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β-2 Adrenergic Stimulation Attenuates Left Ventricular Remodeling, Decreases Apoptosis, and Improves Calcium Homeostasis in a Rodent Model of Ischemic Cardiomyopathy

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Abbreviations: $SERCA_{2a}$, sarcoplasmic reticulum calcium-ATPase; LAD, left anterior descending artery; Clen+Meto, clenbuterol and metoprolol; EDPVR, end-diastolic pressure-volume relationships; LVEDP, left ventricular end-diastolic pressure; dP/dt_{max}, maximum left

ventricular dP/dt; dP/dt_{min}, minimum left ventricular dP/dt; TUNEL, terminal deoxynucleotidyltransferase end labeling; 8-oxoG, 8-oxo-7,8-dihydrodeoxyguanine.

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Abstract

The benefit of the β2-adrenergic agonist, clenbuterol, in left ventricular assist device patients with dilated cardiomyopathy has been reported, but its effect on ischemic HF is unknown. We investigated whether clenbuterol improves left ventricular remodeling, myocardial apoptosis, and has synergy with a β1-antagonist, metoprolol, in a model of ischemic HF. Rats were randomized to: 1)HF only; 2)HF+Clenbuterol; 3)HF+Metoprolol; 4)HF+Clenbuterol+Metoprolol; and 5)rats with sham surgery. HF was induced by LAD artery ligation and confirmed by decreased left ventricular fractional shortening, decreased dP/dt_{max} and elevated LVEDP, compared to Sham rats ($p<0.01$). After 9 weeks of oral therapy, echocardiographic, hemodynamic, and *ex vivo* end-diastolic pressure-volume relationship (EDPVR) measurements were obtained. Immunohistochemistry was performed for myocardial apoptosis and DNA damage markers. Levels of calcium-handling proteins were assessed by Western blot analysis. Clenbuterol-treated HF rats had increased weight gain and heart weights vs HF rats ($p<0.05$). EDPVR curves revealed a leftward shift in clenbuterol rats vs metoprolol and HF rats ($p<0.05$). The metoprolol-treated group had a lower LVEDP and higher dP/dt_{max} vs the HF group ($p<0.05$). Clenbuterol and metoprolol groups had decreased myocardial apoptosis and DNA damage markers and increased DNA repair markers vs HF rats (all $p<0.01$). Protein levels of the ryanodine receptor and $SERCA_{2a}$ were improved in clenbuterol-, metoprolol-, and clenbuterol+metoprolol-treated groups vs HF rats. However, as a combination therapy, there were no synergistic effects of clenbuterol+metoprolol treatment. We conclude that clenbuterol ameliorates EDPVR, apoptosis, and calcium homeostasis but does not have synergy with metoprolol in our model of ischemic HF.

Introduction

Clenbuterol is a ß2-adrenergic receptor agonist first used in the mid-1970s to treat asthma and is approved for this indication in Europe (Salorinne et al., 1975). The drug bears structural similarity to albuterol but has enhanced oral absorption and β-2 selectivity. Clenbuterol also increases muscle bulk (Choo et al., 1992; Maltin et al., 1993; Carter and Lynch, 1994) as a result of an anabolic effect that may be mediated via long-term β2-activation (MacLennan and Edwards, 1989; Choo et al., 1992). As a result of this anabolic action, oral clenbuterol has been abused by athletes to enhance size and strength (Beckett, 1992; Perry, 1993).

Interest in clenbuterol has been recently sparked as a potential treatment for cardiac diseases, specifically in regard to improving cardiac mechanical properties. Petrou et al. (1995) found that clenbuterol led to hypertrophy of latissimus dorsi and cardiac muscle in rats. Subsequent work by this group found that clenbuterol promotes cardiac hypertrophy in rats after banding of the ascending aorta (Wong et al., 1997). Normal rat hearts treated with clenbuterol have also been shown to have elements of "physiologic" hypertrophy, with normal function, morphology, and calcium-handing mRNA levels (Wong et al., 1998). Finally, this group demonstrated improved right ventricular systolic function with clenbuterol therapy after induction of right-sided failure by pulmonary artery banding in sheep (Hon et al., 2001).

