"The value of knowledge is qualified and quantified when knowledge is put into action."

Materials and Methods
4. MATERIALS AND METHODS

The sirolimus eluting coronary stent (Supralimus-Core®) comprised of two regulated components: A device (Co-Cr Alloy coronary stent system) and a drug product (a formulation of Sirolimus contained in biodegradable polymer coating).

The overall studies flow chart of sirolimus eluting coronary stent system (Supralimus-Core®) summarized in Figure 4.1.

Figure 4.1: Overall studies flow chart

4.1 PRECLINICAL EVALUATION

Preclinical studies of Sirolimus eluting stent were conducted at Sree Chitra Thirunal Institute for Medical Sciences and Technology (Biomedical Wing, Kerala, India), RCC (Zelgliweg 1, CH-4452 Itingen/ Switzerland) and SGS Life sciences (Chennai, India).
India). All study protocols used for conducting animal experiments has been reviewed and approved by the Animal Ethics Committee and followed CPCSEA (Committee for the purpose of control and supervision on experiments on animals) guidelines.

4.1.1 BIOCOMPATIBILITY & SAFETY STUDIES

4.1.1.1 In vitro cytotoxicity

4.1.1.1.1 Direct contact method

An in vitro cytotoxicity test using Direct contact method was performed using Supralimus-Core™ stent as per ISO 10993-5. Test sample (Supralimus-Core™ Stent), negative control (Ultra high molecular weight poly ethylene) and positive control (Copper) in triplicate were placed on confluent monolayer of L-929 mouse fibroblast cells. After incubation of cells with test samples at 37±2°C for 24±1 hour, cell culture was examined microscopically for cellular response around test samples. Cellular responses were scored as 0, 1, 2 and 3 accordingly to non-cytotoxic, mildly cytotoxic, moderately cytotoxic and severely cytotoxic.

4.1.1.1.2 Extracts method

As in vitro cytotoxicity test using Test on Extract method was performed using 100 µl of Extracts of test sample (Supralimus-Core™ stent) as per ISO 10993-5, negative control (Ultra high molecular weight poly ethylene) and positive control (Dilute phenol) in triplicate were placed on confluent monolayer of L-929 mouse fibroblast cells. After incubation of cells with test samples at 37±2°C for 24±1 hour, cell culture was examined microscopically for cellular response. Cellular responses were scored as 0, 1, 2 and 3 accordingly to non-cytotoxic, mildly cytotoxic, moderately cytotoxic and severely cytotoxic.

4.1.1.1.3 Indirect agar diffusion method

As in vitro cytotoxicity test using indirect contact method was performed using test samples (Supralimus-Core™ stent) as per ISO 10993-5, negative controls (Ultra high molecular weight poly ethylene) and positive controls (Copper) in triplicate were placed on agar overlaid confluent monolayer of L-929 mouse fibroblast cells. After incubation of
cells with test samples at 37±2°C for 24±1 hour, cell culture was examined microscopically for cellular response around test samples. Cellular responses were scored as 0, 1, 2 and 3 accordingly to non-cytotoxic, mildly cytotoxic, moderately cytotoxic and severely cytotoxic.

4.1.1.2 In vitro cytocompatibility

4.1.1.2.1 Endothelial cell (EC) attachment & proliferation

Human umbilical vein endothelial cells (HUVEC) were used to quantify initial cell attachment. EC in log phase were labelled with tritiated thymidine (³H-thymidine) (American Radio Chemicals, USA) by placing 20 μCi ³H-thymidine/25 cm² flask for 72 hours at 37°C. These cells were harvested and 25000 cells were used for seeding on each stent. After 2 hours of seeding, the unattached cells were removed, and DNA was extracted for ³H counting. An aliquot containing 5 x 10⁴ cells was also processed for ³H counting and was detected using a liquid scintillation counter (TRIATHLER Multi-label tester, HIDEK, Finland). The number of cells attached was calculated based on the counts of unattached cells and the count of known number of ³H-thymidine loaded cells.

To quantify cell proliferation, after removing the unattached cells after 2 hours of seeding, attached cells were fed with complete MCDB 131 medium containing 2 μCi ml⁻¹ ³H-thymidine. After 24 hours and 48 hours medium was changed with fresh medium containing ³H-thymidine and at 72 hours cells were harvested separately from each stent and assayed for ³H, as described above.

A. Endothelial cell cytotoxicity

The endothelial cell suspension with 2 x 10⁴ cells cm⁻² in complete MCDB 131 medium was seeded on the sirolimus eluting stents. The stents seeded with EC were removed from culture medium at 24, 48 and 72 hours after seeding (separate stent for each period) assayed for apoptosis/necrosis using Vybrant Apoptosis Assay Kit (Molecular probes Inc OR. Cat No. V13242). After staining, the cells were washed thoroughly with PBS and were viewed using an inverted fluorescence microscope (Leica, DM IRB) to distinguish live cells with no fluorescence, apoptotic cells with green fluorescence and necrotic cells with red fluorescence. Percentage of apoptosis cells were calculated from
total cells in 10 fields counted under phase contrast microscopy at 10x magnification, and the number of fluorescent cells in the respective fields were viewed using a fluorescence filter.

4.1.1.2.2 Smooth muscle cell (SMC) attachment and proliferation

The smooth muscle cell derived from human peripheral blood stem cells were used (Sreerekha et al 2006). To quantify initial cell attachment, SMC in log phase were labelled with tritiated thymidine ($^3$H-thymidine) (American Radio Chemicals, USA) by placing 20 μCi $^3$H-thymidine/25 cm² flask for 72 hours at 37° C. These cells were harvested and 25000 cells were used for seeding on each stent. After 2 hours of seeding, the unattached cells were removed, and DNA was extracted for $^3$H counting. An aliquot containing 5 x $10^4$ cells was also processed for $^3$H counting and was detected using a liquid scintillation counter (TRIATHLER Multi-label tester, HIDEX, Finland). The number of cells attached was calculated based on the counts of unattached cells and the count of known number of $^3$H-thymidine loaded cells.

