Paper III
Tetradecylthioacetic acid reduces stenosis development after balloon angioplasty injury of rabbit iliac arteries

Karel K.J. Kuiper a,*, Ziad A. Muna b, Knut Ståle Erga a, Endre Dyroy b, Einar Svendsen c, Rolf K. Berge b, Jan Erik Nordrehaug a

a Department of Heart Disease, Haukeland University Hospital, N-5021 Bergen, Norway
b Institute of Clinical Biochemistry, University of Bergen, Haukeland University Hospital, Bergen, Norway
c Department of Pathology, Haukeland University Hospital, Bergen, Norway

Received 19 July 2000; received in revised form 29 December 2000; accepted 3 January 2001

Abstract

Background: tetradecylthioacetic acid (TTA) is a synthetic long-chain fatty acid analogue that inhibits the oxidative modification of low-density lipoprotein particles in vitro. We examined the influence of TTA on the arterial wall response after balloon angioplasty injury in a rabbit iliac model.

Methods and results: 14 rabbits were randomized to receiving either TTA fatty acids 800 mg daily perorally (weight 3.6 ± 0.1 kg) or to normal diet (weight 3.5 ± 0.5 kg, P = NS). Angioplasty was performed via right carotidotomy on both iliac arteries using an oversized balloon catheter, the TTA group being pretreated for 3 weeks. After angioplasty, the lumen diameter was 2.37 ± 0.18 versus 2.36 ± 0.13 mm for the TTA and control groups, respectively (P = NS).

At 10 weeks follow-up angiography, minimal luminal diameter was 1.64 ± 0.27 versus 1.13 ± 0.52 mm for the TTA and control groups respectively (P < 0.05). Histomorphometry did not show significant differences in intimal hyperplasia between the two groups (maximal intimal thickness 0.22 ± 0.04 versus 0.19 ± 0.10 mm, P = NS and intimal area 0.32 ± 0.12 versus 0.36 ± 0.23 mm², P = NS for the TTA and the control groups, respectively). In the heart, the sum of the n-3 fatty acids was 8.9 ± 2.7 in the TTA group versus 4.3 ± 0.0 mol% in the control group (P < 0.05). The anti-inflammatory fatty acid index, calculated as (22:5 n-3 + 22:6 n-3 + 20:3 n-6)/20:4 n-6, was 0.76 ± 0.10 vs. 0.25 ± 0.03 for the TTA and control groups, respectively (P < 0.05). In vitro TTA (100 μM) reduced the proliferation of human smooth muscle cell by more than 50%.

Conclusion: treatment with TTA is associated with positive arterial remodeling after angioplasty injury. The significance of the in vitro inhibition of human smooth muscle cell proliferation needs to be further elucidated. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Tetradecylthioacetic acid; Restenosis; Anti-inflammatory; Angioplasty; Remodeling

1. Introduction

The process leading to restenosis after coronary balloon angioplasty is complex. Subsequent to the initial stretching and vessel trauma, elastic recoil, intima hyperplasia and remodeling occurs. The role of the adventitia in the remodeling of the arterial wall has been increasingly recognized [1–3] and geometric remodeling is now accepted as a more important determinant of late loss than neointimal formation. During angioplasty, reactive oxygen species are formed and may act as vascular smooth muscle cell mitogens [4]. Recently it has been shown that antioxidants reduce restenosis after percutaneous transluminal coronary angioplasty (PTCA) in patients [5,6]. Antioxidants may interrupt the activation of oxidant-sensitive signaling pathways involving the nuclear factor κB [7] and thus mitigate the sustained proinflammatory state that follows angioplasty balloon injury [8–10], resulting in a reduction in restenosis.