An intriguing subsequent report describes the use of clenbuterol (in combination with ACE inhibition, β-1 selective blockade, and spironolactone) in patients with non-ischemic, dilated cardiomyopathy supported with a left ventricular assist device (Yacoub et al., 2001). Ten of 15 patients treated with clenbuterol in this study had significant cardiac improvement, allowing for assist device explantation for recovery (Hon and Yacoub, 2003). This series

represents a rate of myocardial recovery that is more than double that of any previously reported study. This preliminary case series has not yet been confirmed by controlled studies; in addition, the effects of clenbuterol on ischemic cardiomyopathy have not been evaluated to date in any experimental or clinical study.

The interest in the use of β-2 adrenergic agonists, such as clenbuterol, in heart failure also stems from the recent evidence that the toxic effects of β-adrenergic stimulation is mediated primarily via β-1 receptors (Communal et al., 1999; Zhu et al., 2001; Zhu et al., 2003), while β-2 receptor stimulation may be protective (Communal et al., 1999; Chesley et al., 2000; Ahmet et al., 2004). Myocardial apoptosis has been implicated as a possible mechanism in the pathogenesis of heart failure progression (Kang and Izumo, 2000) and has been correlated with the degree of left ventricular remodeling (Abbate et al., 2003). Apoptosis in post-infarction heart failure has been shown to be primarily mediated via β-1 adrenergic receptors (Prabhu et al., 2003). This raises the possibility that combination therapy with a β-1 blocker, such as metoprolol, and a β-2 agonist, such as clenbuterol, may be potentially synergistic in their effects on heart failure.

We utilized a well-established model of ischemic, chronic heart failure in rats to study: (1) the effects of clenbuterol on cardiac function and ventricular remodeling in ischemic cardiomyopathy both alone and in combination with metoprolol, and (2) the underlining effects of clenbuterol on myocardial apoptosis and calcium homeostasis. For this latter objective, we evaluated the effects of clenbuterol on markers of apoptosis, DNA damage, and DNA repair in our chronic model of heart failure. In addition, we studied the effects of clenbuterol on calcium-handling protein expression levels of the ryanodine receptor and sarcoplasmic reticulum calcium-ATPase ($SERCA_{2a}$).

Methods

Induction of Chronic Heart Failure

Studies were performed in compliance with the Guide for the Care and Use of Laboratory Animals and were approved by the Columbia University Institutional Animal Care and Use Committee. Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 250 to 300 g were used for all experiments.

After induction with intraperitoneal ketamine (75 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (5 mg/kg; Lloyd Laboratories, Shenandoah, IA), endotracheal intubation with an angiocathether was performed. Rats were supported by a small animal ventilator (Harvard Apparatus, Holliston, MA). After performing a left thoracotomy, a sham operation (pericardiectomy only) or left anterior descending artery (LAD) ligation was performed, as previously described (Kherani et al., 2004).

A total of 69 rats were used in this study. LAD ligation surgery was performed in 60 rats, of which 37 (62 %) survived 3 weeks post-surgery. Another 9 rats underwent a sham operation (group Sham), with 7 (78 %) survivors. Three weeks post-operatively, echocardiography was performed to establish the baseline level of heart failure (measured by fractional shortening). The LAD ligation rats were divided into 4 treatment groups matched for the degree of heart failure and were randomly assigned to one of 4 therapies for 9 additional weeks: (1) rats receiving no therapy (group heart failure, n = 9); (2) rats receiving high-dose clenbuterol at 1 mg/kg/day (group clenbuterol, n = 9); (3) rats receiving high-dose metoprolol at 200 mg/kg/day (group metoprolol, n = 9); or (4) rats receiving concurrent high-dose clenbuterol at 1 mg/kg/day and high-dose metoprolol therapy at 200 mg/kg/day (group Clen+Meto, n = 10).

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Of 44 surviving rats (37 post-LAD ligation and 7 Sham rats), 39 (89 %) survived the 12 week follow-up period. There were no differences in survival rates among the groups undergoing LAD ligation. There were also no differences in left ventricular infarct size, as expressed as a percentage of the left ventricular circumference, among the groups. The LAD ligation rats all had a myocardial infarction of sufficient size to induce heart failure, spanning ≥ 20% of the left ventricular circumference (mean: 38.1 ± 12.3 %, range: 20 to 63).

Oral Pharmacotherapy

Clenbuterol (ICN Biomedicals, Aurora, OH) was sonicated and dissolved in the drinking water. Metoprolol (Sigma-Aldrich, St. Louis, MO) was dissolved in the drinking water either alone or in combination with clenbuterol. The study drug concentrations were varied to keep the dose delivered within a narrow therapeutic window based on water consumption.

