Title: Effectiveness of β-blockade in experimental chronic aortic regurgitation

Plante et al.: β-blockade in chronic aortic regurgitation

Eric Plante MSc, Dominic Lachance BSc, Martin Gaudreau MSc, Marie-Claude Drolet MSc, Élise Roussel MSc, Marie Arsenault MD and Jacques Couet PhD.
Groupe de Recherche sur les Valvulopathies, Centre de Recherche Hôpital Laval, Institut de cardiologie de Québec, Université Laval, Quebec, Canada.

Word count: 4470
Abstract: 237; Tables and figures: 7

Corresponding author: Marie Arsenault MD
Institut de Cardiologie de Québec,
2725 chemin Sainte-Foy,
Sainte-Foy, (Quebec), Canada, G1V 4G5
Phone: 1-418-656-4510
Fax: 1-418-656-4544
Email: marie.arsenault@crhl.ulaval.ca

This work was supported by operating grants to Dr Couet and Arsenault from the Canadian Institutes of Health Research (MOP-61818), the Heart and Stroke Foundation of Canada and the Quebec Heart Institute.
Abstract

Background: Past studies have suggested that the adrenergic system becomes abnormally activated in chronic volume overload such as in severe aortic valve regurgitation (AR). However the effectiveness of agents directed against this adrenergic activation has never been adequately tested in chronic AR. We therefore tested the effects of metoprolol treatment on the left ventricular function and remodeling in severe chronic AR in rats.

Methods and Results: Severe AR was created in adult male Wistar rats by retrograde puncture of the aortic leaflets under echocardiographic guidance. Two weeks later, animals received or not metoprolol treatment (25/mg/kg) orally for 24 weeks. Left ventricular dimensions, ejection fraction and filling parameters were evaluated by echocardiography. Hearts were harvested at 1, 2, 14 days and 180 days for the evaluation of hypertrophy, β-adrenergic receptor status and extracellular matrix remodeling. We found that metoprolol treatment prevented LV dilatation and preserved the ejection fraction and filling parameters compared to untreated animals. Metoprolol increased the expression of β₁-adrenoreceptor mRNA and reduced G protein receptor kinase 2 levels. Collagen I and III mRNA levels were reduced. Cardiac myocyte hypertrophy was also prevented.

Conclusion: In our experimental model of severe AR, metoprolol treatment had a significant beneficial global effect on LV remodeling and function. These results suggest that the adrenergic system is important in the development of volume overload cardiomyopathy in AR and that adrenergic blocking agents may play a role in the treatment of this disease.
Condensed abstract:

Activation of the adrenergic system occurs in chronic aortic regurgitation but the impact of this activation is not well understood. We therefore tested the effects of beta-adrenergic receptor blocker metoprolol (25/mg/kg/day for 24 weeks) in a model of chronic aortic regurgitation in male Wistar rats. Ventricular function and remodeling were assessed by echocardiography and hearts harvested for tissue analysis. Metoprolol significantly prevented ventricular dilatation and preserved ejection fraction. It also decreased myocyte hypertrophy and collagen expression. Significant alterations in beta-adrenergic receptors and their regulation pathway were observed. We conclude that beta-blockers prevented ventricular dysfunction and remodeling in this animal model.

Key Words

Aortic valve, insufficiency, rat, echocardiography, volume overload, adrenergic system
**Introduction**

Aortic regurgitation (AR) is a chronic volume overload disease that induces progressive left ventricular (LV) dilatation and eccentric hypertrophy. Patients suffering from significant levels of AR will often remain asymptomatic for decades before heart failure develops\(^1\)\(^{-2}\). Current treatment guidelines suggest that they should primarily be treated with vasodilators\(^1\).

Significant alterations of the adrenergic system and adrenergic receptors have been reported in animal models of chronic volume overload\(^3\)\(^{-12}\). Despite these interesting findings, the hypothesis that \(\beta\) adrenergic blocking agents (BB) might be effective to protect the volume-overloaded LV has not been adequately tested. A few studies have suggested that a short-term treatment with BB might be beneficial in volume overload\(^3\), \(^11\), \(^13\), \(^14\). However, the long-term effectiveness of this type of drugs in chronic AR has never been adequately evaluated. In this study, we assessed the effects of a 6-month treatment with the \(\beta\)-blocker metoprolol on the LV function and remodeling of rats with severe chronic AR.
Methods

Animals:

Acute study: 40 male Wistar rats (400-450 g) had severe AR induced for 1, 2 or 14 days until sacrifice to evaluate the acute adaptations of the LV. A sham-operated group was used as control (n=10) 15.

