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Therapeutic and mechanistic explorations of in-stent restenosis in the rat aortic stenting model
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CHAPTER 7
Effect of the paclitaxel and the sirolimus-eluting stent on neointimal formation and neointimal healing in the Zucker diabetic Rat.


submitted
INTRODUCTION

Drug-eluting stents are successful in reducing in-stent restenosis\textsuperscript{1,2}. Diabetes is a risk factor for in-stent restenosis and drug-eluting stents are used more frequently in these patients\textsuperscript{3}. A recent study suggests that the paclitaxel eluting stent and the sirolimus eluting stent might differ in their ability to reduce in-stent restenosis in a diabetic population\textsuperscript{4}. Our first goal was to study if one of those two stents is better in reducing neointimal formation in a diabetic population. Therefore neointimal area was measured in the paclitaxel eluting stent (Taxus) and the sirolimus eluting stent (Cypher) after stent-implantation in diabetic rats. We also measured neointimal area in bare metal control stents (Express 2 and Bx Velocity) to see if stent design affected in-stent restenosis in a diabetic population.

Recently drug-eluting stents have been associated with increased late stent thrombosis\textsuperscript{5}. Joner et al suggested that histologic differences in drug-eluting stent such as increased fibrin deposition and inflammation may be linked with late stent thrombosis\textsuperscript{6}. So our second goal was to determine if the neointima of drug-eluting stents is different from their bare metal controls in a diabetic population. We also studied if differences existed between the neointima of the paclitaxel eluting stent and the sirolimus eluting stent. We measured neointimal cell density, inflammation-score and signs of abnormal healing (hemorrhage and acellularity) at two different time points in the two most used drug-eluting stents (Taxus Express 2 and Cypher) and their bare metal controls (Express 2 and Bx Velocity). Furthermore to assess extracellular matrix formation and tissue strength we measured collagen content in both drug-eluting and bare metal stents.

METHODS

Animals

All procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Specific pathogen-free, Male Zucker (Charles Rivers) diabetic fatty/ rats (ZDF/GmiCrl-fa/fa), characterized by obesity, insulin resistance, and hyperlipidemia, as well as overt hyperglycemia, weighing 320 to 400 g were fed standard rat chow and water ad libitum. Stent implantation was successfully performed in fifty-six rats. Overall mortality was approximately 30%. Most animals died of perforation of the aorta, vena cava puncture or aortic thrombosis during or shortly after stent-implantation.

Animal Protocol

Animals were anesthetized with O\textsubscript{2}, N\textsubscript{2}O, and isoflurane 2\% (Abbot B.V.). Premounted stents all 2.5 x 8 mm were implanted in the abdominal aorta as described previously\textsuperscript{7}. There were 4 groups: one group received the Express 2 stent manufactured by Boston Scientific (the same stent as the Taxus Express 2 stent only without the paclitaxel-eluting polymer), one group received the Taxus Express 2 paclitaxel eluting stent, one group received the Bx Velocity stent manufactured by Cordis Johnson & Johnson (the same stent as the Cypher stent only without the sirolimus-eluting polymer) and one group received the Cypher stent sirolimus eluting stent. All animals received rat chow mixed with clopidogrel (Plavix, Sanophi) 0.33 mg·gram rat chow starting 5 days before stent-implantation until termination of the animals. Clopidogrel intake was estimated by monitoring bodyweight over time (daily oral intake was estimated at 25 gram/day/rat). After 7 or 28 days, animals were anesthetized and heparinized with 500 IU intravenously (Leo Pharma B.V.). Abdominal aortas were harvested, fixed, embedded in methylmetacrylate, sectioned, and stained for histological analysis.

