Methotrexate loaded SAE coated coronary stents reduce neointimal hyperplasia in a porcine coronary model

Y Huang, K Salu, X Liu, S Li, L Wang, E Verbeken, J Bosmans, I De Scheerder

Objective: To evaluate the effect of stent based methotrexate delivery on neointimal hyperplasia.

Methods: Stainless steel coronary stents and biological polymer coated (SAE) stents were randomly implanted in coronary arteries of pigs with a stent to artery ratio of 1.1:1. The pigs were killed after five days (10 stents) or four weeks (20 stents). Second, stainless steel coronary stent stents were dip coated in a 10 mg/ml methotrexate–SAE polymer solution, resulting in a total load of 150 µg methotrexate/stent. SAE coated stents and methotrexate loaded stents were randomly implanted in porcine coronary arteries with a stent to artery ratio of 1.2:1 and followed up to four weeks.

Results: SAE coated stents and bare stents elicited a similar tissue response at five days. At four weeks, neointimal hyperplasia induced by the coated stents was less pronounced than with the bare stents (1.32 (0.66) v 1.73 (0.93) mm², p > 0.05). In vitro drug release studies showed that 50% of the methotrexate was released in 24 hours, and all drug was released within four weeks. No impact on vascular smooth muscle cell proliferation or viability was observed in in vitro cell cultures. Furthermore, as a cytotoxic agent, methotrexate incorporated into a polymer matrix, however, has failed to decrease neointimal hyperplasia in a porcine coronary model. In the present study, we used a biological polymer to deliver methotrexate to the injured coronary artery wall. The biocompatibility of the polymer and the feasibility and efficacy of stent based methotrexate delivery through this polymer were evaluated in a porcine coronary stenting model.

METHODS

Stent and stent coating
Stainless steel balloon expandable stents, 16 mm long, were used for these studies. The bare stents were dip coated in a biological polymer (SAE, Global Medical Systems, Zulte, Belgium) or in 10 mg/ml methotrexate-SAE solution, resulting in a total load of 150 µg methotrexate/stent.

In vitro methotrexate release kinetics
Three methotrexate loaded stents were placed in vials containing 1 ml 0.9% NaCl at 37°C. Ultraviolet absorbance (Cary 4 E spectrophotometer, Varian Inc, Palo Alto, California, USA) was measured at 222 nm each day for the first 14 days and after three and four weeks to determine the methotrexate release. One control polymer-only stent underwent the same procedure and the ultraviolet absorbance values were subtracted from the values of the drug eluting stents.

Impact of methotrexate on vascular SMCs in vitro: SMC proliferation and viability
SMCs were isolated from New Zealand White rabbit aorta, and passaged and cultured (50 000 cells/well) in six-well plates (Corning, Brussels, Belgium). Every third day, 0, 10⁻⁸, 10⁻⁷, 10⁻⁶, and 10⁻⁵ M methotrexate (Sigma, Bornem, Belgium), dissolved in NaCl, or paclitaxel (Bristol-Myers Squibb, Brussels, Belgium), dissolved in 20 µl ethanol, was used.

Abbreviations: IEL, internal elastic lamina; SMC, smooth muscle cell
added in combination with medium changes. After seven
days, SMC proliferation was determined by either cell
counting (Coulter Counter) or protein quantification (BCA
Protein Assay Kit, Pierce, Antwerp, Belgium). Before protein
counting in the protein assay, cell viability was
determined through neutral red uptake. A viability index
for each concentration was calculated as the percentage of
neutral red from the control sample divided by the
percentage of total protein from the control.

Stent implantation
Domestic cross bred pigs of both sexes weighing 20–25 kg
were used. They were fed a standard natural grain diet
without lipid or cholesterol supplementation throughout the
study. All animals were treated and cared for in accordance
with the Belgium National Institute of Health guidelines for
care and use of laboratory animals.

Surgical procedures and stent implantation in the coronary
arteries were performed according to the method described
by De Scheerder and colleagues.

Biocompatibility of the coated stents
To evaluate the biocompatibility of SAE coating, 15 SAE
coated stents and 15 bare stents were randomly deployed in
the right and left coronary arteries of 15 pigs. Arterial
segments were selected to obtain a 1:1.1 stent to artery ratio.

The pigs were killed after five days (10 stents) or four
weeks (20 stents).