Echocardiography

Under mild isoflurane anesthesia, 2-D echocardiography (Sonos-5500, Agilent Technologies, Palo Alto, CA) was performed 3 and 12 weeks post-surgery for pre- and post-treatment measures of cardiac function and analyzed in a blinded fashion. Left ventricular anteroposterior diameter and short-axis area at the papillary muscle level were measured to obtain the left ventricular end-diastolic and end-systolic diameter and area. Fraction shortening and fractional area change were subsequently calculated.

Hemodynamic Measurements

In the terminal experiments 12 weeks post-surgery, rats were anesthetized with inhaled isoflurane. A 2-French Millar catheter (Millar Instruments, Houston, TX) was inserted into the right carotid artery and pressure measurements collected as the catheter was advanced from the aorta into the left ventricle. Hearts were subsequently weighed and used for *ex vivo*

determination of left ventricular end-diastolic pressure-volume relationships (EDPVR). Using Chart 4 (version 4.2.4, ADInstruments, Colorado Springs, CO), left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure, mean aortic pressure, heart rate, and maximum and minimum left ventricular dP/dt (dP/dt_{max} and dP/dt_{min}) were later obtained from hemodynamic recordings in a blinded fashion.

End-Diastolic Pressure-Volume Relationship Determinations

An angiocathether was placed into the left ventricle through the aortic valve. A fine hemostat was placed on the atrial side of the mitral annulus to seal the left ventricle. Left ventricular pressures were measured using a 5-French Millar micromanometer introduced through the angiocathether. While recording left ventricular pressure, saline was infused into the left ventricle in 50 μL increments using a calibrated syringe. The infused fluid was withdrawn and measured to ensure that no leakage had occurred. Using commercial software (Igor Pro, version 4.0.5.1; WaveMetrics, Lake Oswego, OR), values of left ventricular pressure and volume were fitted according to the equation: $\text{Pressure} = \beta\text{Volume}^{\alpha}$, where β is the base constant and α is an index of ventricular stiffness, as previously described (Mirsky, 1976; Kherani et al., 2004). Data were averaged to construct the mean EDPVR tracings for each group, after normalizing left ventricular volumes for differences in heart weight, as reported previously (Amirhamzeh et al., 1997; Rabkin et al., 1998; Burkhoff et al., 2005). Analyses were performed in a blinded manner. Comparisons between groups were made based on normalized volume measurements at left ventricular pressures of 30 mmHg (Burkhoff et al., 2005).

Histological Analysis

A short-axis section of the heart at the point of maximal infarction was fixed in 4% paraformaldehyde solution. Sections were embedded in paraffin and 5 μm slices used for trichrome staining. The infarct size was determined as a percentage of the left ventricular circumference in a blinded fashion. The remaining heart tissue was flash-frozen and stored at –80° C for Western blot analysis.

Assessments of Myocyte Apoptosis, DNA Damage and DNA Repair Enzyme Expression

Five rat hearts from each group were randomly selected and used for analysis of apoptosis and DNA damage and repair markers. A comparable area of the infarct border zone was utilized for these studies. In order to quantify the proportion of cells with DNA fragmentation, an *in situ* terminal deoxynucleotidyltransferase end labeling (TUNEL) assay (Oncor, Gaithersburg, MD) was performed, as previously described (Lin et al., 2003). After deparaffinizing sections, sections were incubated with terminal deoxynucleotidyl transferase buffer (Boehringer Mannheim, Indianapolis, IN) containing TdT enzyme (Boehringer Mannheim) and biotin-16-dUTP (Boehringer Mannheim). Sections were stained with diaminobenzadine and counterstained with hematoxylin. The percentage of TUNEL-positive cells was quantified as an overall percentage of counted cells.

Of all known oxidative DNA damage products, human 8-oxo-7,8-dihydrodeoxyguanine (8-oxoG) has been shown to be the most stable and important (Lin et al., 2003). The degree of DNA damage was therefore determined by staining with monoclonal 8-oxoG antibody, as previously described (Lin et al., 2003). DNA was denatured by soaking paraffin-embedded sections in HCl. Nonspecific staining sites were blocked with 10% fetal bovine serum. Slides were incubated with H_2O_2 to block endogenous peroxidase. Thereafter, slides were incubated with primary anti-8-oxoG monoclonal antibody (Trevigen, Gaithersburg, MD; diluted 1:100) and

with secondary anti-mouse antibody (1:100) conjugated with streptavidin-horseradish peroxidase. Slides were stained with diaminobenzamide and counterstained with methyl green. The percentage of cells staining positive for 8-oxoG was quantified as an overall percentage of counted cells.