Tetradecylthioacetic acid (TTA) {CH3-(CH2)13-S-CH2-COOH} is a 3-thia fatty acid analogue in which a sulphur atom is located in the third position in the carbon chain from the carboxyl end of a normal saturated fatty acid. TTA decreased the plasma triacylglycerol, cholesterol and free fatty acid levels in rats after...
repeated administration [11,12]. TTA administration to rats led to a significant decrease in the m-RNA level of liver apolipoprotein C-III [13]. Some of these effects seemed to be mediated by the activation of peroxisome proliferator-activated receptor-\(\alpha\) (PPAR\(\alpha\)), a ligand-activated transcription factor of the steroid hormone receptor superfamily. PPAR\(\alpha\) is expressed mostly in the liver, but also in human endothelial cells [14]. Recently, it was shown that TTA also activates PPAR\(\gamma\) [13]. Activation of PPAR\(\gamma\) promotes adipogenesis and terminal differentiation of preadipocyte fibroblasts into mature adipocytes [15]. In addition, it was shown that PPAR\(\gamma\) ligands can inhibit the migration of vascular smooth muscle cells [16]. TTA has been shown to possess antioxidant effects, inhibiting the oxidative modification of low-density lipoprotein (LDL) particles in vitro [17]. A potential anti-proliferative effect of TTA has been reported [18]. This fatty acid analogue may therefore have important actions on the arterial wall and could modify the pathogenesis of restenosis.

The objective of our study was to assess the vessel wall reaction after balloon angioplasty injury in rabbits randomized to oral supplements of TTA or no treatment. A secondary objective was to assess the myocardial contents and ratio of n-3 and n-6 fatty acids as an inflammatory index.

2. Methods

2.1. Animals and diets

Fourteen Chinchilla (Chbb:CH) rabbits of either sex, bred locally (Vivarium, Bergen, Norway) were randomized either to receive supplement with TTA 800 mg daily (mean weight 3.6 ± 0.1 kg) or to normal diet (mean weight 3.5 ± 0.5 kg, \(P = \text{NS}\)).

The animals were pretreated with TTA given as oral supplements, dissolved in acetone and sprayed on food pallets, for 3 weeks to ensure accumulation in the tissues. All the rabbits had free access to water and food.

The local ethical committee for animal care and use approved the study protocol.

2.2. Chemicals

TTA was synthesized as previously described [19]. All the other chemicals were from common commercial sources and were of reagent grad.

2.3. Angioplasty procedure

After premedication with 0.5 ml fentanyl (0.315 mg/ml)/fluanisone (10 mg/ml) (Hypnorm\textsuperscript{®}) intramuscularly, the rabbits were anaesthetized by administering diazepam 4 mg/kg intraperitoneally and anaesthesia was maintained by additional 0.3–0.4 ml of a 1:1 mixture of Hypnorm\textsuperscript{®} and diazepam, usually necessary once during the procedure.

After local infiltration of the skin by lignocaine (Xylocaine\textsuperscript{®}) and surgical cut-down, the right carotid artery was cannulated by a 6F sheath. A bolus of 100 U/kg of heparin was administered intraarterially. Before the angioplasty, an angiography was performed in the frontal view by injecting 3 ml of ioxaglate (Hexabrix\textsuperscript{®}) as contrast medium via a Berman\textsuperscript{®} catheter. A semicompliant angioplasty balloon catheter (Express\textsuperscript{®}) 2.5 mm was positioned in the proximal part of each iliac artery and balloon angioplasty was performed with two balloon inflations at the same site in each artery, at 8 and 12 atmospheres for 30 s each to overstretch the artery. The balloon marker, on the middle of the 20-mm long balloon, was placed over the ilio-sacral joint, thus positioning the balloon at identical sites. Inflation was performed using a pressure manometer.

Angiography after dilatation, performed in the same frontal view, showed that all arteries were patent with TIMI flow III. The balloon-artery ratio was 1.48 ± 0.25 versus 1.53 ± 0.31 for TTA group and the control group (\(P = \text{NS}\)), respectively. The carotid artery was ligated and the skin was closed with ligatures. Buprenorfin 0.3 mg (Temgesic\textsuperscript{®}) and penicillin, subcutaneously, were given once daily for the first few days. During follow-up, none of the rabbits had signs of inferior limb arterial insufficiency. A follow-up angiography was performed in the same frontal view after 10 weeks, following the same procedure as initially also using ioxaglate (Hexabrix\textsuperscript{®}) as contrast medium, but now inserting the sheath in the left carotid artery. After angiography a laparotomy was performed and the abdominal aorta was cannulated with an 18G infusion needle. The animals were euthanized by giving an overdose of pentobarbital intravenously via the sheath in the left carotid artery. The iliac arteries were perfusion fixed by infusing 2% glutaraldehyde into the distal aorta at a pressure of 100 mmHg over 15 min, a cannula in the inferior caval vein as efflux. Sections of liver tissue and heart were removed and immediately frozen down in liquid N\textsubscript{2} and stored at \(-80^\circ\text{C}\).