Methotrexate coated stents
Seventeen SAE coated stents and 20 SAE coated stents
loaded with 150 μg methotrexate were randomly deployed in
the coronary arteries of pigs. Arterial segments were selected to
obtain a 1.2:1 stent to artery ratio. Pigs were killed after
four weeks to evaluate the effect of local methotrexate
delivery on neointimal hyperplasia.

Tissue processing and histomorphometric analysis
At five days or four weeks follow up, the pigs were killed and
the stented coronary arteries were perfused with a 10%
formalin solution at 80 mm Hg. The segments were further-
more fixed in a 10% formalin solution. Each segment was
cut into a proximal, middle, and distal stent segment
for histomorphometric analysis. Tissue specimens were
embedded in a cold polymerising resin (Technovit 7100,
Heraus Kulzer GmbH, Wehrheim, Germany). Sections 5 μm
thick, were cut with a rotary heavy duty microtome HM 360
(Microm, Walldorf, Germany) equipped with a hard metal
knife and stained with haematoxylin and eosin, elastic stain,
and a phosphotungstic acid haematoxylin stain. Light
microscopic examination was performed blinded to the type of
stent used. Injury of the arterial wall caused by stent
deployment, peristrut inflammation, and thrombus forma-
tion was evaluated for each stent strut and graded as
seen adjacent to the stent struts. The mean inflammation
score (0.21 (0.21) n 0.28 (0.41), p 0.05) of the SAE coated
stents induced an identical histopathological response. The
drug release curve showed that after 24 hours 50% of the
methotrexate had already been released from the stent. After
the first 24 hours, the release was slower and was 87% at one
week. Finally, after four weeks of incubation in NaCl at 37˚C,
release was complete (fig 1).

Effect of methotrexate on SMC proliferation and
viability
During SMC culture, methotrexate did not have any effect on
cell proliferation, whereas paclitaxel concentration depen-
dently reduced both cell count and protein content. However,
non-paired  t test was used. A probability value of
p < 0.05 was considered to be significant.

RESULTS

In vitro methotrexate release kinetics
The drug release curve showed that after 24 hours 50% of the
methotrexate had already been released from the stent. After
the first 24 hours, the release was slower and was 87% at one
week. Finally, after four weeks of incubation in NaCl at 37˚C,
release was complete (fig 1).

In vivo biocompatibility of SAE coated stents
At five days’ follow up, both the bare and the SAE coated
stents induced an identical histopathological response. The
stenal tissues were well aligned with the vascular wall. The
media was mildly compressed. Arterial injury induced by
stenal implantation was not significantly different between
groups. A thin fibrin layer covering the stent filaments
was observed. A few inflammatory cells trapped within a
thrombotic meshwork were observed (fig 3A). No significant
difference in inflammatory response and thrombus formation
was observed between coated stents and bare stents.

At four weeks’ follow up, the neointima of both coated and
bare stents was well organised and consisted of extracellular
matrix with SMCs (fig 3B, C). A few inflammatory cells were
seen adjacent to the stent struts. The mean inflammation
score (1.02 (0.08) v 1.11 (0.30), p > 0.05) and arterial injury
score (0.21 (0.21) v 0.28 (0.41), p > 0.05) of the SAE coated
and bare stents were not significantly different. The mean
lumen area (5.65 (1.60) mm 2, p > 0.05), and arterial injury
area (5.21 (1.35) mm 2, p > 0.05) were similar in the
two groups.

In vivo effect of stent based methotrexate delivery on
neointimal formation
Histopathological examination found that the lumen surfaces
of both the SAE coated and the methotrexate loaded stents

Statistical analysis
Data are presented as mean (SD). In vitro data (proliferation
and viability assay) were evaluated by means of a one way
analysis of variance and Dunnett’s multiple comparison post
hoc test. For comparison of histomorphometric data between
groups, a non-paired  t test was used. A probability value of
p < 0.05 was considered to be significant.

