Local delivery of 17β-estradiol improves reendothelialization and decreases inflammation after coronary stenting in a porcine model

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Summary
In the current study, we investigated the effect of local intravascular delivery of 17β-estradiol (17β-E) on subsequent in-stent neointimal hyperplasia. Twenty-seven stents were implanted in coronary arteries of juvenile swine. Coronary arteries were randomized to local treatment with 17β-E or no drug therapy (control-vehicle treated). Twenty-eight days post-treatment, angiographic images revealed an improved minimal lumen diameter (2.2 ± 0.2 vs. 1.3 ± 0.2 mm, P < 0.005) and a reduction of late lumen loss (1.7 ± 0.2 vs. 2.3 ± 0.1 mm, P < 0.01) in 17β-E-treated vessels compared to control-vehicle treated. Histological analyses showed a reduction of stenosis (51.49 ± 6.75 vs. 70.86 ± 6.24%, P < 0.05), mean neointimal thickness (0.51 ± 0.07 vs. 0.83 ± 0.14 mm, P < 0.05) and inflammation score (1.29 ± 0.28 vs. 2.85 ± 0.40, P < 0.05) in 17β-E-treated arteries compared to control-vehicle treated arteries. Immunohistochemistry analyses revealed a reduction of proliferating smooth muscle cells and increased in-stent reendothelialization in 17β-E-treated arteries. Finally, we observed a correlation between neointimal hyperplasia and inflammation score, which in turn, was inversely related to reendothelialization. Locally delivered, 17β-E is inhibiting the inflammatory response and smooth muscle cells proliferation and improving vascular reendothelialization which together are contributing to reduce in-stent restenosis in a porcine coronary injury model. Together, these data demonstrate the potential clinical application of 17β-estradiol to improve vascular healing and prevent in-stent restenosis.

Keywords
Coronary disease, hormones, inflammation, restenosis, stents

Introduction
Restenosis following percutaneous transluminal coronary angioplasty (PTCA) occurs in nearly 40% of patients and is predominantly due to neointimal hyperplasia and constrictive remodeling. This latter event can be countered by the use of stents, which may account for the decreased incidence of restenosis observed with their use (1, 2). Despite the advent of stents, restenosis continues to limit the long-term success of PTCA. Stenting may evoke an exuberant proliferative response compared to balloon-catheter angioplasty. The favorable effect of stents in countering constrictive remodeling is therefore in some cases offset by the increased neointimal hyperplasia associated with stenting, thus interfering with our capacity to efficiently prevent arterial reclosure (3). Previous studies reported that systemic administration of 17β-estradiol (17β-E) prevents neointimal hyperplasia post-PTCA (4, 5). More recently, we observed that a single dose of 17β-E administered locally at the injury site was sufficient to reduce neointimal hyperplasia upon PTCA procedure in pigs (6). However, the effects of 17β-E delivered either systemically or locally on processes leading either to in-stent inflammation and reendothelialization, or on vascular healing have not been explored. Thus, the current study was designed to investigate the effect of a local delivery of 17β-E on in-stent neointimal hyperplasia, inflammation and reendothelialization and possible underlying mechanisms.

Methods
Animal preparation
Nine juvenile farm pigs weighing 20–25 kg (four immature females with intact ovaries, five castrated males) were used as pre-
vously described (6). As the female pigs were prepubertal and the male was castrated, there was no need to make distinctions between possible gender effect from the data obtained. The Animal Care and Ethical Research Committee of the Montreal Heart Institute approved the study protocol. Briefly, each animal received 650 mg of acetylsalicylic acid and 30 mg of nifedipine orally one day prior to the initial procedure. After premedication with 6 mg/kg of tiletamine-hydrochloride and zolazepam-hydrochloride (Fort Dodge Animal Health) and administration of 50 µg/kg of atropine (Abbott Laboratories) intramuscularly, the right femoral artery was cannulated percutaneously under general anesthesia. Before the procedure, 100 mg xylocaine (Astapharma Inc) and 250 U/kg heparin (Organon Teknika) were administered intra-arterially. Activated coagulation time was maintained at >350 seconds throughout the procedure, with supplemental heparin given as required.

