J Mater Sci: Mater Med (2011) 22:1521–1528
DOI 10.1007/s10856-011-4335-2

A novel in vitro model for preclinical testing of the hemocompatibility of intravascular stents according to ISO 10993-4

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Received: 23 February 2011 / Accepted: 27 April 2011 / Published online: 21 May 2011

Abstract Subacute stent thrombosis, caused by undesired interactions between blood and the stent surface, is a major concern in the first few weeks following coronary artery stent implantation. The aim of this study was to establish a novel in vitro model for hemocompatibility testing of coronary artery stents according to ISO 10993-4. The model consists of a modified Chandler-Loop design with closed heparin-coated PVC Loops and a thermostated water bath. The tests were performed with anticoagulated human whole blood. After incubation in the loop, blood was analyzed for coagulation and inflammatory activation markers (TAT, β-TG, sP-selectin, SC5b-9 and PMN-elastase). Three different stent types with varying thrombogenicity were tested; statistically significant differences were found between the three stent types in measures of coagulation and platelet activation. The new Chandler-Loop model can be used as an alternative to animal and current in vitro models, especially for the determination of early events after stent implantation.

1 Introduction

During the last decade percutaneous coronary intervention became a common technique worldwide for the treatment of coronary artery stenosis. Since the implantation of the first intravascular stent during the mid 1980s [1], stent thrombosis has been a concern limiting the long-term success of coronary stenting. Subacute stent thrombosis during the first 2 weeks after implantation is mainly caused by thrombus formation through undesired interactions between blood and the stent surface [2, 3]. Adequate medication after stenting, consisting of individually adapted antiplatelet and anticoagulation regimens, is of major importance to avoid these complications [4–8]. In addition to pharmacological prevention methods, minimizing the thrombogenicity of the stent surface is also essential to ensure long-term undisturbed circulation. Improvements to the materials and design of vascular devices, including stent surfaces [9, 10], have been developed to reduce thrombogenicity. However, hemocompatibility of new stent materials should be carefully evaluated prior to clinical application. In addition, since inflammation can stimulate thrombotic events and smooth muscle cell proliferation, leading to stent restenosis [11], the optimal stent should also cause minimal inflammatory response. In vitro and in vivo studies are two different ways to evaluate the biologic response to different types of vascular stents in human coronary arteries. However, the inflammatory and thrombotic response evaluated in animal models is not completely comparable with human blood conditions, due to species differences in blood components, cell populations, and reactions to antiplatelet medications [2]. This study established a new Chandler-Loop-based stent test model which uses fresh human whole blood with arbitrary medication. The new model is therefore more specific for early stage reactions after clinical implantation than an animal model. Using this new model it is possible to incubate different stent types, allowing a negative control with blood from the same donor. The objective of this study was to assess the feasibility of the Chandler-Loop

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model for hemocompatibility testing using three deliberately selected stents that were expected to have different levels thrombogenicity.

2 Materials and methods

2.1 Chandler-Loop

Experiments were performed using an in vitro closed loop model (modified Chandler-Loop) [12]. The modified Chandler-Loop system consists of a thermostated water bath (37°C) and a rotating unit with attached polyvinyl chloride (PVC) loops (Fig. 1). The PVC loops were coated with covalently bonded heparin (CBAS, Carmeda bioactive surface, Medtronic Anaheim, CA, USA) to minimize background activation. The stents were placed in the loop and expanded to 4.2 mm diameter with an inflation pressure of <12 bar. Each loop contained two stents of the same type. The loops were filled with 9 ml blood from one donor and then closed into circuits (tubing length 50 cm, ID 4.3 mm) with a short piece of silicone tubing outside the tubings. The tubing loops were rotated vertically at 30 rpm in the water bath (37°C). After 120 min of circulation the blood was collected in appropriate syringes.