Figure 1 In vitro accumulation of methotrexate released from three SAE
coated coronary stents in 1 ml NaCl at 37˚C.
were covered completely with endothelial cells (table 2). With a greater stent to vessel ratio, medial compression was moderate to severe (fig 3D, E).IEL disruption and medial laceration by stent struts were more frequently observed in the SAE coated stent group. A few sections of the SAE coated stents had a pronounced inflammatory response around some stent struts (fig 3F), although the inflammatory response from the stent struts through the media was rare. The inflammatory response and injury score of the methotrexate loaded stents were significantly lower than those of the SAE coated stents (1.02 (0.03) vs. 1.27 (0.50), p < 0.05 and 0.20 (0.13) vs. 0.41 (0.32), p < 0.05, respectively) (fig 3G), although the mechanical force (oversizing) of the two groups during stent implantation was not significantly different. The ratio of balloon area to IEL area of methotrexate loaded stents was even higher than that of SAE coated stents (1.37 (0.21) vs. 1.28 (0.16), p < 0.05). Furthermore, the neointimal hyperplasia of methotrexate loaded stents was significantly lower than those of the SAE coated stents (1.22 (0.34) vs. 2.25 (1.28), p < 0.01) and area stenosis (21 (8)% vs. 36 (21)%), p < 0.01) of the methotrexate coated stents were significantly lower than those of the SAE coated stents.

**DISCUSSION**

In the present study, metal stents coated with a SAE biological coating did not provoke an increased peristrut inflammatory response at five days and at four weeks' follow up. The neointimal hyperplasia with SAE coated stents at four weeks was even lower than that with bare stents implanted in similar conditions. Impregnation of the SAE coating with 150 µg methotrexate significantly reduced peristrut inflammation and neointimal hyperplasia in an overstretched porcine coronary stenting model. This study showed the efficacy of stent based methotrexate delivery in preventing in-stent restenosis.

**Biocompatible coating: a crucial determinant for the success of drug eluting stents**

Synthetic polymers have been used as a matrix in which to incorporate drugs for local delivery. With improved coating methods, ultrathin and homogeneous stent coating surfaces can be obtained. However, poor biocompatibility with coronary arterial tissue has been observed with some synthetic polymer coatings. An inflammatory response to the polymer coating resulting in increased neointimal

---

**Table 1 In vitro assays of rabbit aortic smooth muscle cell proliferation and viability**

| Drug       | Assay | No. | Concentration | Control | 1% Ethanol | 10⁻¹⁰ mol/l | 10⁻⁹ mol/l | 10⁻⁸ mol/l | 10⁻⁷ mol/l | 10⁻⁶ mol/l | 10⁻⁵ mol/l |
|------------|-------|-----|---------------|---------|------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Methotrexate| Cells | 4   | 100          | NA      | 108 (24)   | 107 (23)   | 94 (18)     | 100 (14)    |
|            |       | 3   | 100          | NA      | 97 (3)     | 97 (6)     | 95 (8)      | 97 (9)      |
|            |       | 3   | 100          | NA      | 99 (6)     | 99 (4)     | 99 (4)      | 98 (5)      |
|            |       | 3   | 100          | NA      | 102 (6)    | 101 (3)    | 104 (10)    | 102 (5)     |
| Paclitaxel | Cells | 4   | 100          | 100 (8) | 55 (13)** | 22 (7)**   | 13 (3)**    | 5 (2)**     |
|            |       | 3   | 100          | 97 (4)  | 95 (4)     | 79 (3)**   | 42 (2)**    | 12 (9)**    |
|            |       | 3   | 100          | 90 (3)**| 88 (1)**   | 66 (4)**   | 32 (2)**    | 5 (1)**     |
|            |       | 3   | 100          | 94 (5)  | 94 (4)     | 83 (7)     | 76 (1)**    | 46 (18)**   |

Data are mean (SD). Cells are the number of smooth muscle cells in culture wells as quantified by Coulter Counter; protein is the amount of total protein in culture wells as quantified by BCA protein assay kit; viability was determined by neutral red staining before total protein quantification; relative viability is the ratio of neutral red staining on total protein quantification. All concentrations are a percentage of the control value.

**Figure 2** Smooth muscle cell (SMC) proliferation determined by means of (A, B) cell counting and (C, D) total protein assay. SMCs were incubated for seven days with (A, C) methotrexate and (B, D) paclitaxel. **p < 0.01; ***p < 0.0005 by one way analysis of variance.**
formation has been reported.12 13 The increased inflammation may counteract the efficacy of drug eluting stents. In the present study, a biological polymer was applied to a metal stent surface to serve as a matrix for local drug delivery. At five days the histopathological response was the same to the SAE coated stents and the non-coated stents. This low inflammatory response to SAE coated stents was also observed at four weeks’ followup. The neointimal hyperplasia and area stenosis were even lower with SAE coated stents than with the bare stents. All these data suggest that the SAE coating is biocompatible with both blood and coronary arterial tissue. SAE coated stents can therefore serve as a vehicle for local drug delivery.