2.4. Determination of fatty acid composition

Total lipids were extracted from plasma, liver and heart as described by Lie et al. [20]. The lipid fractions were evaporated, saponified and esterified prior to separation using a Carlo Erba 2900 gas chromatograph as described elsewhere [12].
2.5. Quantitative angiography

The frames with maximal opacity from baseline angiographies (before and after dilatation), as well as from follow-up angiography were stored for subsequent quantitative analysis. Reference and luminal diameter were measured before and after dilatation as well as balloon to artery ratio using a digital electronic calliper (Sandhill, model EC-l) [21]. At follow-up angiography, minimal luminal diameter, reference diameter and stenosis were determined. Results from both arteries in the same rabbit were averaged to obtain the final results. A 2.5 mm balloon angioplasty catheter (Express® 2.5 mm), placed on the abdomen and dilated at 8 atm (rated balloon diameter 2.5 mm) served for calibration.

2.6. Histology

The dilated segments of the iliac arteries were located by fluoroscopy, using the ilio-sacral joint as anatomical landmark, and dissected in block. Serial sections were processed and the segments were embedded in parafin. Cross sections were stained with hematoxylin-eosin and Verhoeff-van Gieson stains. All the sections were evaluated blinded to the randomisation after digital transition using computer-assisted planimetry (Leica Q500MC, software Qwin 01.02). Areas surrounded by lumen, the internal elastic lamina and by the external elastic lamina were traced. Neointima was defined as the area between the lumen and the internal elastic lamina. Vessel area was defined as the area within the external elastic area. Results of both the arteries in the same rabbit were averaged to obtain the final results.

2.7. Smooth muscle cell proliferation

Human aorta smooth muscle cells (human aorta smooth muscle cells obtained from American Type Culture Collection (ATCC), cell type no. CRL-1999, passage 23) were seeded at a density of 1 × 10⁵ cells per well in Nunclon microwell (96 wells (plate), in 100 μl media. Media consisted of Sigma Ham-F12 medium (N4888), to which 10% Sigma fetal bovine serum (FBS) (F2442), 1% Sigma ITS Liquid supplement 100 × (I3146), 1% endothelial cell growth factor 100 × (E-9640), 1% L-glutamine 200 mM (G7513) and 2% Biowhitaker penicillin (17-603E) were added. After 24 h, 100 μl media (no addition) or 100 μl media and 80 μM BSA with either 100 μM TTA (final concentration, TTA group) or 100 μM palmitic acid (final concentration, palmitic acid group) were added to the cell culture wells. After 0, 3 and 6 days 10 μl of H³-Thymidine 50 μCi/ml was added. Following 60 min of incubation, the plates were placed on ice, the cells were harvested using Packard Filtermate Harvester and the nuclear radioactivity was measured on a Wallac Win Spectral 1414 liquid scintillation counter (Turku, Finland).

2.8. Statistical analysis

All data are presented as mean ± S.D. Results from both arteries in the same rabbit were averaged to obtain the final results. Differences between the two groups are analysed by non-paired t-test. Differences between angiographic diameter after dilatation and at follow-up within the same group were tested with the paired t-test. Relation between angiographic lumen diameter at follow-up angiography and histologic lumen area was evaluated with Pearson’s test for correlation. Results are considered as statistically significantly different when P < 0.05. The data were analysed by using the PC-program SPSS for Windows 8.0.

3. Results

Total occlusion of two arteries in each group had occurred at the final follow-up angiography. Histology confirmed thrombus and these arteries were excluded from further angiographic and histologic quantitation since the wall reaction is different in thrombotic occlusions.

3.1. Angiography

Angiographic measurements were comparable between the two groups at baseline and after dilatation (Table 1). At follow-up, minimal luminal diameter was significantly larger in the TTA group. Stenosis rate was 22.1% compared with 42.4% (P < 0.05) for the control group (Table 1).