Human MutY homologue and 8-oxoG ghycosylase are proteins which have been shown to play important roles in repairing DNA mismatch injury (Slupska et al., 1996; Arai et al., 1997). Their expression patterns were used to evaluate the DNA repair enzyme activity using both immunohistochemistry and immunoblot analysis, as we have recently described (Lin et al., 2003). Sections were blocked in 2% normal horse serum and incubated with primary human MutY homologue or 8-oxoG glycosylase antibody (both 1:100 dilution; Novus Biological, Littleton, OH). After quenching endogenous peroxidase activity with H_2O_2, the slides were incubated with secondary antibody conjugated to horse radish peroxidase (Amersham Biosciences, Piscataway, NJ). The final reaction was achieved by incubating the sections with freshly prepared reagent containing 3-amino-9-ethylcarbazole (Sigma). Mounted sections were counterstained with hematoxylin. Two trained, blinded observers reviewed sections, with at least 3 samples scored per group. Human MutY homologue and 8-oxoG glycosylase expression was evaluated by scoring the percentage of positive staining on the section as we previously described (Wei et al., 1993; Wei et al., 1994). According to this semi-quantitative scoring system, 0 = no staining; 1 = minimal staining (<10% positive); 2 = mild staining (10 to 30% positive); 3 = moderate staining (31 to 50% positive); and 4 = strong staining (>50% positive). The specificity of positive staining was confirmed by substitution of normal rabbit serum for the primary antiserum.

Western blot analysis for human MutY homologue and 8-oxoG glycosylase was performed as described previously (Lin et al., 2003). Protein samples were run on SDS-PAGE using a 10% polyacrylamide gel. After transfer of proteins to nitrocellulose, membranes were placed in 1% powdered milk to block nonspecific binding. After reacting with the primary and secondary antibodies, the membrane was subjected to the Enhanced Chemiluminescence analysis system (Amersham Biosciences). Monoclonal antibody against actin (Ab-6, Oncogene Research Products, MA) was used to control for differences in protein loading. To ascertain specific binding of the anti-human MutY homologue or 8-oxoG glycosylase antibody, a control membrane was studied without this primary antibody.

Calcium-Handling Protein Expression Levels

Five random heart samples from each group were similarly used to analyze calcium-handling protein expression levels. Approximately 150 mg of left ventricular tissue was lysed and samples denatured at 95^0C and size-fractionated using SDS-PAGE using 7.5% separating and 5% stacking gels for $SERCA_{2a}$ and 5% separating and 4% stacking gels for the ryanodine receptor. Proteins were transferred onto nitrocellulose. Blots were blocked in 5% nonfat milk. Blots were incubated with diluted primary antibody diluted (anti-$SERCA_{2a}$: 1:1,000, ABR Affinity BioReagents, Golden, CO; anti-ryanodine receptor: 1:2,500, gift from Dr. Andrew Marks' laboratory; anti-tubulin: 1:1,000, Sigma) . Blots were incubated in the presence of a horseradish, peroxidase-labeled secondary antibody ($SERCA_{2a}$: anti-mouse IgG, Amersham Biosciences; ryanodine receptor: anti-rabbit IgG, Amersham Biosciences) diluted 1:4,000. Blots were developed using ECL reagent (Amersham Biosciences). Optical densities of protein signals were quantified using a densitometer (Molecular Dynamics, Palo Alto, CA) in a blinded

manner. $SERCA_{2a}$ and ryanodine receptor protein levels were expressed relative to levels of tubulin.

Statistical Analysis

All statistical analysis was performed using SPSS 11.5 software (SPSS, Chicago, IL). Comparisons of echocardiographic and body weight data between pre- and post-treatment timepoints were performed with the use of repeated measures analysis of variance, with the group, infarct size, and timepoint as fixed factors. Comparisons between treatment groups for hemodynamic, immunohistochemistry, and protein level data were made using a 2-way analysis of variance, with the group and infarct size [categorized as a large (≥ 30 % of left ventricular circumference) or small infarct (< 30 %)] as fixed factors. For dP/dt_{max} and dP/dt_{min}, non-parametric tests (Kruskal-Wallis) were used because these variables were not normally distributed. The presence of synergy with combination pharmacological therapy was assessed with the use of multivariate analysis of variance, including interaction terms for the combination therapy cohort. Tukey's ad hoc tests were used for all group comparisons. A *p*-value less than 0.05 was considered significant. All data are expressed as a mean ± standard deviation.