2.2 Stent types

Three different stent types were compared: (1) a copper-coated Liberté™ Stent PVD stent (Boston Scientific Corporation, Natick, MA, USA) ("lib cop"), chosen as a positive control because of the high thrombogenicity of copper [13, 14]; (2) a Parlyne C-coated Liberté™ Stent (Boston Scientific Corporation) ("lib par"), chosen exemplary for good hemocompatibility because of the coating with the presumably low thrombogenic Parlyne C [15]; and (3) the commercially available uncoated bare metal Liberté™ Stent (Boston Scientific Corporation). The "lib cop" and "lip par" stents are not designed for clinical use. All three stent types have the same architecture and length and vary only in stent surface properties.

2.3 Blood sampling

Blood was collected from non-medicated, healthy male volunteers ($n = 8$) by venipuncture with 1.4 mm Ø butterfly cannula from a large antecubital vein into sterile and pre-anticoagulated containers. The blood was anticoagulated with 1.2 IU/ml sodium heparin 25000 (Ratiopharm GmbH, Ulm, Germany), to avoid excessive coagulation activation. The experimental results from the "lib par" stent are only based on five donors due to loss of data.

2.4 Sample preparation

For baseline measurement ("t0"), a blood sample (12 ml) was taken from each donor without loop contact. A total volume of approximately 50 ml of blood from one single donor was aliquoted into five samples each containing 9 ml of blood for circulation in the Chandler-Loop model. The first sample was left without a stent, for measuring the background activation caused by the bare loop without any stent. This sample served as a negative control ("ctrl").

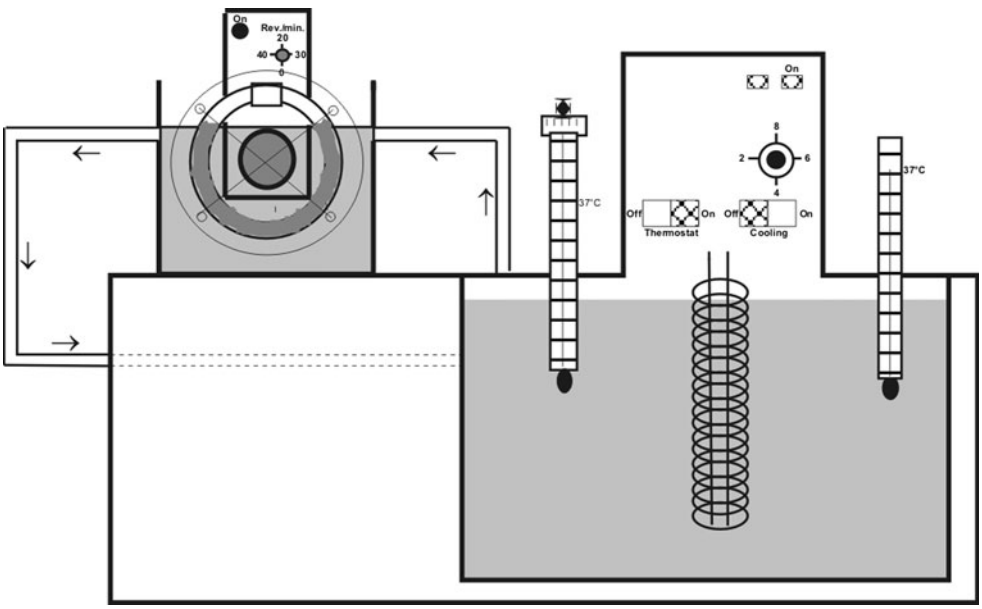

Fig. 1 Modified Chandler-Loop system. The stents were expanded in the tubes which were carefully filled with freshly drawn human whole blood. Then the loops were closed to circuits and placed on the rotating unit

2.5 Blood sampling

After circulating 120 min in the Chandler-Loop model, blood was collected in adequate syringes containing 1.6 mg EDTA/ml blood (EDTA = ethylene diamine tetra acetic acid, 2,7 ml K3E S-Monovette, Sarstedt, Nümbrecht-Rommelsdorf, Germany) for cell counting, sP-selectin and complement (SC5b-9) measurements, 1.0 ml of 0.106 mol/l citrate solution (10.0 ml 9NC S-Monovette, Sarstedt, Nümbrecht-Rommelsdorf, Germany) for thrombin-antithrombin-complex (TAT) measurement or 4,5 ml CTAD-Vacutainer (450 µl of 0,109 M, CTAD = citrate, theophylline, adenosine dipyridamole solution, REF 367599, Becton–Dickinson GmbH, Heidelberg, Germany) for evaluation of β-TG levels.