3.2. Histology and angiographic correlations

Intima/media ratio was the same in the two groups (Table 2). Histological lumen area correlated with angiographic lumen diameter (R = 0.59, P < 0.05) (Fig. 1). There was no correlation between vessel area and angiographic lumen diameter (P = NS).

Remodeling, defined as angiographic late loss minus intimal thickness measured in the histology sections [22], was significantly different between the TTA and the control group (Table 2).

Angiographic late loss was 1.22 ± 0.53 versus 0.70 ± 0.39 mm in the control and TTA groups, respectively (Table 1), while intimal thickness was not different in the two groups (Table 2).
Palmitic acid also decreased H3-Thymidine incorporation into smooth muscle cells and to palmitic acid (100 μM). This indicates that TTA decreases smooth muscle cell proliferation. The fatty acid composition of heart tissue is shown in Table 3. The sum of n-3 fatty acids in the TTA group was approximately doubled compared with controls; there was no change to the sum of n-6 fatty acids. A significant level of TTA was also detected (Table 3). In contrast in the liver tissue there was an increase in saturated fatty acids, a decrease in the sum of the n-3 fatty acids (Ziad A. Muna, data to be published, 2000). The inflammatory fatty acid index (AIFAI), calculated as reported in [23] was significantly increased in both heart (Fig. 2) and liver (Ziad A. Muna, data to be published, 2000). Compared to control, AIFAI was approximately three times as high in the heart tissue of the TTA group.

3.3. Heart fatty acid composition

The fatty acid composition of heart tissue is shown in Table 3. The sum of n-3 fatty acids in the TTA group was approximately doubled compared with controls; there was no change to the sum of n-6 fatty acids. A significant increase in the sum of n-3 fatty acids was accounted for by increase in the 20:5n-3, 22:5n-3 and 22:6n-3. The sum of the saturated fatty acids in the TTA group was decreased, accounted for by a decrease in 15:0, 16:0, 17:0 and 18:0 (data not shown).

A significant level of TTA was also detected (Table 3). In contrast in the liver tissue there was an increase in saturated fatty acids, a decrease in the sum of the n-3 fatty acids (Ziad A. Muna, data to be published, 2000). The anti-inflammatory fatty acid index (AIFAI), calculated as reported in [23] was significantly increased in both heart (Fig. 2) and liver (Ziad A. Muna, data to be published, 2000). Compared to control, AIFAI was approximately three times as high in the heart tissue of the TTA group.

3.4. Smooth muscle cell proliferation

Fig. 3 shows that TTA reduced the H3-Thymidine incorporation significantly both compared with no additives and to palmitic acid (100 μM). This indicates that TTA decreases smooth muscle cell proliferation. Palmitic acid also decreased H3-Thymidine incorporation, although not significantly.

4. Discussion

In this study we found that oral administration of TTA reduced stenosis formation after angioplasty balloon injury by influencing remodeling of the arterial wall. Measurement of n-3 and n-6 fatty acid contents and ratio in the myocardium suggested an anti-inflammatory action of TTA. Furthermore, TTA reduced H3-Thymidine incorporation into smooth muscle cells, indicating an inhibited proliferation of smooth muscle cells cultured in vitro.

Stenosis formation after angioplasty results from remodeling of the vessel with general shrinkage and by smooth muscle proliferation and migration into the intima and increased intimal matrix. TTA may have affected both these mechanisms. There was a smaller loss of arterial lumen in the TTA group while histology did not show a significant difference from controls in intimal thickness, indicating a remodeling of the artery resulting in a reduction in the loss of the lumen diameter. In this normal vessel model, all intima is generated after the injury, and remodeling can be calculated as the difference in late lumen loss and neointima formation [22,24]. The significant correlation between the histology lumen area and the angiographic lumen (Fig. 1) suggests that the remodeling concept using both angiography and histology measurements is valid [22,25].

