Inhibition of in-stent restenosis by oral copper chelation in porcine coronary arteries

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Submitted 8 February 2006; accepted in final form 16 May 2006

Mandinov, L., K. L. Moodie, A. Mandinova, Z. Zhuang, F. Redican, D. Baklanov, V. Lindner, T. Maciag, M. Simons, and E. D. de Muinck. Inhibition of in-stent restenosis by oral copper chelation in porcine coronary arteries. Am J Physiol Heart Circ Physiol 291: H2692–H2697, 2006. First published May 26, 2006; doi:10.1152/ajpheart.00148.2006.—Stress-induced release of IL-1α and fibroblast growth factor-1 is dependent on intracellular copper and is a major driver of neointimal hyperplasia. Therefore, we assessed the effect of tetrathiomolybdate (TTM), a clinically proven copper chelator, on in-stent restenosis. Nine pigs were treated with TTM (5 mg/kg po) twice daily for 2 wk before stent implantation and for 4 wk thereafter, and nine pigs served as controls. In-stent restenosis was assessed by quantitative coronary angiography (QCA), intravascular ultrasound (IVUS), and histomorphometry. Serum ceruloplasmin activity was used as a surrogate marker of copper bioavailability. In TTM-treated animals, ceruloplasmin dropped 70% below baseline levels. Baseline characteristics were comparable in TTM-treated and control animals. At 4-wk follow-up, all parameters relevant to in-stent restenosis were significantly reduced in TTM-treated animals: minimal lumen diameter by QCA was 2.03 ± 0.57 and 1.47 ± 0.45 mm in TTM-treated and control animals, respectively (P < 0.05), percent stenosis diameter was 39% less in TTM-treated animals (27.1 ± 16.6% vs. 44.5 ± 16.1%, P < 0.05), minimal lumen area by IVUS was 60% larger in TTM-treated animals (4.27 ± 1.56 vs. 2.67 ± 1.19 mm2, P < 0.05), and neointimal volume by histomorphometry was 37% less in TTM-treated animals (34.9 ± 11.5 vs. 55.2 ± 19.6 mm3, P < 0.05). We conclude that systemic copper chelation with a clinically approved chelator significantly inhibits in-stent restenosis.

inflammation; stent

MATERIALS AND METHODS

Animal study protocol. This study was approved by the Animal Care and Use Committee of the Dartmouth Hitchcock Medical Center and conformed to the tenets of the American Heart Association on research animal use. The animals were handled according to the National Institutes of Health Guide for Care and Use of Laboratory Animals. Twenty-two male domestic pigs (30–40 kg body wt) were entered into the study; 18 completed the protocol and were used in the analysis. The animals were started on an oral dose of TTM (5 mg/kg) given twice daily (n = 9) or vehicle control (n = 9) 2 wk before stent implantation. Oral aspirin (325 mg) and clopidogrel (300 mg loading dose followed by 75 mg daily) were given on the day of stent implantation and continued daily until the end of the study. Anesthesia was induced by ketamine (20 mg/kg) followed by 1–2% isoflurane in oxygen. Access to the left jugular vein and carotid artery was obtained through 6-F sheaths, and after systemic heparinization (150 U/kg and 10,220.33.5 on June 11, 2017 http://ajpheart.physiology.org/ Downloaded from http://ajpheart.physiology.org/ by 10,220.33.5 on June 11, 2017

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Intracoronary injection of 0.2 mg of nitroglycerin, baseline angiograms of the left anterior descending coronary artery were obtained in two orthogonal projections. After another bolus injection of 0.2 mg of nitroglycerin, motorized intravascular ultrasound (IVUS) pullback images of the target segment were acquired at 0.5 mm/s. A single 2.5 × 15 or 3.0 × 15 mm stent (Multi-Link Zeta, Guidant) was implanted at 8 atm pressure in the proximal left anterior descending coronary artery, resulting in a stent-to-artery ratio of 1.2:1. Angiograms and IVUS pullback were repeated immediately after stent implantation. After 4 wk, the animals underwent repeat angiography in the same orthogonal views, as well as IVUS imaging. After euthanasia, the chest was opened, and the coronary arteries were perfusion fixed for histological analysis.