2.6 Hemocompatibility tests

Blood compatibility tests were performed according to ISO 10993-4 [16, 17], including measures of thrombogenicity, activation of coagulation, number of platelets and platelet activation, hematology, and inflammatory response containing complement activation and secretion of poly-morpho-nuclear-elastase (PMN-elasase) out of neutrophils.

2.7 Analyses of activation markers

The samples were centrifuged immediately, plasma of the blood samples were then aliquoted in 200 µl samples and shock frozen in liquid nitrogen with subsequent storage at −80°C for further investigations. Changes in markers of coagulation and complement activation as well as blood cell release factors were measured by commercially available ELISA kits. Heparin was measured by chromogenic substrate assays (Coatest heparin, Chromogenix, Milano, Italy). Samples were analysed for β-thromboglobulin (Asserachrom β-TG, Diagnostica Stago, Asnieres, France) and sP-selectin (R&D Systems Inc, Minneapolis, MN, USA) as platelet activation markers, and thrombin-antithrombin-III complex (Enzygnost TAT micro, Dade Behring, Schwalbach Germany) to evaluate coagulation. Leukocyte and complement activation were detected by measurements of PMN-elastase (Milenia PMN-Elastase, Milenia Biotec GmbH, Bad Nauheim, Germany) and SC5b-9 (Quidel Corp., San Diego, CA, USA).

2.8 Blood cell count

Cell count was measured in EDTA-blood immediately after sampling using a fully automated cell counter system (micros 60 ABX Hematology, Montpellier, France).

2.9 Scanning electron microscopy (SEM)

After circulation in the loop, the stents were incubated overnight in 2% glutaraldehyde (Serva, Heidelberg, Germany) containing PBS (phosphate buffered saline, Invitrogen Gibco, Karlsruhe, Germany) solution and subsequently rinsed in pure PBS. The remaining water was then removed from the samples using 40–100% of ethanol (Merck, Darmstadt, Germany) in ascending concentrations. Finally all samples were critical point dried (CPD) sputtered with gold palladium and afterwards analysed with SEM (Cambridge Instruments, Cambridge UK, type 250 MK2).

2.10 Statistics

The results are expressed as mean (M) ± standard deviation (SD). Differences between the groups were calculated by analysis of variance (ANOVA). Statistical analysis was performed using the software BIAS for Windows^{TM} Version 9.06 (Epsilon Verlag, Frankfurt, Germany). Data were tested for normal distribution by Kolmogorow–Smirnow test. Homogeneity of variances was tested by Bartlett's test and multiple comparison with Scheffé's method. Differences between different groups were calculated by univariate ANOVA. We considered values of $P < 0.05$ as being significant, $P < 0.01$ and $P < 0.001$ were considered as being highly significant. Values of $P \geq 0.05$ were considered as being not significant (n.s.).

3 Results

3.1 Cell count

After 120 min recirculation in the Chandler-Loop model, the number of blood cells was counted. When blood comes in contact with artificial surfaces, platelet activation and subsequent adhesion takes place. A drop in platelet count is mainly caused by sticking of the platelets to the surface and not by destruction. Platelet counts, as well as red and white blood cell counts were not significantly different among test groups (Fig. 2).

3.2 Activation of the coagulation system

Compared to the control blood sample taken before filling the loops ("t0"; 3.33 µg/l ± 1.5) levels of TAT were significantly elevated in blood from all loops (Fig. 3). The "lib par" stent demonstrated coagulation activation of 7.48 µg/l ± 2.09 slightly higher than the control tube without stent ("ctrl"), which showed TAT values of 6.70 µg/l ± 2.22. In all experiments the highest amount of TAT formation was detected in the copper coated stent (lip cop)