Histology

Histomorphometrical analysis was performed on Lawson (elastin staining) and hematoxyline-eosine stained sections by measurements of the proximal, middle, and distal parts of each stent. Lawson-stained sections were used to measure neointimal formation and injury score. Hematoxyline-eosine
stained sections were used to measure neointimal cell density, inflammation score and look for signs of abnormal healing. The neointimal area, media area and lumen area were measured or calculated as described previously. In short, the areas within the external elastic lamina (EEL), IEL and lumen were measured by using digital morphometry by means of an Olympus BX-50F4 microscope, an Olympus c-3030 zoom digital camera and Olympus DP-Soft version 3.0 software (Olympus, Tokyo, Japan). The lumen area was subtracted from the IEL area to give the neointimal area. The IEL area was subtracted from the EEL area to give the media area. For each stent part the mean of six sections was calculated. The injury and inflammation scores were assessed as described by Schwartz et al and Kornowski et al. Neointimal cell density was determined in hematoxylin-eosin–stained sections at x400 magnification and expressed as x100/mm² as described previously. Signs of incomplete (neointimal) healing were defined as acellularity of neointima or the presence of a hemorrhage in the neointima. Hemorrhage was defined as the presence of more than hundred grouped biconcave discs in the neointima. Acellularity of neointima was defined as absence of cells in an area with an estimated width of half the nearest stent strut and one-third the neointimal thickness. Signs of incomplete healing were scored in the proximal, middle, and distal parts of each stent. Each part was scored four times at 90 degree rotation apart. If none of the parts of the stent showed either a hemorrhage or an acellular neointimal area a score 0 was assigned. If only one of the parts showed either a hemorrhage or an acellular neointimal a score of 1 was assigned. The total score of incomplete healing in each group was averaged, multiplied by 100 and expressed as a percentage. After 1 week the inflammation score, signs of incomplete healing and neointimal cell density were measured. After 4 weeks the same measurements were performed plus the neointimal area, neointimal thickness, media area, lumen area and injury score.

Collagen staining and analysis
0.1% Sirius Red F3B stained sections were used to measure collagen content using computerized image analysis. The expression of collagen was measured using computer-assisted morphometry. A total of 5 fields per slide were evaluated at a magnification of 200 x. Image analysis was performed by a technician blinded to the source of the sample. Image analysis was performed using an automated macro written with the software package Leica Qwin. A background image of a blank area of the slide was obtained and background correction was performed to adjust for subtle irregularities in the illumination of the microscope field. The software was set to subtract background staining from the stained sections, subsequently staining intensity was measured and used as an indirect measure for collagen content.

Statistical methods
Data are expressed as mean ±SEM. Differences between groups were determined by an unpaired samples t test with Bonferonni correction for multiple comparisons (dichotomous variables were tested with Chi-square). All P-values were two-tailed, and a P-value of <0.05 was considered statistically significant. Analyses were performed using SPSS software (SPSS version 12.0, Chicago, IL, USA).

RESULTS

Weight and blood glucose measurements:
Weight and blood glucose measurements of the 1 and 4 week groups are displayed in Table 1. There were no significant differences in weight and blood glucose.
**Histological analysis**

*Neointimal formation*

Neointimal area and control parameters are presented in Table 2.

DES versus DES: There was a significant difference in mean neointimal area between the Taxus and the Cypher stent. Neointimal formation was especially higher at the stent edges in the Taxus stent.

BMS versus BMS: Mean neointimal area was higher in the Express 2 compared to the Bx Velocity, however this difference was not significant. Neointimal area was especially higher at the stent edges in the Express 2 stent. BMS’s versus DES’s: There was no significant difference in mean neointimal area between the Express 2 and the Taxus stent. There was a significant difference in mean neointimal area between the Bx Velocity and the Cypher stent.

*Incomplete healing*

After 1 week no differences exist between bare metal stents and drug-eluting stents in measurements representing incomplete healing of the neointima as shown in Table 3 and Figures 2 and 3. However after 4 weeks drug-eluting stents had a higher inflammation score, lower cell density and more signs of incomplete healing (hemorrhage or acellularity of neointima) compared to bare metal stents as shown in Table 3 and Figures 2, 3 and 4. These differences were mainly observed in the neointima adjacent to the stent struts, but they were also found further from the stent struts. Collagen content was decreased in drug-eluting stents compared to bare metal stents as shown in Table 4.