**TTM preparation and serum chemistry assay.** TTM (Aldrich Chemical, Milwaukee, WI) was dissolved in water, mixed with applesauce, and given to the animals. Because the synthesis of ceruloplasmin is directly regulated by the bioavailability of copper to the liver, we used the serum ceruloplasmin level as a surrogate marker of free copper status (26). Serum ceruloplasmin level was measured by assay for oxidase activity of the enzyme per unit mass of enzyme protein, as described previously (26). In the active treatment group, serum ceruloplasmin oxidase activity was checked at baseline, after 1 wk of TTM administration, and on the day of stent implantation. In the control group, ceruloplasmin was measured 1 wk before stent implantation and on the day of intervention. After stent implantation, ceruloplasmin was measured every week in the TTM-treated group and every other week in the control group (Fig. 1). Blood samples (5 ml), obtained from the jugular vein after ketamine anesthesia, were centrifuged for 10 min at 2,000 g at 4°C, and the serum was frozen at −20°C until assay.

**Quantitative angiographic analysis.** Coronary angiograms were analyzed in end diastole by independent observers blinded to the treatment and IVUS and histomorphometric results. Quantitative analysis of the angiograms was performed using an automatic edge detection algorithm (CRS-PC, GE-OEC Medical Systems, Salt Lake City, UT). The guiding catheter was used as the scaling device for calibration. The minimum lumen diameter (MLD) was defined as the shortest distance between all measured left and right boundaries in the “worst” view. The diameters of the lumen, proximal and distal to the target location, were averaged to obtain a mean reference vessel diameter (RVD). The following angiographic parameters were calculated: percent stenosis diameter at follow-up [(1 − MLDfollow-up/ RVDfollow-up) × 100] and overstretching [(1 − stent diameterpostprocedural/ RVDbaseline) × 100]. Acute gain was defined as the change in MLD from baseline to the end of the procedure, and late lumen loss was defined as the change in MLD from the end of the procedure to follow-up. The stent analysis was confined to the stent itself (in-stent analysis), and the segment analysis included the stent plus a 5-mm segment proximal and a 5-mm segment distal to the stent (segment analysis).

**IVUS imaging protocol and analysis.** Studies were performed with an Oracle In-Vision IVUS Imaging System (EndoSonics/Jomed) and IVUS catheter (2.9F, Avanar, Jomed, Rancho Cordova, CA). Imaging runs started at ≥1 cm distance from the stented segment and ended 1 cm proximal to the stented segment. In three animals, two from the TTM-treated group and one from the control group, IVUS could not be performed because of technical problems with the equipment. A computer-based system was used for analysis of all IVUS records in offline mode by experienced observers blinded to the angiograms, histology, and treatment. The lumen border, stent contour, and external elastic membrane were manually delineated in slices representing five equal segments (segment analysis), each 5 mm long (proximal edge, proximal stent body, central stent articulation, distal stent body, and distal edge), and the following parameters were automatically calculated: lumen area (LA), stent area, and vessel area. The minimal value for LA among all stent segments was identified and used as a minimal LA (MLA) in the analysis. The minimal value for stent diameter among all stent segments was identified and used as a minimal stent diameter in the analysis. Mean stent area was calculated as an averaged value of all stent segments.

**Histomorphological analysis.** Coronary arteries were perfused with 500 ml of 0.9% NaCl and then perfusion fixed for 10 min at 100–150 mmHg pressure using 4% formaldehyde buffered in PBS (pH 7.4). A vessel segment containing the stent and ≥5 mm of the native vessel on both sides of the stent was excised with adjacent tissue and stored in 4% formaldehyde for 24 h. Then the specimen was cut transversely in two equal parts, transferred to JB-4 Plus (Polysciences, Warrington, PA) infiltration solution at 4°C in dark bottles for up to 1 wk, and embedded in JB-4 Plus resin under anaerobic conditions. The stent was cut with a tungsten carbide knife (DDK) into 5-μm-thick sections at 0.4-mm intervals, which resulted in 33–38 samples per stent. The sections were stained with hematoxylin and eosin, and measurements were carried out on each section with Scion software (digital color camera interfaced with a computer and Optimas 6.0 software) through an optical microscope integrated to a digitizing tablet. From each slice, lumen border, stent, and internal and external elastic lamina (IEL and EEL) were traced manually by an investigator blinded to the treatment as well as quantitative coronary angiography (QCA) and IVUS results, and lumen, stent, IEL, and EEL areas were computed for each section. Mean vessel area was calculated from the sum of all EEL areas divided by the number of slices. Neointimal area was calculated as the difference between IEL area and LA for every individual slice. Simpson’s rule was used to calculate neointimal volume for each stent. Minimal and maximal LA were identified for each stent, and an averaged LA from all analyzed slices was calculated as well. With the assumption that the lumen is a perfect circle, MLD was calculated from the MLA and compared with the QCA and IVUS data. The severity of strut-induced injury was averaged over all struts in each vessel and scored as described by Jawien et al. (12): 0, IEL intact; 1, IEL fractured by strut; 2, lacerated media; and 3, strut-ruptured EEL.
Statistical analysis. Statistical analysis was performed using StatView 5.0.1. Continuous variables are expressed as means ± SD. A Student’s t-test was used to examine the differences between the experimental groups. The time courses before and after treatment of the ceruloplasmin levels, RVD, and MLD were compared by ANOVA for repeated measures. The strength of the association of change in ceruloplasmin level remained stable throughout the study, whereas in the TTM-treated animals it decreased sharply by 70 ± 10% within the first 2 wk of TTM application and then remained relatively stable during the 4-wk follow-up.