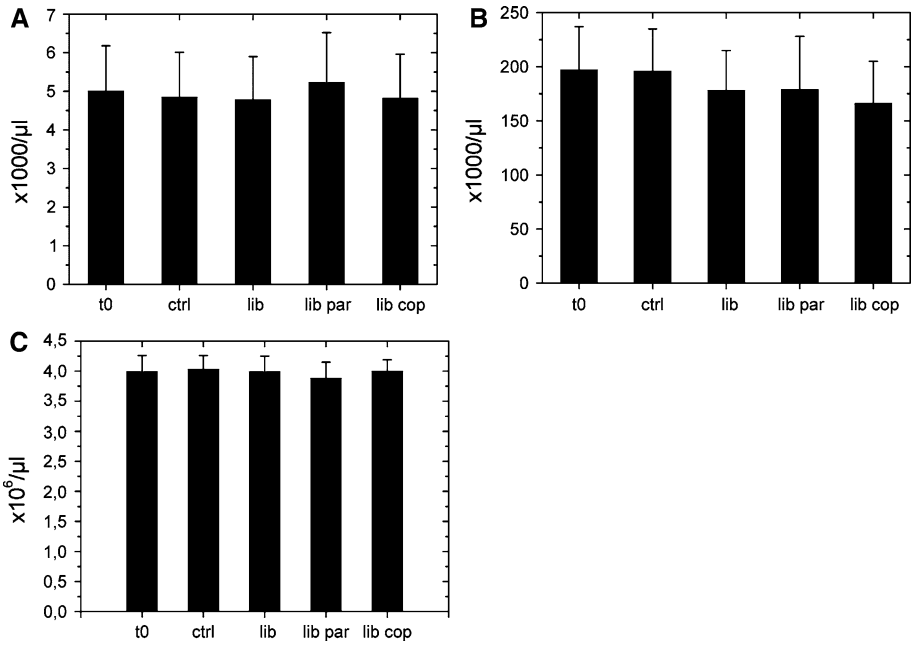

Fig. 2 Number of white blood cells (**a**), platelets (**b**) and red blood cells (**c**) before ("t0") and after 120 min of circulation in the loop without a stent ("ctrl") and with three different stents ("lib"), ("lip par") and ("lib cop"). Values are expressed as mean ± SD

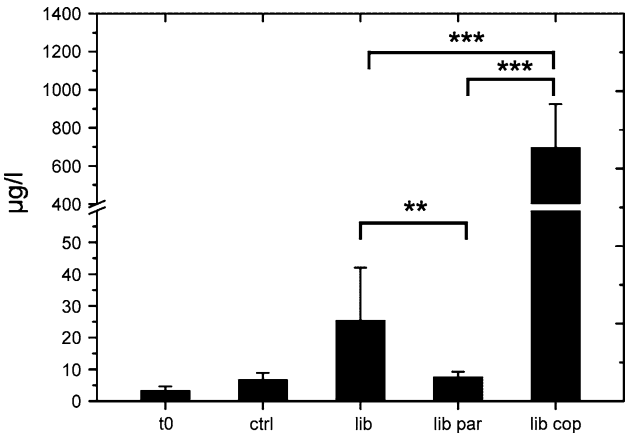

Fig. 3 Concentration of coagulation activation marker thrombin antithrombin complex before ("t0") and after 120 min of circulation in the loop without a stent ("ctrl") and with three different stents ("lib"), ("lip par") and ("lib" cop). Values are expressed as mean ± SD. Statistics between the groups were calculated by univariate analysis of variance (ANOVA). P values: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

containing probe with values of 723 µg/l ± 244.75. The commercially available "lib" stent ranged between these extremes by showing TAT activation of 19.88 µg/l ± 6.93. The differences for coagulation activation were highly significant between all stent types ($P < 0.01$ for "lib" vs. "lib par" and $P < 0.001$ for "lib" vs. "lib cop" and "lib par" vs. "lib cop").