Procedure
Balloon-catheter angioplasty was followed by stent implantation at high pressure to simulate clinical procedures. The left anterior descending (LAD), circumflex (LCX) and right coronary arteries (RCA) of the animals were randomized to receive locally at the injury site either 100 µg/kg 17β-E, 200 µg/kg 17β-E or no drug therapy (control-vehicle). Two 30-second inflations at 10 atm pressure were performed with a standard balloon catheter (estimated inflated balloon diameter: artery ratio was 1.1–1.2:1), with a 30-second interval between inflations. Upon balloon angioplasty, local delivery was performed as previously described (6) using the InfusaSleeve catheter (LocalMed, Inc.) (7). A 7-mm long cuffed tubular stent (Palmaz-Schatz, Johnson & Johnson) was then crimped on the previously-used balloon and deployed at 14 atm pressure for 30 seconds (to achieve a stent:artery ratio of 1.3–1.4:1 at full expansion of the stent). Stent implantation was performed on all three coronary arteries (LAD, LCX, RCA) of each animal.

Coronary angiography
After 28 days, the animals underwent coronary angiography. Images were captured at a speed of 30 frames/s and digitized. To avoid error due to magnification, a segment of contrast-filled guiding catheter was included in every frame for calibration. Quantitative coronary analysis was performed using a computerized edge-detection algorithm. The following coronary diameter measurements were made using diastolic frames by an observer blinded to the treatment allocation of each vessel: 1) basal diameter before injury; 2) diameter at full stent expansion; 3) stent:artery ratio; 4) minimal lumen diameter (MLD) of the stented segment at 4 weeks; 5) %diameter stenosis and 6) late lumen loss (8).

Morphometry
Upon completion of follow-up coronary angiography, the animals were euthanized under general anaesthesia as previously described (6). The stented segments were excised and stored in 10% formalin PBS-buffered solution for 24 h. The specimens were then processed by a modification of the technique as described by Wolf et al. (9). Sections of 8-µm thickness were prepared using a motorized microtome (Olympus) with a D-profile tungsten knife (Delaware Diamond Knife), and mounted on gelatin-coated slides for histological and immunohistochemical staining.

Morphometric analyses were performed on sectioned arteries stained with Verhoeff’s solution, and measurements were made by digital planimetry. Two sections from each stented coronary artery (demonstrating maximum lumen narrowing macroscopically from the proximal and distal halves of each stent) were analyzed and the results averaged. The external elastic laminae area (EEL), internal elastic laminae area (IEL) and lumen area were measured, and the %morphologic stenosis ([IEL-lumen area]/IEL) × 100 was calculated (10). Mean neointimal thickness was derived by the sum of neointimal thickness at each stent strut/total number of struts. Injury score was determined as previously defined (11).

Immunohistochemistry
Immunohistochemical analyses were performed on cross-sections of coronary arteries. For each artery, analysis was performed on the segment of the stent demonstrating maximal neointimal thickness by morphometry. Inflammatory response was assessed by the detection of infiltrated macrophages with rat monoclonal anti-mouse MAC-2 IgG antibodies (Cederlane Laboratories). The percentages of proliferating smooth muscle cells and reendothelialization were assessed respectively by using mouse monoclonal anti-human proliferating cell nuclear antigen (PCNA) IgG (Zymed Laboratories) and goat polyclonal anti-mouse platelet/endothelial cell adhesion molecule-1 (PECAM-1; CD-31) IgG (Santa Cruz Biotechnology Inc.). Non-specific binding of primary antibodies was prevented by pre-incubating the tissues with 5%–serum from the species used to raise secondary antibodies. Purified non-specific rat, mouse IgG or goat IgG were used as primary negative control antibodies. Upon incubation, the primary antibodies were washed with PBS, the slides incubated for 60 min either with a biotinylated goat anti-rabbit or anti-mouse IgG (Vector Labs Inc.). Peroxidase labeling was achieved with an incubation using avidin/peroxidase complex (ABC kit; Vector Labs Inc.), and antibodies visualization established upon exposure to 3,3’-diaminobenzidine solution (DAB kit; Vector Labs Inc.), followed by Mayer’s hematoxylin counterstaining.