Chronic study: 38 male Wistar rats (body weight 400-450g) were divided in 3 groups as follows: #1: normal controls (sham-operated; n=10), #2: untreated AR (n=18) and #3: AR receiving 25 mg/kg/day of metoprolol tartrate (n=10) (Sigma, Oakville Ont. Canada) in drinking water. Drug treatment was started 2 weeks after the surgical procedure described below and continued for 24 weeks thereafter 16.

This protocol was approved by the Université Laval’s animal protection committee and was consistent with the recommendations of the Canadian Council on animal care.

Severe AR was induced in the animals as previously described by retrograde puncture of the aortic valve leaflets 15-17. Echocardiographic studies were performed as described elsewhere 15-17 at each time point of the protocol (0, 1, 2 or 14 days for the acute study and at 0 and 180 days for the chronic study). Cardiac output was calculated by Doppler and indexed to the animal’s body weight. AR was considered severe by echocardiography by the presence of all of the following criteria: color-Doppler ratio of regurgitant jet width to LVOT diameter >50%, retrograde holo-diastolic flow in proximal descending aorta with end-diastolic velocity >18 cm/s, ratio of time-velocity integral of reversed diastolic flow to forward systolic flow in descending thoracic aorta >60%, acute increase in LV dimensions. Echocardiographic criteria of AR severity had to be accompanied by an acute drop of aortic diastolic pressure above 30% to qualify.
Ejection fraction, diastolic filling, relative wall thickness (RWT), LV mass were evaluated as described elsewhere \(^{15-18}\). Normal echocardiographic diastolic filling parameters were previously assessed in a cohort of normal age-matched Wistar rats \(^{15}\).

At the end of the protocol, animals were sacrificed, their hearts were quickly dissected, LVs as well as the other cardiac chambers were weighed and then snap-frozen in liquid nitrogen and kept at -80°C.

Tissue catecholamines determination:

LV tissue total catecholamines were measured by radioimmunoassay (Amersham, Baie d'Urfé, QC, Canada).

\(\beta\)-adrenergic receptors:

LV tissues were homogenized in the following buffer (25mM Tris-HCl, 2mM MgCl\(_2\), 250mM Sucrose, 5mM Hepes (pH 7.4)). Homogenates were centrifuged 30 min (1000xg, 4°C) and the supernatant 30 min at 35000xg. Membranes were suspended in a homogenisation buffer and protein concentration determined. Binding activity was evaluated using \(^{125}\)I-iodocyanopindolol (Specific activity: 2000 Ci/m mole; Amersham). Non-specific binding was determined in presence of propanolol (1µM) (Sigma, Oakville, ON) and subtracted from total count. Total density of beta-adrenergic receptors was determined using increasing concentrations of propranolol (1µM to 0.1nM). \(\beta1/\beta2\) receptor ratio was evaluated using metoprolol. Competition curves and analysis were done using GraphPad Prism version 3.02 (GraphPad Software, San Diego CA).
Immunoblotting:
Crude LV homogenates were separated by SDS-PAGE. Volumes of samples loaded on gel were corrected for the amount of protein. Immunoblotting was performed as described elsewhere\textsuperscript{20}. Membranes were hybridized with primary antibodies directed against GRK2, GRK5, sarcomeric $\alpha$-actin and smooth muscle $\alpha$-actin. Bands were visualized and quantified with a ChemilImager system (Alpha Innotech Corporation, San Leandro, CA).