3.3 Activation of platelets

Platelet activation was assessed through β-thromboglobulin and sP-selectin measurements (Fig. 4), both of which showed the highest activation at the copper coated stent ("lib cop") with β-TG values of 1630.45 IU/ml ± 329.59 and sP-selectin values of 62.84 ng/ml ± 9.46. The least activation of both markers could be found in "t0" baseline samples (76 IU/ml ± 40.49 for β-TG and 30.30 ng/ml ± 5.75 for sP-selectin) and in the bare Loop without a stent ("ctrl"; 241.53 IU/ml ± 59.2 for β-TG and 34.77 ng/ml ± 6.83 for sP-selectin). The other stents tested ranged between these extremes with "lib" showing higher β-TG values of 785.11 IU/ml ± 59.2 and sP-selectin values of 47.78 ng/ml ± 10.13 in comparison with the other stents tested. The "lib par"-containing sample showed moderate thrombogenicity with β-TG values of 493.74 IU/ml ± 233.25 and sP-selectin values of 37.39 ng/ml ± 6.95. Although β-TG and sP-selectin levels showed good accordance among each other, only β-TG values were significantly different for all tested stents with $P < 0.05$ for "lib" vs. "lib par" and $P < 0.001$ for "lib" vs. "lib cop" and "lib par" vs. "lib cop". SP-selectin values were only significant between "lib" and "lib cop" ($P < 0.001$) and "lib par" and "lib cop" ($P < 0.001$).

3.4 Activation of complement system and leukocytes

As expected, complement (SC5b-9) and leucocyte (PMN-elastase) activation markers showed the least amount in baseline samples ("t0") ranging from 158.23 ng/µl ± 31.48 for SC5b-9 and 40.71 µg/l ± 13.11 for PMN-elastase (Fig. 5). Circulation of blood in the Chandler Loop sample ("ctrl") increased complement activation approximately four times to levels of 675.89 ng/µl ± 264.61. Levels of PMN-elastase were only slightly increased,

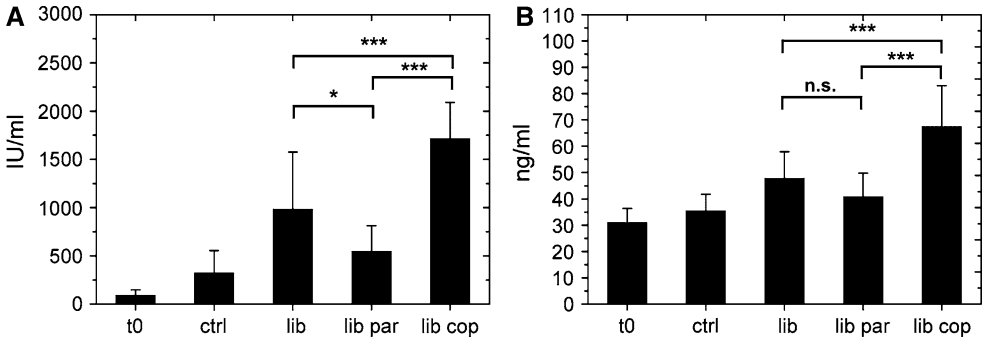

Fig. 4 Concentration of platelet activation markers ß-thromboglobulin (**a**) and sP-selectin (**b**) before ("t0") and after 120 min of circulation in the loop without a stent (ctr) and with three different stents ("lib"), ("lip par") and ("lib cop"). Values are expressed as mean ± SD. Statistics between the groups was calculated by univariate analysis of variance (ANOVA). *P* values:*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Values of $P > 0.05$ were considered as being not significant (n.s.)

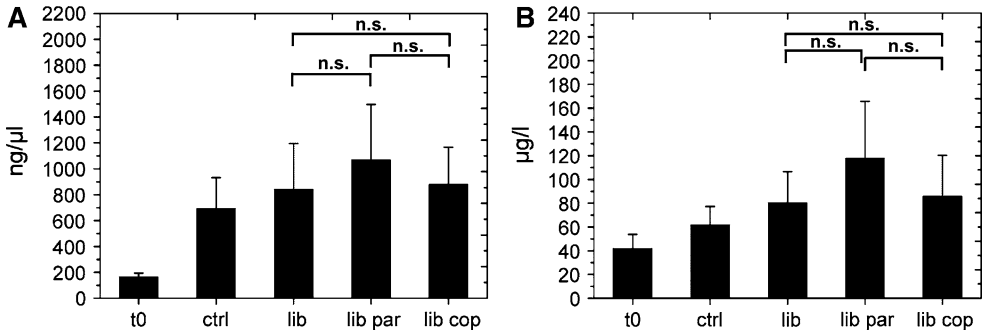

Fig. 5 Concentration inflammatory activation markers, complement system (SC5b-9) (**a**) and leukocytes (PMN-elastase) (**b**) before ("t0") and after 120 min of circulation in the loop without a stent ("ctrl") and with three different stents ("lib"), ("lip par") and ("lib cop"). Values are expressed as mean ± SD. Statistics between the groups was calculated by univariate analysis of variance (ANOVA). *P* values:*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Values of $P > 0.05$ were considered as being not significant (n.s.)