Results

Echocardiographic Data

Figure 1 depicts the echocardiographic data. At 3 weeks post-surgery, there was a significantly lower fractional shortening (17.4 ± 5.22) and fractional area change (29.4 ± 6.20) in the 4 LAD ligation groups, as compared to Sham rats (fractional shortening: 50.8 ± 6.78; fractional area change: 72.3 ± 6.89; $p < 0.001$). There were no differences in the fractional shortening or fractional area change among the 4 LAD ligation groups prior to or after 9 weeks of oral pharmacotherapy.

Hemodynamic Data

Table 1 depicts the direct hemodynamic data obtained in the study animals after 9 weeks of oral pharmacotherapy. Metoprolol-treated animals had a significantly lower heart rate and LVEDP and higher dP/dt_{max} than control heart failure rats. Control heart failure, clenbuterol, and Clen+Meto group had a significantly higher LVEDP than Sham rats, while the metoprolol rats were no different from Sham rats. There were no differences in the systolic or mean left ventricular or aortic pressures among the groups.

Body and Heart Weights

The body weight and heart weight data are shown in Table 2. The percentage change in the body weight was significantly higher in the Sham rats, as compared to the LAD ligation groups. Treatment with clenbuterol alone and in combination with metoprolol, however, led to higher increases in body weight, as compared to the control heart failure and metoprolol-treated groups. For heart weight, Sham rats had lower weights than the LAD ligation cohorts.

Clenbuterol-treated rats had significantly higher heart weights than both control heart failure and metoprolol-treated animals.

End-diastolic Pressure-Volume Relationship Tracings

The *ex vivo*, passive EDPVR curves obtained are shown in Figure 2. There was a rightward shift for heart failure, metoprolol, and Clen+Meto versus Sham rats. In contrast, clenbuterol-treated rats (Volume at 30 mmHg: 0.42 ± 0.05 mL/gm of heart weight) had lower left ventricular chamber volumes than either metoprolol or heart failure rats (Volume at 30 mmHg: 0.51 ± 0.08 and 0.50 ± 0.09 mL/gm of heart weight, respectively; both $p < 0.05$) and were no different from Sham rats (0.36 ± 0.03 mL/gm of heart weight). This signifies that at a given left ventricular pressure, clenbuterol led to lower left ventricular volumes vs right-shifted heart failure rats with dilated and remodeled ventricles.

Apoptosis and DNA Damage and Repair Markers

The quantitative immunohistochemistry data of apoptosis and DNA damage and repair markers are shown in Table 3 and representative images are shown in Figure 3. The staining of TUNEL was increased in all the LAD ligation groups versus the Sham rats, indicating that there was increased apoptosis even 12 weeks after surgery in these animals. Clenbuterol and metoprolol treatment both alone and in combination led to decreased levels of TUNEL staining versus the heart failure group. Similarly, for 8-oxoG staining, the LAD ligation animals had increased levels of this DNA damage marker than Sham rats, though clenbuterol, metoprolol, and Clen+Meto animals had decreased levels versus the heart failure rats. Clen+Meto treatment had an additive effect over clenbuterol or metoprolol therapy alone, as seen by significantly decreased levels of DNA damage with the Clen+Meto group over clenbuterol or metoprolol alone.

For the human MutY homologue and 8-oxoG glycosylase (markers of DNA repair), there was increased levels of both markers versus the Sham group for the clenbuterol, metoprolol, and Clen+Meto animals. Clenbuterol and metoprolol treatment led to improvements both alone and in combination versus the control heart failure group. Interestingly, Clen+Meto treatment led to additive improvement in the 8-oxoG glycosylase staining pattern over either clenbuterol or metoprolol therapy alone. Of note, there was no synergy noted in the effects of Clen+Meto therapy in any of the apoptosis, DNA damage, or DNA repair marker staining patterns.

Calcium-Handling Protein Expression Levels

Figure 4 depicts the calcium-handling protein expression levels for the ryanodine receptor and $SERCA_{2a}$. Heart failure rats had decreased levels of both the ryanodine receptor and $SERCA_{2a}$ versus Sham rats. These levels were upregulated in the clenbuterol, metoprolol, and Clen+Meto groups, though this reached statistical significance only for clenbuterol ($P<0.05$ vs the heart failure group). Ryanodine receptor protein expression levels were normalized in the clenbuterol, metoprolol, and Clen+Meto cohorts, with levels not detectably different from Sham animals. The $SERCA_{2a}$ protein expression level was also partially normalized by clenbuterol, metoprolol, and Clen+Meto treatment.