To quantify cell proliferation, after removing the unattached cells after 2 hours of seeding, attached cells were fed with complete MCDB 131 medium containing 2 μCi ml⁻¹ $^3$H-thymidine. After 24 hours and 48 hours medium was changed with fresh medium containing $^3$H-thymidine and at 72 hours cells were harvested separately from each stent and assayed for $^3$H, as described above.

A. Smooth muscle cell cytotoxicity

The smooth muscle cell suspension with 2 x $10^4$ cells cm⁻² in complete MCDB 131 medium was seeded on the sirolimus eluting stents. The stents seeded with SMC were removed from culture medium at 24, 48 and 72 hours after seeding (separate stent for each period) assayed for apoptosis/necrosis using Vybrant Apoptosis Assay Kit (Molecular probes Inc OR. Cat No. V13242). After staining, the cells were washed thoroughly with PBS and were viewed using an inverted fluorescence microscope (Leica, DM IRB) to distinguish live cells with no fluorescence, apoptotic cells with green fluorescence and necrotic cells with red fluorescence. Percentage of apoptosis cells were calculated from total cells in 10 fields counted under phase contrast microscopy at 10x magnification, and
the number of fluorescent cells in the respective fields were viewed using a fluorescence filter (Leica, DM IRB).

4.1.1.3 In vitro hemocompatibility

4.1.1.3.1 Interaction with whole blood

Interaction of Supralimus-Core® stent with whole blood was done under agitation to analyze the effect of stent on haematology parameters. Blood from human volunteers was collected into the anticoagulant (citrate phosphate dextrose solution). Replicates of Supralimus-Core® stents were placed in separate polystyrene vials and were immersed in phosphate buffered saline for 5 min before they were exposed to blood. To each vial that contains the Supralimus-Core® stents, blood was added and an initial sample was collected immediately for analysis. The remaining blood was exposed to the respective stent for 30 min under agitation at 75±5 rpm using an Environ shaker thermo stated at 35±2°C. The blood sample and the stent were then analyzed. Six empty polystyrene vials were exposed with blood as reference.

A. Consumption of platelets and leukocytes by cell counts

The count reduction in blood was estimated by detecting the counts in initial and 30 minutes samples collected from above step, using Hematology Analyzer Sysmex K 4500. The equipment calibration was verified using traceable control.

B. Plasma coagulation (Fibrinogen and partial thromboplastin time assay)

The initial and 30 minutes samples collected (separated as in step A), were centrifuged at 4000 rpm for 15 minutes and platelet poor plasma was aspirated. Fibrinogen was measured in each PPP (Platelet Poor Plasma) sample. Partial thromboplastin time in each platelet poor plasma sample was detected using a reagent kit obtained from Diagnostica Stago (France) on Start 4, coagulation analyzer.

C. Percentage hemolysis

The total haemoglobin in the initial samples was measured using automatic haematology analyzer (Sysmex K 4500). The free haemoglobin liberated in to plasma (separated as in step B) after 30 minutes exposure was measured in each sample using
Diode Array Spectrophotometer and the percentage haemolysis was calculated using the formula \((\text{Free Hb/Total Hb}) \times 100\).

D. Qualitative analysis of leukocyte adhesion

The stents exposed to blood were rinsed immediately with phosphate buffered saline to remove blood completely, fixed with 1% gluteraldehyde for 1 hour and the cells attached to the stents were identified using Giemsa stain and were analyzed. The stents were cut open and the luminal side was analyzed under light microscope (Leica IMR) for presence of leukocytes. Representative photomicrographs are taken and documented.

4.1.1.3.2 Interaction with platelet rich plasma (PRP)

Interaction of Supralimus-Core\textsuperscript{®} stent with platelet rich plasma was done under dynamic condition to understand the effect of stent on platelets and deposition of platelets to the stents. Blood from human volunteer was collected into the anti coagulant (citrate phosphate dextrose solution). Blood was centrifuged at 2500 rpm for 5 minutes. The platelet rich plasma was collected. The Supralimus-Core\textsuperscript{®} stents that were expanded into the lumen of silicone tube was connected and perfused with phosphate buffered saline for 5 minutes. After draining the PBS, PRP was perfused at a flow rate of 50 ml/min and within 1 minute an initial sample was collected for analysis. After perfusion for 30 minutes, the platelet rich plasma samples were collected for analysis. Empty silicone tubes of the same dimension, into which stents were expanded, were perfused with platelet rich plasma as reference. Stents were rinsed thoroughly and were processed for analysis using Scanning Electron Microscopy.

A. Platelet count reduction

The count reduction was estimated by detecting the counts in platelet rich plasma samples collected from above step, using Hematology Analyzer Sysmex K 4500.

B. Platelet function

The amplitude of platelet aggregation in response to agonists, adenosine diphosphate and collagen were determined in samples from above step as described above.
C. Platelet secretion

The PRP samples collected in step a, were centrifuged and the PPP was collected. The platelet factor 4 (PF4) was analyzed with commercially available ELISA kit (Hyphen Biomed, France). The difference in PF4 between the samples obtained at 1 min and 30 min was estimated to detect platelet activation induced by the stent.

