NAD
	Md8709	TS	NAD	NAD
G2 Female & Polar Test Item Extract	Md8710	TS	NAD	NAD
	Md8711	TS	NAD	NAD
	Md8712	TS	NAD	NAD
	Md8713	TS	NAD	NAD
	Md8714	TS	NAD	NAD
G3, Female & Non-Polar Solvent Control Extract	Md8715	TS	NAD	NAD
	Md8716	TS	NAD	NAD
	Md8717	TS	NAD	NAD

	Md8718	TS	NAD	NAD
	Md8719	TS	NAD	NAD
G4, Female & Non-Polar Test Item Extract	Md8720	TS	NAD	NAD
	Md8721	TS	NAD	NAD
	Md8722	TS	NAD	NAD
	Md8723	TS	NAD	NAD
	Md8724	TS	NAD	NAD

TS: Terminal Sacrifice; NAD: No Abnormality Detected

4. Conclusion

Based on the ISO 10993 Series Biological Evaluation for Medical Devices and the permanent implantation of the left atrial appendage occlusion device, the left atrial appendage (LAA) occlusion device underwent a comprehensive biological evaluation to determine potential local and systemic risks to biological safety as a result of continuous contact with circulated blood. By utilizing an appropriate combination of *in vitro* and *in vivo* studies, the results from the selected biocompatibility test battery indicate that extracts from the device do not result in cytotoxicity in mammalian cell culture systems or in life forms that show dermal irritation or contact hypersensitivity, which implies that there will not be any significant tissue reactions due to any of the device materials. In addition, the device showed no incidence of clinically significant hemolysis through hemocompatibility studies, which affirms positive interactions between the device surfaces and blood elements. There was also no evidence of pyrogenicity or acute systemic toxicity after use of the extracts of the device in systemic effect evaluation assays, thus establishing that the use of the device in simulated physiological testing did not produce any harmful substances that could be leached. In summary, all test results demonstrate that the materials used to manufacture the device and the means of manufacturing the device render the LAA occlusion device to be a stable implant and a product manufactured from materials that exhibit biological compatibility. The study is in compliance with the acceptance criteria established by the ISO 10993 Series and the LAA occlusion device biological risk assessment as part of the overall risk management strategy.

Compliance with ethical standards

Disclosure of conflict of interest

All the authors are an employee of Meril Medical Innovations Private Limited, Vapi, Gujarat – India and declare no competing interests related to this study.

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