**Table 1:** Weight and blood glucose measurements of the 1 and 4 week groups.

<table>
<thead>
<tr>
<th>1 week</th>
<th>A=Express 2</th>
<th>B=Taxus</th>
<th>C=BxVelocity</th>
<th>D=Cypher</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=7</td>
<td>N=5</td>
<td>N=6</td>
<td>N=6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beginweight (g)</td>
<td>360±6</td>
<td>376±1</td>
<td>354±9</td>
<td>372±5</td>
<td>NS /NS /NS /NS</td>
</tr>
<tr>
<td>Endweight (g)</td>
<td>342±3</td>
<td>340±6</td>
<td>334±7</td>
<td>357±6</td>
<td>NS /NS /NS /NS</td>
</tr>
<tr>
<td>Glucose (mmol/l) B</td>
<td>29.8±1.7</td>
<td>30.4±2.15</td>
<td>25.6±2.5</td>
<td>30.0±1.59</td>
<td>NS /NS /NS /NS</td>
</tr>
<tr>
<td>Glucose (mmol/l) 1</td>
<td>30.2±1.39</td>
<td>26.1±2.15</td>
<td>26.9±2.0</td>
<td>30.6±1.67</td>
<td>NS /NS /NS /NS</td>
</tr>
</tbody>
</table>

**4 weeks**

<table>
<thead>
<tr>
<th>N=8</th>
<th>N=8</th>
<th>N=8</th>
<th>N=8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beginweight (g)</td>
<td>354±12</td>
<td>388±12</td>
<td>368±6</td>
</tr>
<tr>
<td>Endweight (g)</td>
<td>354±12</td>
<td>388±14</td>
<td>368±6</td>
</tr>
<tr>
<td>Glucose (mmol/l) B</td>
<td>28.5±1.66</td>
<td>27.2±2.23</td>
<td>28.0±1.66</td>
</tr>
<tr>
<td>Glucose (mmol/l) 4</td>
<td>28.9±1.80</td>
<td>30.5±2.15</td>
<td>28.6±1.78</td>
</tr>
</tbody>
</table>

B=baseline 1=1week 4=4weeks

**Table 2.** Neointimal area, injury score, media and internal elastic lamina area after 4 weeks.

<table>
<thead>
<tr>
<th>Variable</th>
<th>A=Express 2</th>
<th>B=Taxus</th>
<th>C=BxVelocity</th>
<th>D=Cypher</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=8</td>
<td>N=8</td>
<td>N=8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IS (mean)</td>
<td>0.16±0.10</td>
<td>0.34±0.19</td>
<td>0.13±0.12</td>
<td>0±0</td>
<td>NS /NS /NS /NS</td>
</tr>
<tr>
<td>IS (mid)</td>
<td>0±0</td>
<td>0±0</td>
<td>0.17±0.17</td>
<td>0±0</td>
<td>NS /NS /NS /NS</td>
</tr>
<tr>
<td>IS (edges)</td>
<td>0.31±0.21</td>
<td>0.69±0.38</td>
<td>0.09±0.09</td>
<td>0±0</td>
<td>NS /NS /NS /NS</td>
</tr>
<tr>
<td>NA mean (mm²)</td>
<td>0.92±0.06</td>
<td>0.89±0.13</td>
<td>0.77±0.07</td>
<td>0.56±0.05</td>
<td>NS/P&lt;0.05/P&lt;0.05/NS</td>
</tr>
<tr>
<td>NA mid (mm²)</td>
<td>0.77±0.10</td>
<td>0.60±0.07</td>
<td>0.66±0.07</td>
<td>0.52±0.06</td>
<td>NS /NS /NS /NS</td>
</tr>
<tr>
<td>NA edges (mm²)</td>
<td>1.07±0.09</td>
<td>1.18±0.25</td>
<td>0.87±0.09</td>
<td>0.61±0.05</td>
<td>NS/P&lt;0.05/P&lt;0.05/NS</td>
</tr>
<tr>
<td>IEL area (mm²)</td>
<td>3.06±0.16</td>
<td>3.60±0.25</td>
<td>3.12±0.05</td>
<td>3.05±0.11</td>
<td>NS /NS /NS /NS</td>
</tr>
<tr>
<td>Media area (mm²)</td>
<td>0.15±0.01</td>
<td>0.19±0.02</td>
<td>0.17±0.01</td>
<td>0.16±0.01</td>
<td>NS /NS /NS /NS</td>
</tr>
</tbody>
</table>

Abbreviations: IS = Injury Score, NA = Neointimal Area, IEL = Internal Elastic Lamina
Table 3. Incomplete healing: results in the 1 and 4 week groups.