**Methotrexate eluting stents: antirestenotic effects and postulated mechanisms**

It has been found that inflammatory cells and proinflammatory cytokines play an important part in vascular healing. The number of monocytes adhering to the luminal surface of stented arteries correlated linearly with the degree of neointimal hyperplasia.14 In transgenic mice, Rectenwald and colleagues15 showed that tumour necrosis factor α and interleukin (IL) 1 directly participated in the pathogenesis of neointimal hyperplasia. In addition, the anti-inflammatory cytokine rhuIL 10 reduced intimal hyperplasia after both balloon angioplasty and stent implantation in carotid arteries. Increased arterial injury by the perforated balloon catheter delivery and stent based delivery have been explored. Using a perfusion catheter, Muller and colleagues16 found that the mean intimal thickness of the methotrexate treated group was not significantly different from the saline treated control group. With cellulose ester polymer coated stents, Cox and colleagues17 showed that local delivery of methotrexate did not inhibit neointimal proliferation in stented porcine coronary arteries. Increased arterial injury by the perforated balloon, lack of biocompatibility of the polymer coating, too fast drug release, and inadequate tissue concentrations may all have contributed to the failure of these studies. In this study, we found that the release of methotrexate from the SAE coated stents was slow. In the first 24 hours, 50% of the methotrexate was released, and release was complete within four weeks. In the overstretched porcine coronary stenting

| Stent coating | No. | Lumen area (mm²) | Neointimal hyperplasia (mm²) | Area stenosis (%) | Balloon area to IEL area ratio | Inflammation score | Injury score |
|---------------|-----|------------------|-----------------------------|------------------|-------------------------------|-------------------|-------------|
| SAE           | 17  | 4.34 (1.85)      | 2.25 (1.28)                 | 36 (21)          | 1.28 (0.16)                   | 1.27 (0.50)       | 0.41 (0.32) |
| Methotrexate  | 20  | 5.02 (1.42)      | 1.22 (0.34)**               | 21 (8)**         | 1.37 (0.21)                   | 1.02 (0.03)*      | 0.20 (0.13)* |

*p<0.05; **p<0.01 versus SAE coated stents.
Reducing neointimal hyperplasia with coated coronary stents

The SAE coated stents and the methotrexate loaded stents. The lumen surface of methotrexate loaded stents was completely covered by endothelium at four weeks' regeneration. The lumen of methotrexate had no negative effect on endothelial cell proliferation can result in late vascular thrombosis. In our study, methotrexate had no negative effect on endothelial cell regeneration. The difference was noted between the SAE coated stents and the methotrexate loaded stents.

Vascular toxicity with incomplete healing has been observed with paclitaxel eluting stents. Methotrexate, as a chemotherapeutic agent, can also influence the metabolism of cells and has a cytotoxic effect. Inhibition of SMC proliferation has been presumed as a potential mechanism in the effect of methotrexate. In this study, we analysed an in vitro cell culture and compared the effects of methotrexate and paclitaxel on SMCs. The results showed that methotrexate in concentrations from 10^-8 to 10^-6 M had no effect on SMC proliferation and viability. However, paclitaxel was observed to have a dose dependent effect. Therefore, it is not unreasonable to assume that methotrexate is safer and has a wider treatment window than paclitaxel for local treatment. Anti-inflammation, and not antiproliferation, seems to be the main mechanism by which neointimal hyperplasia is decreased.

In summary, the SAE coating was biocompatible with porcine coronary arteries. No retarded endothelial regeneration and other local vascular toxicity were observed. SAE coated stents loaded with methotrexate may eliminate peristrut inflammation, which contributes to reduced neointimal hyperplasia.