Discussion

This is the first study to date evaluating the effects of the β-2 adrenergic agonist, clenbuterol, in ischemic cardiomyopathy and the first to evaluate the *in vivo* effects of combination therapy with a β-1 adrenergic antagonist and β-2 agonist. In our model of chronic, ischemic heart failure, clenbuterol led to improvements in ventricular remodeling, myocardial apoptosis, DNA damage and repair, and calcium homeostasis. Interestingly, we found no evidence of synergy in the use of this β-2 agonist and a selective β-1 antagonist, metoprolol.

Based on echocardiographic and histological data, we were able to achieve a significant degree of heart failure in our experimental model. The size of the left ventricular infarction attained was uniform across treatment arms. The fractional shortening and fractional area change demonstrated a significant decrease in systolic function in our chronic model of heart failure. Finally, repeat echocardiographic and direct hemodynamic data at 3 months demonstrated both systolic and diastolic dysfunction in the control heart failure group versus Sham rats.

As expected, high-dose clenbuterol treatment led to significant increases in heart weight and body weight over 9 weeks of oral pharmacotherapy. Clenbuterol has been previously shown to induce “physiological” myocyte hypertrophy (Petrou et al., 1995; Wong et al., 1998) and is known to have anabolic properties (Beckett, 1992; Maltin et al., 1993; Perry, 1993; Carter and Lynch, 1994). Other β-2 agonists, such as salbutamol, cimaterol, and fenoterol, have also been shown to have anabolic characteristics (Emery et al., 1984; Martineau et al., 1992; Byrem et al., 1998). Long-acting clenbuterol has more potent anabolic effects than short-acting salbutamol unless salbutamol is continuously infused, and these anabolic effects are blocked by selective β-2

antagonists (Choo et al., 1992). The mechanism for this anabolic effect may therefore be mediated by long-acting β-2 adrenergic stimulation (Choo et al., 1992).

In contrast, high-dose metoprolol treatment led to a decreased heart rate and LVEDP and an improved dP/dt_{max} with oral therapy. The improvements demonstrated with metoprolol therapy are similar to prior published reports in rat post-infarction models (Prabhu et al., 2000; Prabhu et al., 2003; Ahmet et al., 2004).

The dosages used for clenbuterol and metoprolol administration in our study were based on prior published reports of use in rodents. Clenbuterol was orally administered at 2 mg/kg/day, a dose similar to prior reports of its anabolic actions in skeletal muscle (Emery et al., 1984; Choo et al., 1992; Carter and Lynch, 1994) and comparable to dosing in larger animals in a recent study of clenbuterol's effects on cardiac function after pulmonary artery banding in sheep (Hon et al., 2001). Although serum clenbuterol levels were not monitored, observed changes in heart weight and body weight with oral clenbuterol pharmacotherapy suggest effective and appropriate drug delivery. In addition, metoprolol was orally administered at 200 mg/kg/day, a dose similar to 2 prior reports of its use in post-infarction rodent models (Prabhu et al., 2000; Prabhu et al., 2003). Although metoprolol drug levels were also not monitored in our experiment, our finding of a decreased heart rate in the metoprolol group is strong evidence of effective therapeutic delivery.

This is the first study demonstrating decreased ventricular remodeling with clenbuterol treatment, as seen by a leftward shift in the EDPVR of the clenbuterol group. Heart failure rats exhibited substantially dilated and remodeled left ventricular chamber volumes relative to non-infarcted Sham rats, with a rightward shift of the pressure-volume curve. Clenbuterol therapy, however, caused a reduction in ventricular cavity dilation, shifting EDPVR leftward, strongly

suggesting that clenbuterol attenuated deleterious post-infarction left ventricular remodeling. Our findings support the report by Hon et al. (2001) who found normal diastolic relaxation and stiffness with clenbuterol therapy in a study of sheep undergoing pulmonary artery banding. Lastly, our results are similar to those by Ahmet et al. (2004), who utilized the β-2 adrenergic agonists finoterol and zinterol in a rodent LAD-ligation model to demonstrate that β-2 adrenergic stimulation improves diastolic function in this experimental study of chronic heart failure.