RESULTS

Baseline characteristics. There were no significant differences in body weight and baseline ceruloplasmin level between the two groups, and procedural characteristics, such as implanted stent size, percent arterial overstretch ratio, and injury score, were also well matched (see online version of this article for supplemental Table I). No acute or subacute thrombosis occurred after stent implantation, and no gross or luminal thrombi were observed. Four of the 22 animals were lost and were not included in the analysis: 1 TTM-treated animal died because of infection within the first week of the study, 1 control animal was lost because of surgical bleeding during the initial procedure, and 1 animal in each group died from ventricular fibrillation during the intervention (Figs. 1 and 2). None of the events could be attributed to TTM treatment.

TTM and serum ceruloplasmin oxidase activity. Serum ceruloplasmin levels in the TTM-treated and control groups at different times are shown in Fig. 1. In the control group, ceruloplasmin level remained stable throughout the study, whereas in the TTM-treated animals it decreased sharply by 70 ± 10% within the first 2 wk of TTM application and then remained relatively stable during the 4-wk follow-up.

QCA, IVUS, and histomorphological analysis of in-stent restenosis. Angiographic, IVUS, and histomorphological data are summarized in Table 1. There were no significant differences in baseline RVD, stent size, and overstretching between the TTM-treated and control animals. RVD remained comparable between the two groups at the 4-wk follow-up. In-stent MLD was similar in the two groups immediately after stent implantation (Fig. 2); however, it decreased more rapidly during follow-up in control (Fig. 2A) than in TTM-treated (Fig. 2B) animals, so that at 4 wk after stent implantation, MLD was significantly larger in TTM-treated than in control animals: 2.03 ± 0.57 vs. 1.47 ± 0.45 mm (P < 0.05; Fig. 2C).

In-stent stenosis diameter by QCA, MLA by IVUS, and neointimal volume by histomorphometry are summarized in Fig. 3. Stenosis diameter decreased 39% in TTM-treated animals: from 44.4 ± 16.1% (control) to 27.1 ± 16.6% (P < 0.04; Fig. 3A). Although the acute gain within the stented segment was similar in the two groups, the late lumen loss was 34% less

Table 1. Postprocedural and follow-up stent and vessel measurements by QCA, IVUS, and histology

<table>
<thead>
<tr>
<th></th>
<th>Groups</th>
<th>TTM</th>
<th>Control</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td>QCA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>9</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RVD, mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.76±0.18</td>
<td>2.67±0.36</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>Postprocedure</td>
<td>2.76±0.18</td>
<td>2.67±0.36</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>Follow-up</td>
<td>2.79±0.2</td>
<td>2.74±0.34</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>MLD, mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postprocedure</td>
<td>3.10±0.21</td>
<td>3.00±0.33</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Follow-up</td>
<td>2.03±0.57</td>
<td>1.47±0.45</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Stenosis, Diameter %</td>
<td>27.1±16.6</td>
<td>44.5±16.1</td>
<td>&lt;0.04</td>
<td></td>
</tr>
<tr>
<td>Acute gain, mm</td>
<td>0.35±0.18</td>
<td>0.31±0.18</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>Late lumen loss, mm</td>
<td>1.04±0.47</td>
<td>1.57±0.56</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Minimal CSA, (follow up), mm²</td>
<td>3.48±1.93</td>
<td>1.86±1.01</td>
<td>&lt;0.04</td>
<td></td>
</tr>
</tbody>
</table>

| IVUS | | | | |
| n | 7 | 8 | | |
| MLA, (follow-up), mm² | 4.27±1.56 | 2.67±1.19 | <0.05 |
| MLD, (follow-up), mm² | 2.15±0.34 | 1.62±0.42 | <0.03 |
| MSA, (follow-up), mm² | 7.50±0.9 | 6.80±1.4 | 0.25 |
| MSD, (follow-up), mm² | 3.00±0.2 | 2.92±0.3 | 0.26 |