ranging at 61.35 µg/l ± 17.54, approximately 30% higher above sample "t0". Highest values of SC5b-9 were found in "lib par" (930.87 ng/µl ± 420.73); the same stent also showed the highest detected values of PMN-elastase (104.18 µg/l ± 51.56). In contrast to the coagulation and platelet activation data, the "lib cop" sample was not found to provoke significantly greater inflammatory response than the other stents. The values were 817.52 ng/µl ± 256.24 for SC5b-9 and 81.92 µg/l ± 36.69 for PMN-elastase. The lib-containing Loop showed SC5b-9 values of 842.27 ng/µl ± 352.48 and PMN-elastase values of 80.31 µg/l ± 26.22. It was nearly equivalent with the copper coated stent ("lib cop") which achieved similar values in both categories. In summary, inflammation markers were all higher in stent samples, but not significantly distinguishable (Fig. 4).

3.5 Scanning electron microscopy

After circulation in the Chandler-Loop, cell deposition and protein adsorption could be found on the surface of all tested stents (Fig. 6).

4 Discussion

The potential for stent thrombosis limits the long-term success of stent implantation. Within the first weeks after implantation, thrombotic occlusion can occur (subacute stent thrombosis), mainly caused by thrombus formation through undesired interactions between blood and the stent surface. The stent can activate coagulation and platelets and induce proinflammatory responses via complement and leucocyte activation. These early stage reactions after stenting are the result of blood material contact and disruption of flow through the stent [18]. In contrast to animal models, in our modified Chandler-Loop model we are able to measure coagulation, platelet activation, inflammatory response, and cell deposition under human whole blood conditions. Thus, the Chandler-Loop model may be a valuable, alternative to animal models, especially for early-stage events after stent implantation.

The objective of this study was to assess the feasibility of the Chandler-Loop model for hemocompatibility testing using three deliberately selected stents that were expected to have different levels thrombogenicity.