We found no evidence of systolic improvements, however, in the clenbuterol-treated cohort. There was no change in echocardiographic measurements or dP/dt_{max} after 9 weeks of clenbuterol therapy. This is in contrast to the findings of Hon et al. (2001), who reported improved contractile and systolic function with clenbuterol treatment of sheep after pulmonary artery banding, but similar to the findings of Wong et al. (1997), who found no significant changes in systolic function with clenbuterol-treated rats after pulmonary artery banding.

In addition, we demonstrate significantly lower levels of calcium-handling proteins with the induction of chronic, ischemic heart failure and upregulation with clenbuterol, metoprolol, and combinational clenbuterol and metoprolol treatment, as seen by the protein expression levels of the ryanodine receptor and $SERCA_{2a}$. This is the first report to evaluate the effects of clenbuterol on calcium-handling protein levels in a model of heart failure. Our study is consistent with prior experimental reports evaluating the effects of clenbuterol on $SERCA_{2a}$ mRNA levels in pressure-overloaded rodents (Wong et al., 1997). Furthermore, this is consistent with a recent study reporting improved calcium-handling in isolated myocytes obtained from a small series of DCM patients treated with clenbuterol while supported with a left ventricular assist device (Terracciano et al., 2003). This upregulation of calcium-handling proteins carries considerable clinical significance, as altered calcium homeostasis has been implicated in the

pathogenesis of heart failure (Yano et al., 2003; Braz et al., 2004), and represents one possible mechanism by which clenbuterol may lead to the observed attenuation of left ventricular remodeling.

We also demonstrate that clenbuterol led to decreased myocardial apoptosis, DNA damage and increased DNA repair, as seen with the quantitative immunohistochemistry staining of key markers of apoptosis, DNA damage, and DNA repair. In addition, Western blot analysis of 8-oxoG glycosylase and human MutY homologue protein levels confirmed these findings. Decreased myocardial apoptosis via TUNEL staining has been previously shown in both *in vivo* experimental models (Ahmet et al., 2004) and cultured myocytes (Communal et al., 1999; Chesley et al., 2000; Zhu et al., 2001) with therapy with other β-2 adrenergic agonists. To our knowledge, this is the first study to evaluate the effects of β-2 adrenergic stimulation on DNA damage and repair. These improvements pose another plausible mechanism by which clenbuterol therapy may attenuate left ventricular remodeling, as myocardial apoptosis has been implicated in the remodeling process (Abbate et al., 2003). Inhibition of apoptosis pathways has also been recently shown to decrease left ventricular remodeling (Chandrashekhar et al., 2004).

Interestingly, the use of clenbuterol led to similar improvements in apoptosis, DNA damage, and DNA repair as metoprolol therapy. Prior reports have demonstrated that β-adrenergic receptor-mediated apoptosis is largely independent of β-2 stimulation and mostly mediated via β-1 receptor pathways (Zaugg et al., 2000). In addition, prior studies have confirmed that β-1 adrenergic antagonists (Ahmet et al., 2004) and β-2 adrenergic agonists (Communal et al., 1999; Chesley et al., 2000; Zhu et al., 2001; Ahmet et al., 2004) lead to deceased myocardial apoptosis.

The combination of the β-2 adrenergic agonist clenbuterol and the β-1 antagonist metoprolol did not lead to evidence of synergy in this study. We present evidence of additive improvements in the 8-oxoG and 8-oxoG glycosylase staining patterns in the Clen+Meto group, indicating that clenbuterol and metoprolol led to independent improvements in DNA damage and repair, but these effects were not synergistic. Chesley et al. (Chesley et al., 2000) have shown that β-2 adrenergic stimulation decreases apoptosis through a pertussis toxin-sensitive, phosphatidylinositol-3'-kinase-dependent pathway in cultured myocytes. In contrast, β-1 adrenergic-mediated apoptosis has been recently shown to occur via a calmodulin kinase II pathway (Zhu et al., 2003; Zhang et al., 2005). The independence of these pathways is further supported with the presence of an additive, but not synergistic, effect in our study. Combination therapy with clenbuterol and metoprolol, however, did not lead to the same attenuation of ventricular remodeling as with clenbuterol alone (seen with EDPVR) or to the systolic improvements observed with metoprolol therapy alone (seen with dP/dt_{max}), suggesting that combination therapy antagonized or counteracted each of these beneficial effects.