Messenger RNA accumulation:
cDNA synthesis and RT-PCR analyses were carried out as described elsewhere\textsuperscript{16} using the following amplimer pairs: glyceraldehyde phosphate dehydrogenase (GAPDH), 5'-ATCCCATCACCCTCCTCAG-3' and 5'-CCATCACGCCACAGTTTCC-3'; $\beta_1$-adrenoreceptor $\beta_1$-AR: 5'-GCACACTGGCAATGTAATGC-3' and 5'-GTTGAACAGAAGTGACC-3'; $\beta_2$-AR: 5'-GCCTGCAAGTGAAGTGATTT-3’ and 5’-TTAACAGTGCTTTGCTCC-3’; collagen type 1 (Col1): 5'-TGTTCGTGGTTCTCAGGG TAG-3' and 5'-TTGTCGTAGCAGGGTTCTTTC-3'; Col3: 5'-CGAGGTAACAGAGGTGAAGA-3' and 5'-AACCCAGTATTCTCCGCTTTT-3' and pre-Matrix metalloprotease 2 (MMP2): 5'-CTATTCTGTAGCAGCACTTTGGA-3' and 5'-CAGACTTTGGTTCTCCA-3’. Denaturation, annealing and amplification temperatures were 94, 60 (50 and 55 for Col3 and MMP2, respectively) and 68\degree C, respectively.
Cardiomyocyte cross-sectional area:
LV sections fixed in paraffin were stained with Trichrome-Masson. Myocyte cross-sectional area (CSA) was measured\textsuperscript{16}. Sections from at least 10 animals per group were studied. Results are mean ± SEM in arbitrary units.

Immunohistochemistry:
The number of fibronectin-positive cells/field was evaluated in LV sections as previously described\textsuperscript{16}. The mean of sham controls were arbitrarily fixed to 100 and the results of the other groups are expressed relative to the sham controls.

Statistical analysis:
Results are presented as mean ± SEM unless specified otherwise. One-way analysis of variance was performed to compare serial data. Statistical significance was set at a p value of 0.05 or less using post-hoc Tukey’s test. Data and statistical analysis were performed using GraphPad Prism.
Results

Clinical data:

Drug treatment was well tolerated. All animals were alive at the end of the protocol. No animals developed clinical signs of heart failure in either group. Lung weight at sacrifice tended to be slightly increased in untreated AR rats but this did not reach statistical significance. Metoprolol treatment had no significant effect on lung weight (not shown).

Hemodynamics:

All rats remained normotensive (Table 1). Cardiac index was significantly increased in all AR compared to sham animals (+36%, p<0.05). We noted a trend towards lower heart rates in the metoprolol group that did not reach statistical significance. The stroke volumes and cardiac indexes of all metoprolol AR animals were comparable and remained significantly higher than normal controls.

Echocardiographic evaluation of LV function and remodeling

Eccentric hypertrophy was present before drug treatment was started 14 days after AR and ejection fraction was still in the normal range at that time (Table 2). After 180 days, all non-treated animals had developed significant LV hypertrophy compared to normal controls (sham) (LV mass index AR: 2.8 mg/g ± 0.1 mg/g vs. sham: 1.8 mg/g ± 0.1 mg/g, p<0.01). Metoprolol decreased the eccentric remodeling, eccentricity being defined as a decrease in RWT (RWT AR-metoprolol: 0.36±0.02 vs. RWT in untreated AR: 0.30±0.01, p<0.05). There was a trend towards a decrease in calculated and measured LV mass in metoprolol treated animals. AR resulted in a significant dilatation of the LV cavity as shown in Figure 1. End diastolic and end systolic diameters increased in non-
treated animals when compared to controls. Metoprolol decreased the end-systolic dimensions but had a milder effect on diastolic dimensions. LV ejection fraction decreased significantly in untreated animals (Fig. 1) but remained normal and similar to controls in rats treated with metoprolol. Diastolic filling parameters (Doppler E/A wave ratio of the mitral valve outflow) remained normal in 80% of the animals treated with metoprolol compared to 60% in the non-treated group (p<0.05).