D. Analysis of platelet adhesion by scanning electron microscopy

The stents exposed to platelet rich plasma were rinsed with phosphate buffered saline to remove platelet rich plasma completely, fixed with 2 % gluteraldehyde and dehydrated. The stents were cut open and the luminal side was analyzed for platelet deposition. Before analysis test stents were critical point dried, gold sputter coated and were analyzed under scan electron microscope.

4.1.1.3.3. Interaction with radio labelled platelet rich plasma (125I-PRP) with radioscntigraphy

Interaction of Supralimus-Core® stent with radio labelled platelets was done to quantify the number of platelet adhered to the stent during the exposure period. Blood from human volunteer was collected into the anticoagulant (CPD). Blood was centrifuged to obtain platelet rich plasma at 2500 rpm for 5 minutes. Platelets from PRP was collected, washed and labelled with 1-125. The labelled platelets were re-suspended in the same donor's platelet poor plasma and the count was adjusted to get 2 x 10^8 to 2.5 x 10^8 per ml PRP. The sirolimus eluting stents were exposed to 1-125 labelled platelets for 30 minutes under agitation at 75 ± 5 rpm using an Environ shaker thermo stated at 35 ± 2° C.

A. Radioscintigraphy

The exposed sirolimus eluting stents were rinsed with PBS to remove the PRP completely and fixed with gluteraldehyde. The sirolimus eluting stents were then dried and phosphor imaged to detect the number of platelets deposited on the sirolimus eluting stents. Aliquots of radio labelled platelets with known number of platelets were imaged to make the calibration curve. The intensity of radio images of each sirolimus eluting stents were used for estimation of platelet adhesion on to it based on the calibration curve.
4.1.1.4 Local tolerance

4.1.1.4.1 Intracutaneous (intradermal) reactivity test

The physiological saline (NS) extract of the sirolimus eluting strips/stent was aseptically injected into 5 sites (0.2 ml/site) on the upper left hand side of 2 Albino rabbit (adult, not less than 2 kg). The Physiological saline (control extract) alone was injected into 5 sites on the lower left hand side of the same rabbits. The grading of erythema and oedema of test and control sites of all animals at 24, 48 and 72 hours was recorded.

The requirements of the tests are met if the difference between the test sample mean score and the control mean score is 1.0 or less. The test would have been repeated, if at any observation period the average reaction to the test sample is questionable greater than the average reaction to the control.

Table 4.1: Grading system for intracutaneous (intradermal) reactions

<table>
<thead>
<tr>
<th>Erythema</th>
<th>Score</th>
<th>Oedema</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No erythema</td>
<td>0</td>
<td>No oedema</td>
<td>0</td>
</tr>
<tr>
<td>Very slight erythema (barely perceptible)</td>
<td>1</td>
<td>Very slight oedema (barely perceptible)</td>
<td>1</td>
</tr>
<tr>
<td>Well-defined erythema</td>
<td>2</td>
<td>Well-defined oedema (edges of area well-defined by definite raising)</td>
<td>2</td>
</tr>
<tr>
<td>Moderate erythema</td>
<td>3</td>
<td>Moderate Oedema (raised approximate 1mm)</td>
<td>3</td>
</tr>
<tr>
<td>Severe erythema (beet-redness to eschar formation preventing grading of erythema)</td>
<td>4</td>
<td>Severe oedema (raised more than 1mm and extending beyond exposure area)</td>
<td>4</td>
</tr>
</tbody>
</table>

Total possible score for irritation 8

4.1.1.4.2 Closed patch test for delayed hypersensitivity

Total 15 healthy adult Guinea pigs were selected for the experiment. Prior to each application period, clip the hair on the upper back (dorsal) on either side of the vertebral column of each animal.
A. Induction Phase

The skin was lightly swabbed using 70% alcohol and air dried. A saturated patch of four ply gauze soaked in the test solution (physiological saline extract of stent) was applied topically on the clipped upper back region of 10 animals. Similarly a saturated (Physiological saline alone) patch of four-ply gauze was topically applied as control to the other 5 animals. This was then covered with occlusive dressings. The occlusive dressings and patches were removed after 6 hours. Repeat this procedure on three weeks. The skin reactions were observed for the evidence of any erythema or oedema after removal of dressing.

B. Challenge phase

Fourteen days after the last application (induction period) all the test and control animals were challenged with the test solution. For this, the hair on the animals flank area (untested area) were removed and lightly swabbed with 70% alcohol and topically applied with a saturated patch of test solution (physiological saline extract of stent) and secured with occlusive dressings. Removed dressings and patches were after 6 hours.

Grades of 1 or greater in the test group generally indicate sensitization provided grades of less than 1 are seen on control animals. If grades 1 or greater noted on control animals, then the reactions of test animals that exceed the most severe control reaction are presumed to be due to sensitization. If the response is equivocal, a retest is recommended to confirm the results from the first test.

Table 4.2: Magnusson and Kligman Scale

<table>
<thead>
<tr>
<th>Patch test reaction</th>
<th>Grading Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>No visible change</td>
<td>0</td>
</tr>
<tr>
<td>Discrete or patchy erythema</td>
<td>1</td>
</tr>
<tr>
<td>Moderate and confluent erythema</td>
<td>2</td>
</tr>
<tr>
<td>Intense erythema and swelling</td>
<td>3</td>
</tr>
</tbody>
</table>
4.1.1.5 Pyrogen Test

Healthy, mature Albino rabbits housed individually in an area of uniform temperature and free from disturbances likely to excite them. They were acclimatized for seven days in the same controlled temperature between 22±3°C in a rabbit restrainer. Daily rectal temperature was measured using a thermometer. This study we used only those rabbits whose control temperatures does not vary by more than 1° C from each other, and not used any rabbit having a temperature exceeding 39.8° C.