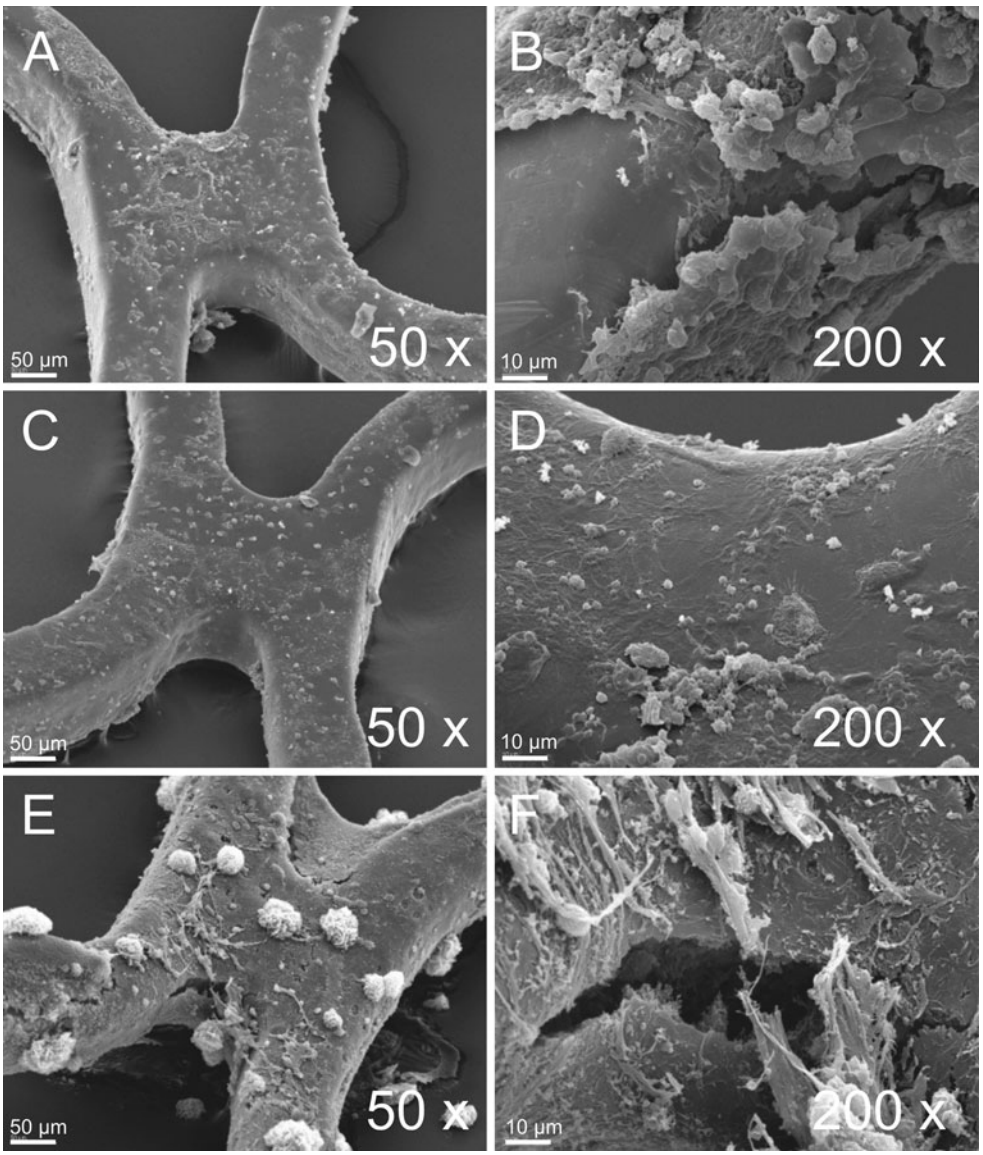

Fig. 6 Scanning electron micrographs of the tested stents after circulation in the Chandler-Loop: (**a**) "lib" sample, magnification ×50; (**b**) "lib" sample, magnification ×200; (**c**) "lib par" sample magnification ×50; (**d**) "lib par" sample, magnification ×200; (**e**) "lib cop" sample, magnification ×50; (**f**) "lib cop" sample, magnification ×200

4.1 Thrombogenicity

Stents are made of metallic compounds that have inherent thrombogenic properties [19]. Contact of blood with artificial stent surfaces leads to platelets activation and intrinsic coagulation, with subsequent excretion of platelet activation markers like sP-selectin and β-thromboglobulin [20–22]. Coagulation activation is represented by TAT complex formation. In the Chandler-Loop model it is possible to distinguish between more or less thrombogenic stents by measuring the concentration of these markers. In order to show the complete potency of coagulation activation, we tested a copper coated stent which was chosen because of its strong thrombogenic response (this stent is not appropriate for medical use). As expected, the copper-coated stent (lib cop) was found to provoke a very strong response in coagulation activation measured by TAT generation. Activation of platelets, represented by sP-selectin and β-TG values within this stent, were also found to be higher in the copper-coated stent than in the other stents that were tested. In contrast, the "lib par" stent demonstrated the lowest thrombogenicity according to the Chandler-Loop model, causing only slightly higher activation (TAT, sP-selectin, β-TG) compared to the control group, consistent with the presumed low thrombogenicity of Parlyne C [15]. TAT levels in the copper coated stent were about a hundred times higher than "ctrl" and "lib par".