Adrenergic system and regulatory pathways:
Myocardial total catecholamines were elevated in all AR rats compared to normal controls (Fig. 2A). Metoprolol treatment did not affect tissue catecholamines levels. A decrease in total βAR density was recorded 2 days after AR induction but this parameter returned to normal at day 14 and remained normal after 6 months (Fig. 2 B). Metoprolol treatment had no effect on β-receptor density and β1/β2 ratio in the LV remained relatively stable (not shown). β1 adrenoreceptors (β1AR) mRNA expression by RT-PCR was similar in non-treated AR animals compared to normal controls whereas metoprolol treatment increased β1AR expression. β2AR receptor mRNA levels remained unchanged (Fig. 3).
In acute AR, GRKs 2 and 5 were up-regulated in the LV (Fig.4). GRK2 protein content remained elevated after 6 months whereas GRK5 levels were not statistically different than controls. Metoprolol treatment decreased the LV protein content of GRK 2 (Fig.4) as well as the GRK2 mRNA levels (not shown). A similar trend was observed for the expression of the Gs protein. Gs protein mRNA levels were significantly elevated in AR
compared to sham (+45%, p<0.05) and Metoprolol treatment tended to attenuate this increase (-17% vs AR, p=ns).

Myocyte hypertrophy and extracellular matrix remodeling (at 180 days):
Myocyte cross-sectional area tended to be larger in AR animals compared to controls (+9%, p=ns). Metoprolol treatment decreased myocyte hypertrophy (-49% vs. untreated AR, p=0.001)

Sarcomeric α-actin protein content was only moderately increased in AR left ventricles (at 180 days) (Fig.5). On the other hand, smooth-muscle α-actin protein levels were greatly increased in AR LVs. Metoprolol treatment did not reverse the effect of AR on both types of α-actins. The density of fibronectin-positive cells in the LV doubled in all AR animals. Metoprolol treatment had no effect on this parameter. AR rats had higher LV mRNA levels of fibronectin, collagens I and III as well as MMP2 than sham-operated controls (Figure 5). Metoprolol treatment significantly decreased collagen I and III mRNA levels compared to untreated AR animals. β-blockade normalized fibronectin mRNA levels but did not alter MMP2 expression in AR rats.
Discussion

In this study we demonstrate for the first time in an animal model of chronic aortic valve regurgitation that a long term treatment with a β-blocker can help prevent LV remodeling, maintain normal LV ejection fraction and filling parameters, prevent myocyte hypertrophy and inhibit some aspects of extracellular matrix remodeling.

In heart failure, β-blockers protect the failing myocardium against catecholamine toxicity and counteract the neurohormones by improving βAR density and affinity, decreasing fibrosis and extracellular matrix remodeling. Significant abnormalities of the adrenergic system have also been identified in humans and animals submitted to chronic volume overload. Others have reported significant decreases in β-adrenoreceptor density in the LV of patients with chronic volume overload compared to pressure overload and normal controls. In subjects with clinical heart failure from severe AR, intense adrenergic activity is present as in other causes of heart failure. β-blockers have been proven effective in the treatment of residual LV dysfunction in patients who underwent aortic valve replacement for symptomatic AR. However, the adrenergic system status and the efficacy of preventive β blocker treatment in subjects with compensated AR are unknown. Small anecdotal studies in a few subjects with Takayasu arteritis and AR suggest β blockers are well-tolerated and may decrease LV hypertrophy.

β-blockers have never been adequately tested in a chronic animal model of AR. In rabbits with severe AR, significant changes in catecholamine levels were reported. βAR are also significantly affected as well as the activity of adenylate cyclase. It has been shown in a similar model that sympathectomy can protect against volume overload.
thereby suggesting an important role of the sympathetic system in this hemodynamic state. In acute AR in rabbits, short term β-blocker treatment (7 days) was well tolerated and decreased cardiac remodeling. β-blockers have been studied in dogs with chronic mitral regurgitation, another form of volume overload cardiomyopathy. In this model, β-blockers alone or in combination with an angiotensin converting enzyme inhibitor significantly improved LV function.

β-blockers are traditionally avoided in AR because it occurs in diastole and it is feared that bradycardia might increase the regurgitant volume. β-blockers are usually not given in patients with AR in fear of this bradycardia but this relies on little scientific proof. Although regurgitant volume/beat may increase with severe bradycardia, total regurgitant volume/minute usually remains unchanged since the number of cardiac cycles/minute also decreases. There is a potential risk of increased afterload due to an increased stroke volume. In one study however, bradycardial pacing in a model of severe AR unexpectedly improved cardiac work and myocardial capillary growth.