**Authors’ affiliations**

Y Huang, X Liu, S Li, L Wang, E Verbeken, I De Scheerder, Department of Cardiology, Department of Pathology, University Hospitals, Leuven, Belgium

K Salu, J Bosmans, Department of Cardiology, University of Antwerp, Antwerp, Belgium

**REFERENCES**

1. Edelman ER, Rogers C. Pathobiologic responses to stenting. *Am J Cardiol* 1998;81:4E–6E.
2. De Scheerder I, Wang K, Wilczek K, et al. Local methylprednisolone inhibition of foreign body response to coated intracoronary stents. *Circulation* 1996;7:161–6.
3. Sousa JE, Costa MA, Abizaid A, et al. Lack of neointimal proliferation after implantation of single coated stents in human coronary arteries: a quantitative coronary angiography and three-dimensional intravascular ultrasound study. *Circulation* 2001;103:192–5.
4. Hu SK, Mitcho V, Cronksy AJ, et al. Studies on the effect of methotrexate on macrophage function. *J Rheumatol* 1988;15:206–9.
5. Kutolo M, Sulli A, Pizzorni C, et al. Anti-inflammatory mechanisms of methotrexate in rheumatoid arthritis. *Ann Rheum Dis* 2001;60:729–35.
6. Cox DA, Anderson PG, Roubin GS, et al. Effect of local delivery of heparin and methotrexate on neointimal proliferation in stented porcine coronary arteries. *Coron Artery Dis* 1992;3:237–48.
7. Seye CI, Godreau AP, Delattre JM, et al. Overexpression of P2Y2 purinoceptor in intimal lesions of the rat aorta. *Arterioscler Thromb Vasc Biol* 1997;17:3602–10.
8. Bruijn IH, Bull H. Effects of local cytochalasin D delivery on smooth muscle cell migration and an collagen-induced intimal hyperplasia in the rabbit carotid artery. *Br J Pharmacol* 2001;134:473–83.
9. Bonin PD, Singh JP, Gomill RB, et al. Inhibition of fibroblast and smooth muscle cell proliferation and migration in vitro by a novel aminochromone U-67154. *J Vasc Res* 1993;30:108–15.
10. Schwartz RS, Huber KC, Murphy JG, et al. Restenosis and the proportional neointimal response to coronary artery injury: results in a porcine model. *J Am Coll Cardiol* 1992;19:267–74.
11. Huang Y, Wang L, Verweire I, et al. Optimization of local methylprednisolone delivery to inhibit inflammatory reaction and neointimal hyperplasia of coated coronary stents. *J Invasive Cardiol* 2002;14:505–13.
12. van der Giessen WJ, Uincoff AM, Schwartz RS, et al. Marked inflammatory sequelae to implantation of biodegradable and non-biodegradable polymers in porcine coronary arteries. *Circulation* 1996;94:1690–7.
13. De Scheerder IK, Wilczek KL, Verbeken EV, et al. Biocompatibility of polymer-coated oversized metallic stents implanted in normal porcine coronary arteries. *Atherosclerosis* 1995;114:103–14.
14. Rogers C, Edelman ER. Endovascular stent design dictates experimental restenosis and thrombosis. *Circulation* 1995;91:2995–3001.
15. Recenvald JE, Moldawer LI, Huber TS, et al. Direct evidence for cytokine involvement in neointimal hyperplasia. *Circulation* 2000;102:1697–702.
16. Feldman LJ, Aguirre L, Ziol M, et al. Interleukin-10 inhibits intimal hyperplasia after angioplasty or stent implantation in hypercholesterolemic rabbits. *Circulation* 2000;101:908–15.
17. Murphy JG, Schwartz RS, Edwards WD, et al. Methotrexate and azathioprine fail to inhibit porcine coronary restenosis [abstract]. *Circulation* 2000;102:822 AppI III:342.
18. Muller DW, Topol EJ, Abrams GD, et al. Intramural methotrexate therapy for the prevention of neointimal thickening after balloon angioplasty. *J Am Coll Cardiol* 1992;20:460–6.
19. Hirata S, Matsuura T, Saora R, et al. Inhibition of in vitro vascular endothelial cell proliferation and in vivo neovascularization by low-dose methotrexate. *Arthritis Rheum* 1989;32:1065–73.
20. Forb A, Heller PF, Shroff S, et al. Pathological analysis of local delivery of paclitaxel via a polymer-coated stent. *Circulation* 2001;104:473–9.
21. Liang GC, Nemickas R, Madayag M. Multiple percutaneous transluminal angioplasties and low dose pulse methotrexate for Takayasu’s arteritis. *J Rheumatol* 1989;16:1370–3.