Selected three rabbits were restrained in a rabbit restrainer under conditions mentioned above. Control rectal temperature of each rabbit was recorded 30 minutes prior to the test using a thermometer. This is the base for the determination of any temperature increase resulting from the injection of a test solution. The stent extract was injected intravenously into the marginal ear vein of each rabbit at a dose of 10 ml/kg body weight. We recorded the rectal temperatures at 30 minutes interval between 1 and 3 hours subsequent to the injection. The rise in temperature from the control temperature was noted at the end of the experimental period. If no rabbit shows an individual rise of 0.5° C or more above its respective control temperature, the stents meets the requirements for absence of pyrogen.

4.1.1.6 Biofunctional Study

The purpose of this study was to evaluate sirolimus eluting stents for in vivo stent occlusion, patency and other associated cardiac events in porcine coronary artery model in comparison to bare metal stent at 26 weeks and polymer coated stent was studied at 4 weeks.

A. Four weeks group

A total of 5 polymer alone stents were deployed in right coronary artery and left anterior descending coronary artery of 3 animals. The stents were deployed 3 to 9% native vessel over-stretch. The stents used belonged to sizes of 2.5x11mm and 3x11mm.

B. Twenty six weeks group
A total of 24 sirolimus eluting stents and bare metal stents were deployed in right, left anterior descending and left circumflex coronary arteries of 11 animals at native vessel over-stretch of 0 to 8.6%. Eight animals survived the duration of the study, yielding 14 sirolimus eluting stents and 5 bare metal stents for further evaluation.

The haematological parameter like complete blood counts, haemoglobin, packed cell volume; bleeding time and clotting time were estimated before implantation and explantation of the stent in the study. The biochemical parameters like BUN and total protein were estimated before implantation and explantation of the stent in the study.

4.1.1.7 Histopathological evaluation

A total number of 7 stents in situ were studied. The segment of carotid artery containing the sirolimus eluting stent was received for Histopathology Laboratory. Each blood vessel was cut into three pieces; a piece containing stent (stent-segment), proximal references segment and a distal reference segment. The stent-segment was embedded in resin and others were embedded in routine paraffin wax. Representative tissue sections from each were stained with Toludine Blue and Harri's Haematoxylin and Eosin. The inflammation, angiogenesis, thrombus or deposition of fibrin/fibrinoid stent, haemorrhage and necrosis parameters were studied in all sections. Lumen patency was observed in all. Each parameter was assessed in the intima, media and adventitia and graded as per the criteria given in table 4.4.

Table 4.3: Basis for grading the nature/severity of lesion

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Basis for grading the nature/severity of lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No lesion</td>
</tr>
<tr>
<td>Inflammation</td>
<td>0</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>0</td>
</tr>
<tr>
<td>Thrombus/fibrin deposits</td>
<td>0</td>
</tr>
<tr>
<td>Haemorrhage</td>
<td>0</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0</td>
</tr>
</tbody>
</table>
4.1.1.8 Genotoxicity (salmonella typhimurium reverse mutation assay)

Salmonella Reverse Mutation Assay was conducted using Histidine auxotrophic strains of Salmonella typhimurium tester strains viz. TA97a, TA 98, TA 100, TA 1535 and TA 102 (Source: Bruce Ames Laboratory, Molecular and Cell Biology, University of California, 401, Barker Hall Berkeley, CA 94720-3202, U.S.A.). The test substance (sirolimus eluting stent) was tested at the concentrations of 61.72, 185.18, 555.55, 1666.67 and 5000µg/plate using Dimethyl sulphoxide as solvent. All the strains were stored in liquid nitrogen (-160° C), in cryocans. Strains were maintained as master plates and checked periodically for viable counts and genotype characteristics. For assay, cultures were grown for 16 hours in nutrient broth at 37° C. The cell density of cultures, 1-3 x 10^9 cells per ml was assessed by cell count. For 7 culture media, total incubation period was 48-72 hours. The Culture medias are a) Minimal glucose agar medium (Vogel-Bonner minimal medium E) b) Soft agar (overlay agar) containing 0.5 mM histidine and biotin c) Minimal glucose agar with biotin d) Minimal glucose agar with histidine and biotin e) Minimal glucose agar with histidine, biotine and ampicillin f) Nutrient agar g) Nutrient broth. The bacterial strains were cultured in nutrient broth. Vogel Bonner minimal medium E was choosen as the selective medium. The top agar contained 0.6% agar, 0.5% NaCl and 0.05 mM histidine-biotin (Maron and Ames, 1983).

For preparation of S-9 mix, male rat species was used. The strain was Sprague Dawley. The age and weight of animal was 8-10 weeks and 175-200 grams respectively. The mixed function oxidase systems in the rat liver were stimulated following an intraperitoneal injection of sodium Phenobarbital (diluted in corn oil) at a dosage of 80 mg/kg/day for five consecutive days. On the 6th day of induction, following an overnight fasting, the rats were killed and livers aseptically removed (Bionetics Laboratory Products, Ong et. al 1980).

The preparation of liver homogenate was carried out with sterile glassware and solutions at 0-4°C. Excised livers were transferred to a beaker containing 0.15M KCl. (3ml KCL:1 gram liver) minced with sterile scissors and homogenized. The homogenate was centrifuged for 10 minutes at 9000 gram and the supernatant divided into small aliquots. These were stored at -160° C in cryocan and tested with mutagen 2-Aminofluorene (2AF)
before use to test the enzymatic activity of S-9 preparation. The total protein per ml of S-9 homogenate was determined by using Lowry’s method. Protein content determined was 4.3 grams/100ml.