Our animals tolerated very well the long-term β-blocker treatment at a relatively small dose for rats. Metoprolol treatment was effective against LV remodeling and helped preserve a normal ejection fraction and better filling parameters. It is not known if higher doses of Metoprolol would have the same beneficial effects. These beneficial effects of metoprolol may be attributable to several factors. We observed only minor changes in the LV β-adrenergic density in AR rats although metoprolol treatment increased β₁ receptor mRNA expression. This suggests an increased turnover of the receptor in this situation. However, most of the changes we observed took place downstream in the signaling pathway. The GRK system is
considered to play a pivotal role in the desensitization and down-regulation of G protein-coupled receptors. We were thus interested to investigate GRK 2 and 5 protein contents in the LV of our AR rats. Changes in the expression of the GRK2, GRK5 and Gs levels suggest an activation of the adrenergic system not only during the acute but also in the chronic phase since high levels of GRK2 persist even after 6 months. The presence of elevated LV tissue catecholamines concentration is also an indicator of neurohormonal activation. Metoprolol normalized the GRK2 expression. High levels of GRK2 have been associated to cardiac hypertrophy and heart failure. Inhibition of GRK2 activity in these models was shown to improve cardiac function and to elicit changes similar to β-blockade. Since β-adrenergic receptor internalization is necessary for activation of pathways that elicit cellular growth, the effect of metoprolol on GRK2 levels is of significant importance. The minimal effects of beta-blockade on beta-adrenergic receptor density in our model seem to come in contrast with previous observations made in animals and humans with congestive heart failure where beta-blockade usually results in a significant increase in receptor density. Our animals were not in overt heart failure however and were still relatively early in the evolution of their disease. For these reasons, we did not expect dramatic variations in receptor density and in fact there were little variations of the overall density of β-adrenergic receptors throughout the protocol even in untreated animals. Our data suggests that β-blockade favorably affects the receptor’s turnover and its regulatory pathways. However, the exact mechanisms by which this occurs remain to be further explored. In our study, metoprolol treatment also reduced the cross-sectional area of cardiomyocytes. Although such effects of β-blockers have been shown in other models of cardiac diseases, this is the first time it
has been shown in a model of chronic AR. Metoprolol had also a beneficial effect on extracellular matrix remodeling by inhibiting collagen and fibronectin expression. These parameters were increased in AR rats. An increase in the expression of MMP2 is also an indicator of an active remodeling process. The increase in the expression of collagens and fibronectin as well as smooth muscle α–actin suggest an increased number of fibroblasts in the AR rats. Metoprolol had no effect on the number of fibroblasts present in AR rats. We previously observed that the amount of fibroblasts in the LV of AR rats rises abruptly in the first two weeks post AR thus before the beginning of treatment in our study. Nevertheless, our results suggest that β-blockade alone can inhibit LV remodeling by inhibiting the expression of several extracellular matrix components despite the presence of an increased amount of fibroblasts in the LV.

Study limitations:
Great care must be taken before results of animal studies are transposed to humans. Our model of AR was not designed to evaluate long term morbidity and mortality and has significant differences compared to the disease in humans as most animal models do. Drug dosage in rats and humans are radically different and cannot be interchanged. The doses of metoprolol we used induced only marginal reductions of heart rate in our animals. The effects of higher doses potentially resulting in significant bradycardia need to be assessed. Since most patients with severe AR who are not yet candidates for valve surgery use oral vasodilators, the combination of these drugs with beta-blockers needs to be evaluated. Further studies will be performed to answer those questions.
Conclusion: In our animal model of chronic AR, a six-month treatment with β-blocker metoprolol prevented LV dilatation, preserved LV ejection fraction, helped maintain normal filling parameters and prevented myocyte hypertrophy and inhibited some aspects of extracellular matrix remodeling. These results suggest an important role of the adrenergic system in the development of volume overload cardiomyopathy associated with severe chronic AR.
References


Figure legends:

Figure 1: Evolution of LV dimensions and systolic function by echocardiography in AR rats treated or not with Metoprolol (180 days). Results in the non-treated AR (squares) or rats treated with Metoprolol (circles) are presented as mean ± SEM (n=10; 18 for AR). *: P<0.05 and **: P<0.01 vs. untreated AR animals.