| Histomorphometry | | | | |
| n | 9 | 9 | | |
| LA, mm² | | | | |
| Minimal | 3.27±1.72 | 1.89±0.92 | <0.05 |
| Maximal | 5.10±1.35 | 3.37±1.21 | <0.02 |
| Mean | 4.15±1.33 | 2.70±1.21 | <0.03 |
| Mean VA, mm² | 6.30±0.96 | 6.40±0.59 | 0.71 |
| Neointima volume, mm³ | 34.9±11.5 | 55.2±19.6 | <0.02 |
| MLD, mm | 1.98±0.54 | 1.50±0.40 | <0.05 |

Values are means ± SD. TTM, tetrathiomolybdate; QCA, quantitative coronary angiography; RVD, reference vessel diameter; MLD, mean lumen diameter; CSA, cross-sectional area; IVUS, intravascular ultrasound; MLA, minimal lumen area; MSA, mean stent area; MSD, minimal stent diameter; LA, lumen area; VA, vessel area.
in the TTM-treated animals (1.04 ± 0.47 vs. 1.57 ± 0.56 mm, P < 0.05; Table 1). No significant differences in the proximal and distal 5-mm segments beyond the stent were found between the two groups. IVUS analysis confirmed the beneficial effect of TTM treatment on lumen size at follow-up: a 60% larger MLA in the TTM-treated animals (4.27 ± 1.56 vs. 2.67 ± 1.19 mm², P < 0.05; Fig. 3B). In addition, there was good agreement between the angiographic and IVUS analysis in MLD estimation at follow-up (r = 0.89, P < 0.0001).

Finally, despite a similar degree of injury from the stent struts (see supplemental Table I), there was marked neointimal formation in the control animals (Fig. 4A) and sparse neointimal hyperplasia in the TTM-treated animals (Fig. 4B). Histomorphometric reconstruction of neointimal volume showed a significant 37% reduction in neointimal volume in the TTM-treated animals (34.9 ± 11.5 vs. 55.2 ± 19.6 mm³, P < 0.02; Fig. 3C). In addition, the agreement between MLD estimations by angiography and histology (r = 0.98, P < 0.0001) and
IVUS and histology \((r = 0.85, P < 0.0001)\) was highly significant.  

Relation of neointimal growth to extent of copper chelation. Because all implanted stents were 15 mm long and stent area and vessel area were similar in the two groups, the difference in total neointimal growth can be attributed to TTM treatment and the resulting change in copper bioavailability. Indeed, neointimal volume 4 wk after stent implantation correlated significantly \((r = 0.56, P < 0.02)\) with percent change in serum ceruloplasmin level on the day of the procedure vs. baseline level (see supplemental Fig. I). A weaker, but still significant, relation was found between total neointimal volume at 4-wk follow-up and percent ceruloplasmin change vs. baseline at 2 wk \((r = 0.55, P < 0.02)\) and 4 wk \((r = 0.48, P < 0.05)\) after stent implantation. In general, more aggressive copper chelation resulted in less neointimal growth. In addition, although percent stenosis diameter (QCA) at 4-wk follow-up was strongly related to artery overstretching during stent implantation in control animals \((r = 0.82, P < 0.01)\), this relation was completely abolished in the TTM-treated animals \((r = 0.07, P = 0.86; \text{see supplemental Fig. II})\).  