Table 1: Angiographic data analyses of porcine coronary arteries at basal, post-stent implantation, and at 28 days post-procedure.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control-vehicle treated</th>
<th>Low-dose 17β-estradiol (100 µg/kg)</th>
<th>High-dose 17β-estradiol (200 µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal diameter (mm)</td>
<td>2.8 ± 0.2</td>
<td>3.1 ± 0.1</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>Post-stent diameter (mm)</td>
<td>3.7 ± 0.2</td>
<td>3.9 ± 0.1</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>Stent:artery ratio</td>
<td>1.34 ± 0.03</td>
<td>1.27 ± 0.03</td>
<td>1.28 ± 0.04</td>
</tr>
<tr>
<td>Follow-up MLD (mm)</td>
<td>1.3 ± 0.2</td>
<td>2.2 ± 0.21</td>
<td>2.3 ± 0.21</td>
</tr>
<tr>
<td>% diameter stenosis</td>
<td>65.8 ± 4.7</td>
<td>42.8 ± 4.17</td>
<td>41.2 ± 4.31</td>
</tr>
<tr>
<td>Late lumen loss (mm)</td>
<td>2.3 ± 0.1</td>
<td>1.7 ± 0.24</td>
<td>1.7 ± 0.24</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. *P < 0.01 vs. control, †P < 0.005 vs. control. MLD: minimal lumen diameter.
Immunohistochemical measurements were made under microscopic high-power magnification (×1000). Inflammation score (the extent and density of macrophage infiltrate) was determined as follows: grade 0 = no macrophages surrounding the strut, grade 1 = presence of very few macrophages surrounding the strut, grade 2 = few, grade 3 = moderate, and grade 4 = many macrophages surrounding the strut. The inflammation score for each section was obtained by dividing the aggregate of inflammation score around each strut by the total number of struts.

The proliferating SMC index (%) was calculated as ΣPCNA-positive SMC/ΣSMC in each high-power magnification field. To standardize comparisons among the treatment groups, high-power magnification measurements for each section were obtained at four fixed locations separated apart by 90°, and the results averaged. Only cells with distinct staining of nuclei were considered positive.

The lumen circumference and the sum-total of the luminal border staining positively for PECAM-1 expression were measured for each section. The degree of reendothelialization was evaluated by the percentage of vascular lumen covered by endothelial cells staining positively for PECAM-1. To exclude any possible role of bias, an examiner with no knowledge of the treatment groups to which the sections belonged, performed morphometric and immunohistochemical measurements.

### Statistical analyses

Values are expressed as mean ± S.E.M. Baseline and follow-up angiographic parameters, morphometric, and immunohistochemical data among the three treatment groups were compared with one way repeated measures of variance (ANOVA). Post-hoc comparisons were obtained with Dunnett adjustment. For the purpose of data presentation, treatments with 100 μg/kg or 200 μg/kg of 17β-E were considered as low-dose and high-dose therapy respectively. Results were considered significant if \( P < 0.05 \).

### Results

#### Follow-up post-procedure

All nine animals recovered uneventfully; there was no mortality and no incidence of acute or subacute stent thrombosis. One coronary artery randomized to treatment with high-dose 17β-E developed extensive dissection following manipulation of an accidentally displaced guide wire after predilatation; drug delivery and stent implantation were not performed, and the artery was excluded from analysis. No changes in heart rate, ECG, or blood pressure were noted during local delivery of 17β-E.

#### Coronary angiography and morphometric analyses

Among the three treatment groups, no significant differences were noted in basal diameter, stent to artery ratio, and post-stent implantation diameter (Table 1). At follow-up, in control-vehicle group, the degree of stenosis was 65.8 ± 4.7%. Treatment with 17β-E (100 or 200 μg/kg) reduced significantly the degree of stenosis to 42.8 ± 4.1% and 41.2 ± 4.3%, respectively. In addition, treatment with 17β-E (randomized low and high doses) reduced the late lumen loss observed in control-vehicle group by 26% (Table 1, Fig. 1). To ascertain that stent deployment in control-vehicle and 17β-E-treated groups was equivalent, we measured the EEL and IEL areas and injury score, and did not observe any statistical differences between the three groups (Table 2). However, treatment of coronary arteries with the low-dose of 17β-E reduced the percentage of morphologic stenosis (51.49 ± 6.75% vs. 70.86 ± 6.24% for control-vehicle treated group, \( P < 0.05 \)) and neointimal thickness (0.51 ± 0.07 mm vs. 0.83 ± 0.14 mm for control-vehicle treated group, \( P < 0.05 \)). Treatment with high-dose of 17β-E was slightly less effective, failing to achieve statistical significance (Table 2). We then performed a correlation analysis to assess if the beneficial effect of 17β-E on the reduction of neointimal hyperplasia correlated with the extent of coronary arterial injury. To maximize the power analysis of our study, the groups of animals treated with 17β-E (100 and 200 μg/kg) were pooled. We observed, both in