Figure 2: Total catecholamine levels and β-adrenoreceptor density in LV homogenates from sham-operated and AR rats treated or not with Metoprolol. Results are means ± SEM (n=10 per group) A) Total catecholamine levels at 180 days. B) Total β-adrenoreceptor density in LV of rats after 1, 2, 14 or 180 days of AR without treatment. Relative proportion of type 1 and 2 receptors was unchanged (not shown). *: P<0.05 vs. sham-operated animals (day 0).

Figure 3: Semi-quantitative evaluation by RT-PCR of β-adrenoreceptor subtypes gene expression in the LV of sham-operated of AR rats treated or not with Metoprolol at 180 days (mean ± SEM (n=10 per group)). Messenger RNA levels in sham-operated animals were arbitrarily set to 100. *: P<0.05 vs. untreated AR.

Figure 4: G-protein receptor kinases 2 and 5 protein levels in the LV of AR rats. A) GRK2 protein content during acute and chronic AR and effect of metoprolol treatment as evaluated by immunoblotting (mean ± SEM (n=10/group) relative to day 0 animals (fixed arbitrarily to 100)). *: P<0.05 and **: P<0.01 vs. sham-operated animals (day 0); ¶:
p<0.05 vs. day 180. B) Representative immunoblottings for GRK2 and GRK5. C) GRK5 protein content during acute and chronic AR evaluated by immunoblotting. *: P<0.05 vs. sham-operated animals.

Figure 5: LV and extracellular matrix remodelling at 180 days. A) Sarcomeric and smooth muscle cell α-actin protein content in crude LV homogenates from sham-operated, untreated and Met-treated AR rats. B) Fibronectin-positive cells/field in the LV of AR rats. C) Semi-quantitative evaluation by RT-PCR of fibronectin mRNA. D) Collagen type 1 (Col1) mRNA expression by RT-PCR. E) Collagen type III (Col3) mRNA expression. F) pro-MMP2 (MMP2) mRNA expression. Results are expressed as mean ± SEM (n=10/group). *: P<0.05 and **: P<0.01 vs. untreated AR animals.
Table 1. Hemodynamics (day 180).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham (n=10)</th>
<th>AR (n=18)</th>
<th>Met (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse pressure (mmHg)</td>
<td>50.7 ± 4.6*</td>
<td>69.7 ± 6.6</td>
<td>69.2 ± 6.7</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>249 ± 5</td>
<td>239 ± 9</td>
<td>232 ± 12</td>
</tr>
<tr>
<td>Stroke volume (ml)</td>
<td>0.29 ± 0.01**</td>
<td>0.43 ± 0.02</td>
<td>0.44 ± 0.03</td>
</tr>
<tr>
<td>Cardiac output (ml/min)</td>
<td>70.8 ± 2.8**</td>
<td>101.7 ± 4.1</td>
<td>102.4 ± 9.3</td>
</tr>
<tr>
<td>Cardiac index (ml/min/g)</td>
<td>0.110 ± 0.010*</td>
<td>0.145 ± 0.007</td>
<td>0.152 ± 0.015</td>
</tr>
</tbody>
</table>

Values are means ± SEM. *: p<0.05 and **: p<0.01 vs. AR control rats. Stroke volume is from pulsed Doppler measurement in the left ventricular outflow tract.
Table 2. Echo parameters baseline at week 2 prior to the initiation of Metoprolol vs. vehicle alone (n=28).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline</th>
<th>Week 2</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>End diastolic diameter (mm)</td>
<td>7.8 ± 0.10</td>
<td>9.6 ± 0.10</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>End systolic diameter (mm)</td>
<td>4.0 ± 0.08</td>
<td>5.5 ± 0.10</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>72.3 ± 1.49</td>
<td>66.9 ± 1.11</td>
<td>0.01</td>
</tr>
<tr>
<td>Relative wall thickness</td>
<td>0.39 ± 0.010</td>
<td>0.34 ± 0.006</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LV mass index (mg/g)</td>
<td>1.9 ± 0.11</td>
<td>2.8 ± 0.16</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values are means ± SEM.
Fig. 1
Plante et al.
Fig. 2
Plante et al.
Fig. 3
Plante et al.
Fig. 4
Plante et al.
Fig. 5
Plante et al.