Sterility of S-9 preparation was tested by adding 0.5 ml of S-9 to top agar and plating the same on minimal glucose agar incubated at 37° C for 48 hours. Plates examined contained no microbial contamination. The S-9 mix was prepared immediately prior to its use in experimental procedure. The microsomal enzyme reaction mixture contained following components 10 ml (S-9 fraction – 1.00ml, 0.4 M MgCl₂ – 1.65 M KCl salt solution – 0.20 ml, 1.0 M G-6-P - 0.05 ml, 0.1 M NADP - 0.40 ml, 0.2 M Phosphate buffer pH 7.4 - 5.00 ml, Distilled water - 3.35 ml)

The study was conducted without and with metabolic activation (S9 fraction) prepared from sodium Phenobarbital induced rat liver. The solvent control and appropriate positive controls (Methyl methane sulphonate, Sodium azide, 4-Nitroquinolene-N-Oxide for without metabolic activation and 2-Aminofluorene, 2-Aminoanthracene and Danthron for with metabolic activation) were tested simultaneously. Two experiments were carried out using each tester strain with plating in triplicates at each concentration. The test substances were considered to be toxic if there were a decrease in the number of revertants and/or thinning or absence of background lawn.

4.1.2 PHARMACOKINETIC STUDY IN ANIMALS

Total 15 animals (Male New Zealand White Rabbit, age 14-15 weeks with weight of 3032.1-3351.3 g) divided in 5 groups. One sirolimus eluting stent was deployed in each animal. The sirolimus eluting stent was checked by angiographic control.

<table>
<thead>
<tr>
<th>Allocation Groups</th>
<th>Animal No.</th>
<th>Termination After (Day/Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1-3</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>4-6</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>7-9</td>
<td>7</td>
</tr>
<tr>
<td>D</td>
<td>10-12</td>
<td>15</td>
</tr>
<tr>
<td>E</td>
<td>13-15</td>
<td>28</td>
</tr>
</tbody>
</table>

Ashok Thakkar

L.M. College of Pharmacy, Ahmedabad, India
After medication with Pentobarbital (Vetanarcol®) at a dose sufficient to induce appropriate induction of anesthesia (usually 16-32mg Pentobarbital per animal i.v. at a dose volume of 2-4 mL), the level of anesthesia was maintained with Isoflurane (Forene®)/Oxygen via mask. Prior to surgery, the animals were shaved and the incision site was disinfected with standard solution of 10% povidone-iodine (Betadine®). An immediately preoperative 0.15 g Cephazoline (Kefzol®) was injected i.m.

A peri-interventional injection of approx. 100 U Heparin (Liquemine)/Kg body weight in physiological saline solution was performed, as appropriate. All sheets and catheters were rinsed with diluted heparin solution during intervention. After the animals have been narcotized, and inguinal access to the iliac artery has been identified and intravascular access maneuver was performed. An appropriate monorail balloon catheter (approx. 11 mm of length and approx. 3 mm of diameter) was implanted. Appropriate pressure was applied in order to achieve a circumferential expansion and an optimal apposition to the vessel wall. The application instruments were withdrawn, hemostasis was achieved and the wounds were closed.

Postoperative analgesia consisted of subcutaneous injections of a single injection of Buprenorphine (Temgesic®) at a dose of 0.02 mg every 8-12 hours for at least 12 hours after operation. Inhibition of thrombocyte aggregation was performed with lysine-acetylsalicylic acid (Aspegic® corresponding to acetylsalicylic acid 0.07 mg/mL) added to drinking water from the acclimatization until necropsy. Clopidogrel (Plavix® corresponding to 0.01 mg/mL) was added to tap water one day prior to surgery until the end of the postoperative observation period.

Blood samples for hematology and clinical biochemistry were collected from the ear vein from all animals during the acclimatization period and just before necropsy. The animals were fasted for approximately 18 hours before blood sampling (except during acclimatization to reduce the stress induced by the transport) but allowed access to water ad libitum. Blood samples were collected early in the working day to reduce biological variation caused by circadian rhythms.

The venous bloods (approx. 2mL) from the rabbits were collected in heparinised tubes after 1,3,7,15 and 28 days prior necropsy. The full blood was stored at -78° C to -83°
C until its transport on dry ice to determination of sirolimus level in blood. Sirolimus was determined using protein precipitation technique for the determination of Sirolimus level in whole blood of rabbits. Measurements were performed using a LC/MS/MS (liquid chromatography coupled with tandem spectrometric determination) method which was previously developed and the reliability were checked. For this study, Food and Drug Administration (Guidance for industry: bio analytical method validation, May 2001) and International Conference on Harmonization (Topic Q2B: Validation of analytical procedures: Methodology) test guidelines followed.

4.2 CLINICAL EVALUATION

4.2.1 RECOIL STUDY

4.2.1.1 Recoil study in human

4.2.1.1.1 Study population

This was a prospective, single-centre, non-randomized study. A total of 19 patients were treated with sirolimus-eluting coronary stent system implantation for de novo native coronary artery lesions at Shri B.D. Mehta Mahavir Heart Institute, Surat by Investigator. This study was approved by the local ethics committee. Repeat angiography was performed in all patients within 24 hours after the index procedure.