### Table 2: Morphometric analyses of porcine coronary arteries at 28 days post-procedure.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control-vehicle treated</th>
<th>Low-dose 17β-estradiol (100 μg/kg)</th>
<th>High-dose 17β-estradiol (200 μg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEL area (mm²)</td>
<td>7.97 ± 0.84</td>
<td>8.69 ± 0.68</td>
<td>8.45 ± 0.59</td>
</tr>
<tr>
<td>IEL area (mm²)</td>
<td>7.19 ± 0.76</td>
<td>7.69 ± 0.67</td>
<td>6.97 ± 0.65</td>
</tr>
<tr>
<td>Injury score</td>
<td>1.99 ± 0.09</td>
<td>1.99 ± 0.15</td>
<td>2.02 ± 0.13</td>
</tr>
<tr>
<td>% morphologic stenosis</td>
<td>70.86 ± 6.24</td>
<td>51.49 ± 6.75</td>
<td>54.74 ± 5.42</td>
</tr>
<tr>
<td>Neointimal thickness (mm)</td>
<td>0.83 ± 0.14</td>
<td>0.51 ± 0.07</td>
<td>0.58 ± 0.10</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. \( *P < 0.05 \) vs. control, \( **P < 0.005 \) vs. control. EEL: external elastic laminae, IEL: internal elastic laminae.
Control-vehicle and 17β-E-treated coronary arteries, a positive correlation between the injury score and the neointima hyperplasia (Fig. 2). More importantly, we observed that the beneficial effect of 17β-E was significantly increased in function of the injury score (grade 2 and 3), providing up to 40% reduction of neointimal thickening as compared to control-vehicle-stented arteries (Fig. 2).

Beneficial effect of 17β-E on the vascular healing process
In a recent study, we defined the capacity of 17β-E to promote cultured endothelial cells proliferation and reduce smooth muscle cells proliferation (12). In addition, since a treatment with 17β-E may provide anti-inflammatory activities, we then wanted to assess if a local treatment with 17β-E would reduce the inflammatory response and favour the reendothelialization process in stented coronary arteries. By immunohistochemical analysis, we observed a marked infiltration of macrophages in control-vehicle treated group, mainly localized around the stent struts. On the other hand, a single bolus intravascular delivery of 17β-E within the stented coronary injury sites led to a profound reduction of macrophages infiltration, lowering the score of inflammatory cellular infiltration from 2.85 ± 0.40 to 1.29 ± 0.28 (P < 0.05) as compared to control-vehicle treated arteries (Figs. 3, 5).

We then assessed the proliferating state of SMCs and the degree of reendothelialization. By PCNA staining of native uninjured coronary arteries, we observed that the basal level of proliferative SMCs was 0.89 ± 0.30%. At day 28, post-stent implantation, the %-proliferating SMCs values were lower in the groups exposed to 17β-E treatment. Arteries treated with the randomized low- and high-dose of 17β-E showed an average of 5.64 ± 1.18% of PCNA-positive SMCs, respectively, as compared to 11.51 ± 2.34% (P = 0.04) in control-vehicle treated coronary arteries (data not shown). Finally, we addressed the percentage (%) of reendothelialization by the immunohistochemical detection of endothelial PECAM-1 expression in stented arteries. Treatment with the randomized low- and high-dose of 17β-E provided a significant reendothelialization of the injured stented-arteries increasing it from 22.32 ± 4.9% in control-vehicle treated coronary arteries to 65.83 ± 14.61% (P < 0.05) (Figs. 4, 5).

Discussion
In the current study, we observed that the local delivery of 17β-E at the time of initial PTCA and stent implantation reduces SMCs proliferation, limits the degree of inflammation, and enhances vascular reendothelialization, which together are contributing to the reduction of in-stent restenosis 28 days post-procedure.
Previous studies have reported the beneficial effects of 17β-E following arterial injury. Prolonged systemic therapy with 17β-E by either subcutaneous or intramuscular delivery has been observed to inhibit neointimal hyperplasia following balloon arterial injury in animals (4, 5, 13, 14), whereas we reported that a single dose of locally delivered 17β-E was sufficient by itself to provide such inhibitory activity on neointimal hyperplasia post-PTCA in pig coronary arteries (6).