4.2 Inflammatory response

In addition to thrombogenic reactions, inflammatory responses (through complement and leucocyte activation) may also occur due to contact with the artificial surfaces of the stents or the Loops [23]. Thrombosis and inflammation are undesired events which, especially when combined, may lead to stent occlusion. For this reason the optimal stent would need to minimize both coagulation and inflammation (rather than either alone). In addition, because factors affecting thrombogenicity and inflammation can be mutually conflictive within the same stent, it is important to test both factors independently. The modified Chandler-Loop model was able to successfully demonstrate that the copper coated stent (lip cop) provokes a very high coagulation activation, as indicated through TAT- β-TG- and sP-selectin values, whereas the thrombogenicity of the Parlyne C sample ("lib par") was comparatively low. In contrast, the inflammatory markers SC5b-9 and PMN-elastase demonstrated opposite results, with the copper-coated stent showing significantly less complement and leucocyte activation than the Parlyne C-coated stent.

4.3 Scanning electron microscopy

SEM was used as an indicator of cellular adhesion and fibrinogen adsorption. SEM served only as general representation of cell and protein deposition. After 120 min of whole blood incubation, cell and protein deposition was visible on all stent surfaces. Cells could mainly be identified as platelets and leucocytes as well as erythrocytes, which were covered and connected by fibrin fibers attached to cells and directly deposited to the stent surface.

It is important to determine the blood compatibility of the stents without influencing the potential thrombogenic and inflammatory responses caused by the large surface of the PVC Loop. For this reason CBAS heparin coated tubes were chosen, this surface shows very good hemocompatibility and therefore limits background activation. The additional rise of activation markers in comparison to controls before filling the Loops is still low enough to observe further increases in stent-containing Loops. In contrast to the original Chandler Loop design, which utilized static Loops and an actively-driven roller pump, in the modified version the blood circulates passively by rotation of the Loop itself. This modification prevents the traumatizing effect of an actively pumping system and is an advantage over the active-pumping in vitro model suggested by Beythien et al. [24]. Passive circulation also ensures that platelet activation is mainly caused by the exposed stent surface and not by pumping effects. Another in vitro test model introduced by Hong et al. consists of a rotating disc with a slide chamber mounted to the outward rim of the disc [25]. With a slide chamber volume of only 1.65 ml, this model is at a disadvantage compared with the Chandler Loop model that can hold a bigger blood volume, which is essential for running several blood tests out of the same sample. An additional advantage of the modified Chandler-Loop model is the determination of background activation, allowing a measurement of the effect of the loop alone without any stent.

5 Limitations and design considerations

Admittedly an upper limit of thrombogenicity of stents in the Chandler-loop model has not been defined yet. It is also argued that a certain degree of thrombogenicity like extracellular matrix protein adhesion to the stent is advantageous for the re-endothelialisation of the stent surfaces [26]. Compared with animal models, in vitro models also have some disadvantages. Because PVC tubings are not comparable to real vessel walls they are not appropriate for long-term testing for late stent thrombosis through increased neointimal proliferation. To improve statistical evaluation, an increase of sample size, and numbers of blood donors seems to be a promising strategy to enhance statistical significance.

6 Conclusion

Within this study we established a novel in vitro model based on a modified Chandler-Loop system. This system could be helpful to test the hemocompatibility of new stent designs or stent coatings before they are clinically introduced. The main advantages of this new stent test model are the high standardization of test conditions and the potential to compare data from different stent types directly. The new Chandler-Loop model may be an alternative to animal models and current in vitro test systems, especially for early events after stent implantation. Because they are fast and fairly inexpensive, Chandler-Loop experiments should be included in conjunction with animal models when testing new stents, allowing direct comparison of in vitro and in vivo data.

Acknowledgments The authors thank Karl-Heinz Hellmer for the excellent support during the SEM procedures. Tests and stent delivery systems were sponsored by Boston Scientific Corporation, Natick, MA, USA. Furthermore the authors thank Barbara A. Huibregtse and Michael J. Eppihimer (Boston Scientific Corporation, Pre-clinical department, 1 Boston Scientific Place, Natick, MA 01760 USA), Kristin L. Hood (Boston Scientific Corporation, Scientific communications, 100 Boston Scientific Way, Marlborough, MA 01752-1234 USA) and Nic Van Dyck (Boston Scientific Corporation, Clinical department, Gaetano Martinolaan 50, 6229 GS Maastricht Netherlands) for their valuable work of reviewing the manuscript.

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