Patients with de novo native coronary artery lesions located in epicardial vessel who qualify for percutaneous coronary intervention were included according to the inclusion and exclusion criteria. In a study patients included were; Age ≥18 years, Eligible for percutaneous coronary intervention, Acceptable candidate for CABG, Clinical evidence of ischemic heart disease and/or a positive territorial functional study, Documented stable angina pectoris or unstable angina pectoris with documented ischemia (Braunwald Class IB-C, IIB-C or IIIB-C), or documented silent ischemia, The target lesion is de novo coronary artery lesion with ≥ 50% and < 100% stenosis in one of the major epicardial territories (LAD, LCX or RCA), A second target lesion in another major epicardial vessel could be treated and this second lesion should fit with the inclusion/exclusion criteria and will receive the same type of stent, The target lesion must be covered by one study stent,
preferably with a margin of 3 mm on each side of the lesion. The target lesion must be \( \leq 34 \) mm in length by visual estimate. The target reference vessel diameter must be \( \geq 2.50 \) mm and \( \leq 4.00 \) mm. Patient or patient’s legal representative has been informed of the nature of the study and agrees to its provisions and has provided written informed consent as notified/approved by the Institutional Review Board/Ethics Committee of the clinical site.

In a study patients excluded were; Female of childbearing potential, Documented left ventricular ejection fraction (LVEF) \( \leq 25\% \). Evidence of an acute Q-wave or non-Q-wave myocardial infarction within 72 hours preceding the index procedure, Known allergies to the following: aspirin, clopidogrel bisulfate, heparin, sirolimus, cobalt chromium, contrast agent (that cannot be adequately pre-mediated), A platelet count \(<100,000\ \text{cells/mm}^3\) or \(>700,000\ \text{cells/mm}^3\) or a WBC \(<3,000\ \text{cells/mm}^3\) or a WBC \(<3,000\ \text{cells/mm}^3\). Acute or chronic renal dysfunction (creatinine \(>2.0\ \text{mg/dl}\) or \(>150\ \text{μmol/L}\)), Target vessel has evidence of thrombus, Target vessel is excessively tortuous which makes it unsuitable for proper stent delivery and deployment, Previous bare metal stenting (less than 1 year) anywhere within the target vessel, Previous drug-eluting stenting anywhere within any epicardial vessel, The target lesion requires treatment with a stent other than PTCA prior to stent placement (e.g. but not limited to, directional coronary atherectomy, excimer laser, rotational atherectomy, etc.), Significant (>50%) stenosis proximal or distal to the target lesion that might require revascularization or impede run-off, Heavily calcified lesion and/or calcified lesion which cannot be successfully predilated, Target lesion is located in or supplied by an arterial or venous bypass graft, Ostial target lesion, Patient is currently participating in an investigational drug or device study, including its follow-up period. Within 30 days prior to procedure, patient has undergone a previous coronary interventional procedure of any kind. Within 48 days post-procedure, patient requires planned interventional treatment of any non-target vessel. Planned intervention of the target vessel after the index procedure is not allowed, CVA within previous 6 months, Unprotected Left Main (LM) coronary artery disease (stenosis >50%), In the investigator’s opinion, patient has a co-morbid condition(s) that could limit the patient’s ability to participate in the study, compliance with follow-up requirements or impact the scientific integrity of the study, Planned surgery within 48 days.
after the index procedure, Life expectancy less than 1 year, Any contraindication to blood sampling.

4.2.1.1.2 Treatment

Sirolimus eluting coronary stent system is indicated for use in-patients with symptomatic ischemic heart disease due to coronary artery lesions with a reference vessel diameter ranging from 2.5 mm to 4.0 mm and is intended to improve coronary luminal diameter.

4.2.1.1.3 Adjunctive Medications

All patients were on Aspirin in a dose of 75-150mg at least 24 hours prior to the procedure. A loading dose of 300 mg of clopidogrel was given 24 hours prior to procedure or 600 mg on the day of the procedure was given to patients before procedure. Clopidogrel was prescribed minimally for 12 months or longer as per physician’s decision.

4.2.1.1.4 Study endpoint

The primary endpoint was immediate (post-procedure) and early (within 24 hours) recoil following sirolimus eluting coronary stent system implantation for de novo native coronary artery lesions.

A total of 19 patients, who underwent elective stent implantation for single de novo native coronary artery lesions, were enrolled: all patients treated with the sirolimus-eluting cobalt chromium coronary stent system (Supralimus-Core®). Acute absolute recoil, assessed by quantitative coronary angiography, was defined as the difference between mean diameter of the last inflated balloon at the highest pressure (X) and mean lumen diameter of the stent immediately after the last balloon deflation (Y). Acute percent recoil was defined as (X - Y)/X and expressed as a percentage. The recoil also measured in 24 hours of stent implantation (mean lumen diameter post – mean lumen diameter follow-up).

4.2.1.2 In vitro recoil study

The dilation behaviours of four coronary stents (No. 46 to No. 49) at the diameter of 3.5 mm and after recoil were examined at EndoLab® Mechanical Engineering GmbH (Seb.-Tiefenthaler Str. 13, D-83101 Thansau / Rosenheim). The pressure in the balloon
catheter was increased to the nominal pressure level of 6 bars and the diameter was determined. Additionally the recoil of the stent was determined. The outer diameter of the stent/catheter system was determined by a diameter scanning stent as shown in figure 4.3 and 4.4. The balloon catheter with the stent was mounted on a linear stepper motor. The balloon-stent system was moved in an infrared diameter scanning stent to scan the diameter along the axis of the stent. After scanning of the 6 bar pressure level (stent diameter of 3.5 mm) the recoil diameter without internal pressure in the balloon has been determined. This procedure has been performed with balloon catheters of a nominal diameter of 3.5 mm. The recoil of each stent was determined at five different points of the scanned diameter curve by using below equation.

\[
R = \frac{d(p_{\text{bar}}) - d(p_{\text{bar}})}{d(p_{\text{bar}})} \cdot 100\%
\]

The Pressure sensor for dilation was Burster 8201-5020-P013A4 (20 bar) No.: 137 while IR extensiometer CCD camera GK29 (MT Lang) No.: 14. The Stepper Motor was Isel A2411-9212-A2 No.: 139. The PC-AD system of National Instruments AT-MIO-16XE-50 No.: 103 and Catheter was Balloon Ø3.5 mm (nominal pressure 6 bar).