More recently, New et al. demonstrated the potential benefit of estrogen-coated stents to prevent neointimal hyperplasia (15). These observations were supported as well by the Estrogen And Stents To Eliminate Restenosis (EASTER) trial using a 17β-E-eluted BiodVysio stent (Biocompatibles Ltd.), in which a low rate of binary restenosis and revascularization were observed (16). In the present study, we assessed the efficacy of a local bolus endovascular delivery of 17β-E in preventing restenosis upon implantation of non-coated stents.

The role of inflammatory cell infiltration in neointimal hyperplasia following arterial injury is well recognized (17, 18). Thus, the ability of 17β-E to inhibit the adhesion and chemotaxis of inflammatory cells within impaired vasculature (19, 20) might play a pivotal role in the prevention of neointimal hyperplasia. Kastrati et al. have shown that a polymorphism of the gene of interleukin (IL)-1 receptor antagonist, a protein that regulates inflammatory response, was associated with reduced restenosis (21). In our study, we observed a significant reduction in inflammatory cells infiltration (macrophages) (Fig. 4).

The induction of stent reendothelialization mediated by 17β-E may also contribute to the cytoprotective effect of 17β-E to reduce neointimal hyperplasia. The cytoprotective activity of the neointima is probably supported by the capacity of the estrogen to enhance endothelial NO synthesis (22, 23), which concurs with a previous report in which we detailed that a local therapy with 17β-E during PTCA conferred endothelial protection by enhancing reendothelialization and up-regulating eNOS expression at the PTCA site (24). Based on our in vitro study, 17β-E enhances reendothelialization by promoting endothelial cell migration and proliferation upon ERα activation (12).

The capacity of 17β-E to prevent neointimal hyperplasia is also gaining by the capacity of NO to inhibit SMCs proliferation (25), neointimal hyperplasia (26), but also by its direct capacity to prevent SMCs migration and proliferation through ERβ activation (12).

Thus, multiple protective mechanisms might be involved in the beneficial effects of local delivery of 17β-E.

**Dose-related effects of locally delivered 17β-E**

Overall, the effects mediated with a low (100 µg/kg) and high dose (200 µg/kg) of 17β-E were relatively similar and no significant differences were observed between the two groups. It is possible that the low-dose of 17β-E was already sufficient to saturate the estrogen receptors preventing any additional effects of a higher dose of 17β-E. Furthermore, it is noteworthy to mention that the doses chosen in this stent-study were higher than those previously used in our initial angioplasty-study (600 µg) (6). Therefore, the use of lower doses would be required to define a possible 17β-E dose-response effect.

**Study limitations**

In the present model, stent implantation was performed in normal porcine coronary arteries. It may not be appropriate to extrapolate our results to atherosclerotic coronary arteries. It is acknowledged that systemic spillover of 17β-E could occur during local delivery. Should an effect due to systemic spillover exist, it should apply equally to all three treatment groups, as all three treatment arms were performed in a randomized fashion in each animal. In addition, 17β-E that enters the circulation is rapidly eliminated, mostly by hepatic metabolism (27). Thus, the results of the study clearly show a difference between the estrogen-treated and control-vehicle treated arteries. We used standard metal stents and the results could have been different if drug-eluting stents had been used as control group. However, it as been proposed that sirolimus- or paclitaxel-eluting stents could delay reendothelialization and be related to late thrombosis when clopidogrel is discontinued prematurely (28).

In summary, we have shown that a single dose of 17β-E delivered locally at the time of stent implantation has the potential to reduce subsequent in-stent restenosis in a porcine coronary arterial injury model. The ability of locally delivered 17β-E to inhibit SMC proliferation, and the inflammatory response to injury, as well as to enhance reendothelialization could possibly contribute to its beneficial effects in preventing neointimal hyperplasia and late stent thrombosis. These results are consistent with existing evidence demonstrating the inhibitory effects of prolonged systemically administered 17β-E on neointimal hyperplasia following arterial injury, but extend these findings to instant restenosis and indicate that a single locally-administered dose of 17β-E may be sufficient for substantial benefit. The approach of preventing in-stent restenosis by a single dose of 17β-E administered locally at the time of the initial procedure could be promising for the improvement of the outcome of clinical PTCA with stent implantation, and merits further investigation.

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