|                  | A=Express 2 | B=Taxus | C=BxVelocity | D=Cypher | P-value  
|------------------|------------|---------|--------------|----------|---------- 
| Inflammation score | 0.44±0.05  | 0.44±0.09 | 0.41±0.03    | 0.53±0.09 | NS/NS/NS/NS |
| Neointimal cell density (× 100/mm²) | 20.31±1.9   | 20.39±4.9   | 21.14±2.8   | 18.17±2.6   | NS/NS/NS/NS |
| Signs of incomplete healing (% of rats) | 100±0       | 100±0     | 100±0        | 100±0      | NS/NS/NS/NS |

**4 weeks**

|                  | N=8        | N=8      | N=8          | N=8       | P-value  
|------------------|------------|----------|--------------|-----------|---------- 
| Inflammation score | 0.03±0.02  | 0.19±0.06 | 0.04±0.02    | 0.16±0.04 | P<0.05/P<0.05/NS/NS |
| Neointimal cell density (× 100/mm²) | 44.62±2.59 | 14.30±1.87 | 43.37±2.84  | 17.91±3.39 | P<0.05/P<0.05/NS/NS |
| Signs of incomplete healing (% of rats) | 25±16      | 100±0    | 25±16        | 100±0     | P<0.05/P<0.05/NS/NS |

1 week: DES versus DES (B:D): There was no significant difference in inflammation score, cell density and signs of incomplete healing between the Taxus® Express²™ and the Cypher® Stent.

BMS versus BMS (A:C): There was no significant difference in inflammation score, cell density and signs of incomplete healing between Express® and the Bx Velocity® Stent.

BMS’s versus DES’s (A:B and C:D): the Express® did not differ from the Taxus® Express²™ Stent in inflammation score, cell density and signs of incomplete healing. The Bx Velocity® did not differ from the Cypher® Stent in inflammation score, cell density and signs of incomplete healing.

4 weeks:DES versus DES (B:D): There was no significant difference in inflammation score, cell density and signs of incomplete healing between the Taxus® Express²™ and the Cypher® Stent.

BMS versus BMS (A:C): There was no significant difference in inflammation score, cell density and signs of incomplete healing between Express® and the Bx Velocity® Stent.

BMS’s versus DES’s (A:B and C:D) the Taxus® Express²™ had a higher inflammation score, lower cell density and more signs of incomplete healing compared to the Express® Stent. The Cypher® Stent had a higher inflammation score, lower cell density and more signs of incomplete healing compared to the Bx Velocity® Stent.

Figure 1: Neointimal area (4 weeks mean, mid, end) in the four different groups.
Figure 2: Inflammation score and neointimal cell density in the four different groups (1 and 4 weeks). After 1 week there are no differences in inflammation score and neointimal cell density between bare metal and drug-eluting stents. However after 4 weeks there is more inflammation and lower cell density in the neointima of drug-eluting stents.

Table 4. Collagen content: results in the 4 week groups. In Group E (bare metal stents) collagen content was significantly greater compared with group F (drug-eluting stents).

<table>
<thead>
<tr>
<th>Variable</th>
<th>A=Express 2</th>
<th>B=Taxus</th>
<th>C=BxVelocity</th>
<th>D=Cypher</th>
<th>E=A+C</th>
<th>F=B+D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=8</td>
<td>N=8</td>
<td>N=8</td>
<td>N=8</td>
<td>N=16</td>
<td>N=16</td>
</tr>
<tr>
<td>Collagen content</td>
<td>4.4±0.9</td>
<td>1.5±0.7</td>
<td>2.1±0.6</td>
<td>0.4±0.4</td>
<td>3.25±0.6</td>
<td>1.0±0.4</td>
</tr>
</tbody>
</table>

No significant differences in total collagen content were found when all 4 groups were seperately compared however there was a trend towards less collagen content in the drug-eluting stents especially in the sirolimus-eluting stent. However after we combined both drug-eluting (E) and bare metal stent (F) groups and compared the two groups (E:F) a significantly lower collagen content was found in the drug-eluting stents (Table 4).
DISCUSSION

Neointimal formation
In the diabetic Zucker rat, no reduction in neointimal area was seen with the Taxus stent. The Cypher stent had decreased neointimal formation compared to its bare metal control. It is not likely that this difference in neointimal area can be explained by differences in cytostatic effects of both drugs, because the effect on cell density was similar with both drugs.