**Figure 4.2:** Setup for the stent dilation
4.2.2 PHARMACOKINETIC STUDY IN HUMAN

4.2.2.1 Study population

This was a multi-centric, interventional, non-randomized, open label, uncontrolled, single group assignment, Pharmacokinetics study. Approximately 20 patients were enrolled in the study at 4 centres. Among 20 patients, 8 patients enrolled at Shree B.D. Mehta Mahavir Heart Institute, Surat, 5 patients enrolled at Baroda Heart Institute & Research Centre, Vadodara, 4 patients enrolled at Life Care Institute of Medical Science and Research, Ahmedabad and 3 patients enrolled at Bankers Heart Institute, Vadodara. This study was approved by the respective local ethics committee. Patients were followed for 48 days post-procedure. Patients with de novo native coronary artery lesions located in epicardial vessel who qualify for percutaneous coronary intervention were included according to the inclusion and exclusion criteria.

In a study patients included were; Age ≥18 years, Eligible for PCI, Acceptable candidate for CABG, Clinical evidence of ischemic heart disease and/or a positive territorial functional study. Documented stable angina pectoris or unstable angina pectoris with documented ischemia or documented silent ischemia, The target lesion is a single de
novo coronary artery lesion with ≥ 50% and < 100% stenosis in one of the major epicardial territories, a second target lesion in another major epicardial vessel could be treated and this second lesion should fit with the inclusion/exclusion criteria and will receive the same type of stent. The target lesion must be covered by one study stent, preferably with a margin of 3 mm on each side of the lesion. The target lesion must be ≤ 34 mm in length by visual estimate, The target reference vessel diameter must be ≥ 2.5 mm and ≤ 3.5 mm, Patient or patient’s legal representative has been informed of the nature of the study and agrees to its provisions and has provided written informed consent as notified / approved by the Institutional Review Board/Ethics Committee of the clinical site.

In a study patients excluded were; Female of childbearing potential, Documented LVEF ≤ 25%, Evidence of an acute Q-wave or non-Q-wave myocardial infarction within 72 hours preceding the index procedure. Known allergies to the following: aspirin, clopidogrel bisulfate, heparin, sirolimus, cobalt chromium, contrast agent (that cannot be adequately pre-medicated), A platelet count <100,000 cells/mm³ or >700,000 cells/mm³ or a WBC <3,000 cells/mm³, Acute or chronic renal dysfunction (creatinine >2.0 mg/dl or >150 μmol/L), Target vessel has evidence of thrombus, Target vessel is excessively tortuous which makes it unsuitable for proper stent delivery and deployment, Previous bare metal stenting (less than 1 year) anywhere within the target vessel, Previous drug-eluting stenting anywhere within any epicardial vessel, The target lesion requires treatment with a device other than PTCA prior to stent placement (e.g. but not limited to, directional coronary atherectomy, excimer laser, rotational atherectomy, etc.), Significant (>50%) stenosis proximal or distal to the target lesion that might require revascularization or impede run-off, Heavily calcified lesion and/or calcified lesion which cannot be successfully pre-dilated, Target lesion is located in or supplied by an arterial or venous bypass graft, ostial target lesion, Patient is currently participating in an investigational drug or device study, including its follow-up period. Within 30 days prior to procedure, patient has undergone a previous coronary interventional procedure of any kind. Within 48 days post-procedure, patient requires planned interventional treatment of any non-target vessel. Planned intervention of the target vessel after the index procedure is not allowed, CVA within previous 6 months, Unprotected LM coronary artery disease (stenosis >50%), In the
investigator’s opinion, patient has a co-morbid condition(s) that could limit the patient’s ability to participate in the study, compliance with follow-up requirements or impact the scientific integrity of the study, Planned surgery within 48 days after the index procedure, Life expectancy less than 1 year, Any contraindication to blood sampling.

4.2.2.2 Treatment

Sirolimus eluting coronary stent system is indicated for use in-patients with symptomatic ischemic heart disease due to coronary artery lesions with a reference vessel diameter ranging from 2.5 mm to 3.5 mm and is intended to improve coronary luminal diameter.

Table 4.5: Stent diameter, length and corresponding dose

<table>
<thead>
<tr>
<th>Stent length (mm)</th>
<th>Stent diameter (mm)</th>
<th>Sirolimus dose (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>2.5 – 3.5</td>
<td>38</td>
</tr>
<tr>
<td>12</td>
<td>2.5 – 3.5</td>
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</tr>
<tr>
<td>16</td>
<td>2.5 – 3.5</td>
<td>77</td>
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<td>20</td>
<td>2.5 – 3.5</td>
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</tr>
<tr>
<td>24</td>
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<td>153</td>
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<td>36</td>
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</tr>
<tr>
<td>40</td>
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<td>192</td>
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</tbody>
</table>

4.2.2.3 Adjunctive Medications

All patients were on Aspirin in a dose of 75-150mg at least 24 hours prior to the procedure. A loading dose of 300 mg of clopidogrel was given 24 hours prior to procedure or 600 mg on the day of the procedure was given to patients before procedure. During the procedure initial dose of 70-100 IU/kg bolus of heparin was given to the patient. Additional heparin was used if necessary during procedure to achieve activated coagulation time > 250 seconds. Administration of GP IIb/IIIa inhibitor was left to the investigator’s discretion. Angiographic success was defined as ≤ 20% stenosis by visual estimation.
4.2.2.4 Visit specific blood sampling schedule

In each patient, 14 venous blood samples were drawn from before implantation of the first stent (baseline, 0-minutes time point). To follow the early release in time, we scheduled the samples at 10, 30 and 60 minutes after stent placement. Thereafter, blood samples were taken at regular intervals 2, 4, 6, 12, 24, 72 (day 3), 168 (day 7), and 336 (day 14), 672 (day 28) and 1152 (day 48) hours to follow the build-up in blood up to a maximum and the subsequent distribution and elimination from the blood.