DISCUSSION  
Here we corroborate our earlier data proving the critical role of copper in neointimal hyperplasia (16). Our earlier data were obtained from the rat carotid artery model, and the present pig model is more relevant to human disease, because coronary anatomy in pigs is the same as in humans, coronary artery stenting is not possible in rats, and, structurally, the common carotid artery in the rat is a transitional vessel between a muscular artery and a large-conductance vessel, such as the aorta, which has a primarily elastic component. The elastic component of the common carotid artery is greater than that of coronary arteries; thus the response to injury may differ between these artery types. Finally, many pharmacological approaches to inhibit neointimal formation were successful in the rat model but later proved to be ineffective in clinical trials (3). Thus, on the basis of the present study, we propose that reduction of biologically available copper by oral copper chelation therapy may be a clinically valid approach for prevention of in-stent restenosis.  
Our objective was to reduce ceruloplasmin by 80% and to maintain this level within 20 ± 5% of the baseline value. In the present study, the ceruloplasmin level was decreased by 70% within the first 2 wk of treatment, and this reduction was sustained during follow-up. In some animals, ceruloplasmin was reduced more profoundly, to 90% below its baseline level, which was associated with temporary painful joint swelling. This symptom was completely reversible without recurrence after temporary interruption of TTM administration for 1 or 2 days, but it attests to the variability in the daily oral uptake of TTM, which caused fluctuations in the ceruloplasmin level. These fluctuations remained minor throughout the study and did not reach statistical significance. Joint swelling in the presence of very low ceruloplasmin levels seems to be specific to juvenile pigs, in which copper demand is increased because of the intensive development of connective tissue and elastin in the growing bones and joints. Clinically, there is considerable experience with TTM indicating that ceruloplasmin reduction up to 80% is very well tolerated in patients (6, 25). Importantly, side effects can occur only if ceruloplasmin levels are reduced beyond the goal of any copper chelation treatment, as in bone marrow suppression, connective tissue defects, decreasing bone density, and neuropathy (2). Fortunately, all side effects are completely reversible after cessation of TTM administration (2). The joint swelling in the present study at ceruloplasmin levels 90% below baseline prevented study of higher doses; we have, however, studied the effect of the duration of treatment in the rat model and found that the duration of treatment before arterial injury and the duration of TTM administration after injury significantly affect neointimal hyperplasia (16). In a comparison of 2 wk, 1 wk, and no pretreatment, there was a stepwise reduction of the inhibitory effect on neointimal hyperplasia depending on the duration of pretreatment. Accordingly, posttreatment for 4 days had a significantly less profound effect on the prevention of neointimal growth than posttreatment for 10 days.  
Our major finding is that oral TTM significantly inhibited neointimal formation, as revealed by angiographic, IVUS, and histomorphometric indexes. Specifically, the late lumen loss, diameter stenosis, and neointimal volume were ~35% less in TTM-treated than in control animals. Furthermore, copper chelation abolished the relation of restenosis to severity of mechanical injury. TTM has been shown to inhibit crucial steps in the inflammation-triggered restenotic cascade (16, 17). Indeed, we found a blunted inflammatory response at 28 days after stent implantation in the vessel wall of TTM-treated animals compared with controls. Parastruts and neointimal and/or adventitial inflammation was mild to moderate and consisted predominantly of mononuclear cells in the control animals but was not observed in the TTM-treated animals (Fig. 4). We previously showed in the rat carotid artery, at 4 and 7 days after balloon injury, that TTM inhibits mononuclear cell infiltration into the vessel wall (16).  
Stented vessel segments were embedded in JB-4 Plus medium, a glycol methacrylate-based monomer that blocks antigen sites for most antibody reactions. Although our inability to perform immunohistochemistry can be considered a study limitation, the underlying molecular mechanism for the anti-restenotic effect of TTM has been extensively studied and reported previously (16, 22). Most notably, TTM-treated rats demonstrated up to 60% reduction in neointimal development after balloon injury of the carotid artery, concomitant with a significant decrease in FGF-1 and IL-1α release (16). Our observation in the porcine model that inflammatory cell infiltration is strongly reduced in the TTM-treated animals, as was seen in the rat model, in our interpretation supports an anti-inflammatory mechanism due to impaired IL-1α release caused by a lack of biologically active copper. Taken together, these data suggest that copper chelation may attenuate the restenotic response to injury via inhibition of FGF-1 and IL-1α release into the extracellular compartment.  
In recent years, the spectrum of coronary interventions has shifted from balloon angioplasty to stent implantation (1); however, restenosis remains a key challenge, especially in patients with diabetes, small vessels, long lesions, and in-stent restenosis (11). Drug-eluting stents (DES) are known to reduce in-stent restenosis in various subsets of patients and lesions (8, 27). The major limitations of DES are delayed reendothelialization and high cost. As observed previously, there is a substantial acceleration of reendothelialization after balloon injury in
rats treated with TTM, although the mechanism is not completely understood (16). The high efficacy in reducing neointimal growth after stent implantation shown in this study, along with the low cost of TTM and its ability to accelerate reendothelialization, makes copper chelation with TTM or alternative molecules a very attractive strategy for systemic treatment of restenosis. Furthermore, it might be considered an agent for local delivery from DES.

Thus copper chelation, which has a prominent antiproliferative and anti-inflammatory effect, can effectively reduce neointimal formation after stent implantation and may be considered for future clinical application.

REFERENCES