Table 2
Histomorhormophometry of balloon injured iliac artery segments

<table>
<thead>
<tr>
<th></th>
<th>Control group (n = 7)</th>
<th>TTA group (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal intima thickness (mm)</td>
<td>0.19 ± 0.10</td>
<td>0.22 ± 0.04</td>
</tr>
<tr>
<td>Intimal area (mm²)</td>
<td>0.36 ± 0.23</td>
<td>0.32 ± 0.12</td>
</tr>
<tr>
<td>Intimal/medial area ratio</td>
<td>0.76 ± 0.43</td>
<td>0.85 ± 0.59</td>
</tr>
<tr>
<td>Luminal area (mm²)</td>
<td>1.00 ± 0.38</td>
<td>1.01 ± 0.18</td>
</tr>
<tr>
<td>Remodeling (mm)</td>
<td>1.03 ± 0.47</td>
<td>0.52 ± 0.32*</td>
</tr>
</tbody>
</table>

* Data are mean ± S.D., Remodeling is defined as angiographic late loss minus intimal thickness. TTA is tetradecylthioacetic acid.
* P < 0.05.

Table 1
Angiographic measurements

<table>
<thead>
<tr>
<th></th>
<th>Before dilatation</th>
<th>After dilatation</th>
<th>At follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control group (n = 7)</td>
<td>TTA group (n = 7)</td>
<td>Control group (n = 7)</td>
</tr>
<tr>
<td>Diameter artery (mm)</td>
<td>1.85 ± 0.20</td>
<td>1.92 ± 0.18</td>
<td>2.36 ± 0.13</td>
</tr>
<tr>
<td>Reference diameter (mm)</td>
<td>1.85 ± 0.20</td>
<td>1.92 ± 0.18</td>
<td>1.86 ± 0.25</td>
</tr>
<tr>
<td>Acute gain (mm)</td>
<td>0.51 ± 0.25</td>
<td>0.45 ± 0.21</td>
<td>1.98 ± 0.24</td>
</tr>
<tr>
<td>Dilatation (%)</td>
<td>29.3</td>
<td>32.3</td>
<td>1.22 ± 0.53</td>
</tr>
<tr>
<td>Late loss (mm)</td>
<td>42.4</td>
<td>22.1</td>
<td></td>
</tr>
</tbody>
</table>
| Stenosis (%)          |                  |                 |              | 3.3. Heart fatty acid composition

The fatty acid composition of heart tissue is shown in Table 3. The sum of n-3 fatty acids in the TTA group was approximately doubled compared with controls; there was no change to the sum of n-6 fatty acids. The increase in the sum of n-3 fatty acids was accounted for by increase in the 20:5n-3, 22:5n-3 and 22:6n-3. The sum of the saturated fatty acids in the TTA group was decreased, accounted for by a decrease in 15:0, 16:0, 17:0 and 18:0 (data not shown).

A significant level of TTA was also detected (Table 3). In contrast in the liver tissue there was an increase in saturated fatty acids, a decrease in the sum of the n-3 fatty acids (Ziad A. Muna, data to be published, 2000). The anti-inflammatory fatty acid index (AIFAI), calculated as reported in [23] was significantly increased in both heart (Fig. 2) and liver (Ziad A. Muna, data to be published, 2000). Compared to control, AIFAI was approximately three times as high in the heart tissue of the TTA group.

3.4. Smooth muscle cell proliferation

Fig. 3 shows that TTA reduced the H3-Thymidine incorporation significantly both compared with no additives and to palmitic acid (100 μM). This indicates that TTA decreases smooth muscle cell proliferation. Palmitic acid also decreased H3-Thymidine incorporation, although not significantly.

4. Discussion

In this study we found that oral administration of TTA reduced stenosis formation after angioplasty balloon injury by influencing remodeling of the arterial wall. Measurement of n-3 and n-6 fatty acid contents and ratio in the myocardium suggested an anti-inflammatory action of TTA. Furthermore, TTA reduced H3-Thymidine incorporation into smooth muscle cells, indicating an inhibited proliferation of smooth muscle cells cultured in vitro.