4.2.3 FIRST-IN-MAN (FIM) STUDY

4.2.3.1 Study population

This study (MAXIMUS study) was a single-centre, prospective and non-randomized. This study conducted at Max DDHV Institute, New Delhi by Investigator in accordance with the International Conference on Harmonization guidelines Good Clinical Practices, Declaration of Helsinki, and Medical Ethics Committee requirements. This study was approved by the local ethics committee. FIM study which included clinical follow-up data was collected at 1, 8 and 12 months after the procedure. The study included 105 patients with de novo native coronary artery lesions including multi-vessel disease treated with sirolimus eluting stent. Repeat angiography was performed 8 months post stent implantation and analyzed by independent core laboratory.

Patients included in the study were more than 18 years of age, with symptomatic ischemic heart disease with de novo stenotic coronary lesion with reference vessel diameter of ≥ 2.5 and ≤ 3.5mm. Patient or patient’s legal representative has been informed of the nature of the study and agrees to its provisions and has provided written informed consent as notified/approved by the Ethics Committee of the clinical site.

Patients were excluded if any of the following conditions were present; Women of childbearing potential, Impaired renal function (creatinine > 2.0 mg/dl or 177 µmol/l), Any patient who has a platelet count < 100,000 cells/mm$^3$ or > 700,000 cells/mm$^3$, a WBC of < 3,000 cells/mm$^3$, or documented or suspected liver disease (including laboratory evidence of hepatitis), Recipient of heart transplant; Restenotic or lesion in graft, Patient with a life expectancy less than 12 months, Known allergies to aspirin, clopidogrel
bisulphate, heparin or cobalt chromium; Currently participating in an investigational drug or another device study, or subject to inclusion in another investigational drug or another device study during follow-up. Unprotected left main coronary artery disease with ≥50% stenosis, Angiographic evidence of thrombus (thrombus larger than half the diameter of the vessel and/or requiring other interventions such as angiojet, exciser, thrombolysis, etc.), Ejection fraction ≤30%.

4.2.3.2 Treatment

Sirolimus eluting coronary stent system is indicated for use in-patients with symptomatic ischemic heart disease due to coronary artery lesions with a reference vessel diameter ranging from 2.5 mm to 3.5 mm and is intended to improve coronary luminal diameter.

<table>
<thead>
<tr>
<th>Event</th>
<th>Screening</th>
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<th>8 Month</th>
<th>12 Month</th>
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<td>X²</td>
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- 1 Month follow up Time Window ± 7 days
- 8 Month follow up Time Window ± 30 days
- 12 Month follow up Time Window ± 45 days
  1 Within 72 hours prior to procedure
  2 Within 24 hours post-procedure or at discharge
  3 Within 24 hours prior to procedure
  4 Within 6 to 8 hours post-procedure
  5 24 hours post-procedure or at discharge
4.2.3.3 Adjunctive Medications

All patients were on Aspirin in a dose of 75-150mg at least 24 hours prior to the procedure. A loading dose of 300 mg of clopidogrel was given 24 hours prior to procedure or 600 mg on the day of the procedure was given to patients before procedure. During the procedure initial dose of 70-100 IU/kg bolus of heparin was given to the patient. Additional heparin was used if necessary during procedure to achieve activated coagulation time > 250 seconds. Administration of GP IIb/IIIa inhibitor was left to the investigator's discretion. Angiographic success was defined as ≤ 20% stenosis by visual estimation.

4.2.3.4 Study Endpoints and Definitions

MACE was defined as the primary safety endpoint which was defined as the incidence of cardiac death, myocardial infarction, emergent cardiac surgery and clinically justified TLR at 30 days following index procedure. MI was defined as Q-wave MI (development of new pathological Q waves in 2 or more leads with CK-MB levels elevated above normal) or non-Q-wave MI (elevation of CK levels to 2 times upper normal limit with CK-MB levels elevated above normal). The primary efficacy endpoint was in-stent binary restenosis rate at 8 months which determined by offline QCA. Binary angiographic restenosis was defined as a DS ≥ 50% at follow-up angiography.

Secondary efficacy endpoints were assessed as acute gain, post procedure MLD, post procedure % diameter stenosis and late loss at follow-up, clinically justified TLR, angiographic and procedural success. Late loss was defined as the difference between MLD post-procedure and MLD at follow-up. TLR was clinically justified if diameter stenosis was ≥ 50% (by off-line QCA) or if the patient exhibited one of the following symptoms: (1) recurrent angina pectoris, (2) ischemia at rest or during exercise (3) Abnormal results of any invasive functional diagnostic test (e.g. Doppler flow velocity reserve, fractional flow reserve). Secondary safety endpoint was defined as device related SAEs and angiographic sub acute and late stent thrombosis up to 12 months (Standard definition of stent thrombosis using standard ARC criteria used). The stent thrombosis is defined as acute if it occurred between 0 and 24 hours, subacute between 25 hours and 30
days, late between 31 days and 1 year, and very late beyond 1 year after stent implantation. With respect to probability, stent thrombosis is defined as definite, probable, or possible.