A more pronounced edge effect with the Express 2 stent is the most likely explanation which is not counteracted by paclitaxel. The trend towards an increase in neointimal formation seen in the Taxus stent design (Express 2 and Taxus) was largely attributed to an increase in neointimal formation at the edges of the stent (Table 2, Figure 1). Increased injury to the artery is correlated with more neointimal formation\(^1\). Interestingly in the Taxus and Express (same stent) there was also a trend towards more injury at the edges of the stent (Table 2). Diabetic arteries differ from non diabetic arteries: they contain more hyaluronan, type IV collagen, and fibronectin in the media\(^2\). So the Taxus and Express 2 stent design may induce more neointimal formation at the edges and a diabetic population may be especially at risk due to differences in vessel characteristics with vulnerability to injury. There is some evidence for this Iakovu et al reported in a retrospective analysis of 977 patients (26% diabetics) who received a Taxus stent a high incidence of in-stent restenosis at the stent edges\(^3\). This might indicate that stent design is still important for in-stent restenosis even in drug-eluting stents possibly because of its effect on local drug concentrations\(^4\).

Figure 3: Signs of delayed healing in the four different groups (1 and 4 weeks). After 1 week there are no differences in signs of delayed healing between bare metal and drug-eluting stents. However after 4 weeks there are more signs of delayed healing present in the neointima of drug-eluting stents.
Incomplete healing
Our results show no differences in neointimal cell density, inflammation score and signs of incomplete healing after 1 week between bare metal and drug-eluting stents (Table 3, Figure 2 and 3). However after 4 weeks neointimal is low and signs of incomplete healing and low inflammation are present in the drug-eluting groups (Table 3, Figure 2 and 3). Also collagen content is lower in drug-eluting stents compared with bare metal stents after 4 weeks (Table 4).

Figure 4: Examples of hemorrhage in Taxus en Cypher stents.

These results demonstrate that incomplete healing exist in a diabetic population with drug-eluting stents. This might be relevant since drug-eluting stents are often used in diabetic patients because they are more prone to develop in-stent restenosis. Increased inflammation, incomplete reendothelization and fibrin deposition were reported by others studying incomplete healing in drug-eluting stents. In this study we report also low neointimal cell density, acellularity and hemorrhage (Figure 4) and low collagen content as being characteristic for incomplete healing in drug-eluting stents in diabetic rats. We found also incomplete healing in non-diabetic rats (data not shown here). This suggests that incomplete healing is not specific for diabetic animals but rather a generic effect possibly due to the inhibiting effect of both sirolimus and paclitaxel on proliferating cells which than induces low neointimal cell density, impaired extracellular matrix formation and prolonged chronic inflammation. The precise mechanism underlying incomplete healing in drug-eluting stents is still unknown. Incomplete neointimal coverage has been associated with subclinical thrombus formation. So it would be interesting to study if our findings on incomplete neointimal healing in drug-eluting stents in a diabetic population are associated with thrombus formation and late stent thrombosis.

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References


ABSTRACT
Aims: Some studies suggest that the paclitaxel eluting stent and the sirolimus eluting stent might differ in their ability to reduce in-stent restenosis in diabetic patients. We wanted to determine in diabetic rats which drug-eluting stent (paclitaxel or sirolimus eluting stent) is better in reducing in-stent restenosis and secondly to assess if the neointima of drug-eluting stents is different compared to bare metal control stents in a diabetic population. Methods: Male Zucker rats were randomized to one of four groups: Express 2 stent, Taxus Express 2 stent, Bx Velocity stent and Cypher stent. Stents were implanted in the abdominal aorta. Histological analyses were performed after 1 and 4 weeks. After 1 week the inflammation score, neointimal cell density and signs of incomplete healing were measured. After 4 weeks the same measurements were performed plus the neointimal area, media area and lumen area. Results: After 4 weeks neointimal area was reduced in the sirolimus eluting compared to the paclitaxel eluting stent. No differences were observed after 1 week between bare metal stents and drug-eluting stents. After 4 weeks neointimal cell density was lower, inflammation-score was higher, signs of incomplete healing were increased and collagen content was lower in the drug-eluting stents. No differences in neointima healing were found between the paclitaxel and the sirolimus eluting stent. Conclusions: The sirolimus eluting stent reduced neointimal formation compared to the paclitaxel eluting stent. Secondly incomplete healing is also found in diabetic animals with drug-eluting stents but with no clear differences between the paclitaxel and the sirolimus eluting stent.