Stenosis formation after angioplasty results from remodeling of the vessel with general shrinkage and by smooth muscle proliferation and migration into the intima and increased intimal matrix. TTA may have affected both these mechanisms. There was a smaller loss of arterial lumen in the TTA group while histology did not show a significant difference from controls in intimal thickness, indicating a remodeling of the artery resulting in a reduction in the loss of the lumen diameter. In this normal vessel model, all intima is generated after the injury, and remodeling can be calculated as the difference in late lumen loss and neointima formation [22,24]. The significant correlation between the histology lumen area and the angiographic lumen (Fig. 1) suggests that the remodeling concept using both angiography and histology measurements is valid [22,25].
Other studies have reported that increased oxidative stress leads to proliferation of vascular smooth muscle cells [26]. Our in vitro experiment did suggest reduced proliferation of smooth muscle cells in the presence of TTA. This was not consistent with our histological findings in which the intimal area in the TTA group was only numerically, but not significantly, thinner than the controls. The reason for this discrepancy could be due to a lower concentration of TTA present in the arterial wall with oral administration, compared with the concentrations used in the in vitro experiments. Another explanation could arise from the fact that smooth muscle cells from different embryonic origins might respond differently to different cytokines, mitogens, chemotactic factors or extracellular matrixes [27]. Moreover, extracellular matrix changes may possibly cancel out any effects that TTA may have had on cell proliferation in vivo.

It has been shown that oxidative stress increases after angioplasty, leading to impaired endothelial function and relaxation and to an inflammatory response. Treatment with antioxidants might counteract the deleterious effect of the reactive oxygen species. Both the experimental and clinical studies have reported the reduction of restenosis after administration of the antioxidant drug probucol [5,6,28]. In vitro studies have shown that TTA has antioxidant properties, inhibiting the oxidative modification of LDL particles [17,29]. As in the probucol study [5], TTA was started 3 weeks before the procedure to assure saturation of the tissue.

Although we have no data on the fatty acid composition of the iliac arteries, the increase in n-3 fatty acids in the heart tissue is probably of great significance. This change had a direct effect on the n-3/n-6 ratio that was evident in the calculated anti-inflammatory fatty acid index (Fig. 2). The benefit of n-3 PUFAs on atherosclerotic thrombotic events has been well-documented [30–32]. Elevated circulating levels of inflammatory substances are found in patients with angina, particularly in those with unstable disease [33,34] and subsequent to coronary angioplasty activation of platelets and leukocytes was reported [35]. PPARs are expressed in different immunological and vascular wall cell types where they exert anti-inflammatory and proapoptotic activities [36]. This might potentiate the fatty acid-mediated anti-inflammatory activity of TTA since it is a potent PPAR activator [13]. Therefore, it seems plausible that TTA changed the fatty acid composition in

Table 3
Fatty acid composition (mol%) in total homogenates of heart tissue*  

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Control group (n = 7)</th>
<th>TTA group (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td>35.2 ± 1.0</td>
<td>26.7 ± 3.9*</td>
</tr>
<tr>
<td>MUFA</td>
<td>22.9 ± 1.1</td>
<td>21.0 ± 5.0</td>
</tr>
<tr>
<td>PUFA</td>
<td>39.3 ± 2.1</td>
<td>45.0 ± 7.0</td>
</tr>
<tr>
<td>n-3</td>
<td>4.3 ± 0.2</td>
<td>8.9 ± 2.7*</td>
</tr>
<tr>
<td>n-6</td>
<td>35.0 ± 1.9</td>
<td>36.0 ± 4.6</td>
</tr>
<tr>
<td>TTA</td>
<td>0.0 ± 0.0</td>
<td>2.7 ± 0.6*</td>
</tr>
</tbody>
</table>

* Data are mean ± S.D. TTA is tetradecylthioacetic acid. SFA is saturated fatty acids; MUFA is monounsaturated fatty acids; PUFA is polyunsaturated fatty acids.

* P < 0.05.
the extra-hepatic tissue and activates PPARα resulting in a milder inflammatory reaction that is triggered by angioplasty, leading to a reduced stenosis. Further studies are in progress to clarify molecular mechanisms of TTA’s anti-inflammatory properties.

5. Conclusions

TTA reduces stenosis formation after angioplasty balloon injury. Analysis of n-3 and n-6 fatty acid content and ratio in the myocardium and liver suggests an anti-inflammatory action of TTA, which has previously also been shown to have antioxidant properties. The reduced stenosis formation seems to be due to remodeling of the artery while a reduction of the intimal thickness was not seen compared to controls. In vitro, TTA also inhibits smooth muscle proliferation, although this was not confirmed in this animal model.

Acknowledgements

This work was supported by a grant from the Bergen Heart Foundation, Bergen, Norway and by The University of Bergen, Norway. The authors are grateful to Kari Williams for excellent technical assistance.

References