Limitations. The dosages used for clenbuterol and metoprolol were high-dose, raising the possibility that synergy was not seen with combination therapy because of the counteraction of β-2 adrenergic effects with the use of an imperfectly selective β-1 antagonist. As mentioned above, drug serum levels were also not measured. In addition, the mechanisms by which clenbuterol attenuated ventricular remodeling were not elucidated by this study. Improvements in calcium-handling protein levels, apoptosis, DNA damage, and DNA repair are only implicated as possible mechanisms. Future studies will better characterize the effects and pathways of chronic β-2 adrenergic stimulation in ischemic heart failure, both alone and in combination with β-1 adrenergic blockade.

Conclusions. We demonstrate that clenbuterol attenuates ventricular remodeling and ameliorates myocardial apoptosis, DNA damage and repair, and calcium homeostasis in an experimental model of ischemic heart failure. These changes did not have synergy with metoprolol therapy. Recently initiated trials studying the effects of clenbuterol on cardiac recovery will determine if ischemic heart failure patients will sustain benefits from this therapy.

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Acknowledgments

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Footnotes

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Figure Legends

Figure 1. Echocardiographic data, as expressed by fractional shortening and fractional area change. Each of the LAD ligation groups had a significantly lower fractional shortening and fractional area change than the Sham animals. There were no significant changes in fractional shortening or fractional area change between the baseline and endpoint parameters for any of the groups.

Figure 2. *Ex vivo* end-diastolic pressure-volume-relationship (EDPVR) curves, after normalization of left ventricular volumes for differences in heart weights. Clenbuterol-treated rats were shifted to the left vs both control heart failure and metoprolol rats and were no different from Sham rats. This signifies that at a given left ventricular pressure, clenbuterol led to lower left ventricular volumes vs right-shifted heart failure rats with dilated and remodeled ventricles. In contrast, heart failure, metoprolol, and Clen+Meto rats had higher passive left ventricular volumes than Sham rats.

Figure 3. Representative immunohistochemistry staining patterns for the apoptosis, DNA damage, and DNA repair markers (original magnification 1000x). A. TUNEL, B. 8-oxoG (DNA damage), C. 8-oxoG glycosylase (DNA repair), D. Human MutY homologue (DNA repair). Representative autoradiographs of the DNA repair protein expression levels are shown (E and F) in comparison to β-actin levels. 8-oxoG glycosylase and human MutY homologue levels were increased in the clenbuterol, metoprolol, and Clen+Meto groups, as compared to the heart failure

and Sham cohorts. Combination therapy with clenbuterol and metoprolol also led to higher 8-oxoG glycosylase and human MutY homologue levels than metoprolol therapy alone.

Figure 4. Calcium-handling protein expression levels of the ryanodine receptor (A) and sarcoplasmic reticulum Ca^{2+}-ATPase ($SERCA_{2a}$) (B), expressed relative to tubulin expression levels. Representative autoradiographs, with the corresponding tubulin blotting signals, are also depicted. Ryanodine receptor and $SERCA_{2a}$ levels were decreased in the heart failure group, as compared to the Sham group. Clenbuterol-, metoprolol -, and Clen+Meto-treated rats had increased levels of the ryanodine receptor and $SERCA_{2a}$ vs control heart failure rats.

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Table 1. Hemodynamic Data

	Sham	Heart Failure	Clenbuterol	Metoprolol	Clen+Meto
Heart Rate (bpm)	291±52.2	302±19.1	281±11.2	243±19.4‖	275±18
LVEDP (mmHg)	5.92±1.85	22.4±11.6‡	21.1±11.0†	11.1±5.29§	20.1±9.53†
LVSP (mmHg)	116±17.3	113±10.3	109±10.7	107±9.27	108±11.1
MAP (mmHg)	39.8±5.56	47.2±8.85	44.2±6.06	42.7±5.38	43.4±4.77
Maximum dP/dt (mmHg/s)	6700±1706	4609±583†	4933±596*	5462±541§	5327±1270
Minimum dP/dt (mmHg/s)	-6458±1799	-3854±563‡	3901±694†	-4032±830†	-4117±1035†

*$p < 0.05$ vs Sham. †$p < 0.01$ vs Sham. ‡$p < 0.001$ vs Sham. §$p < 0.05$ vs heart failure. ‖$p < 0.01$ vs heart failure.
Clen+Meto, clenbuterol and metoprolol; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; MAP, mean arterial pressure.

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Table 2. Body and Heart Weight Data

	Sham	Heart Failure	Clenbuterol	Metoprolol	Clen+Meto
% Δ Weight[a]	76.1±19.3	33.8±4.79‡	49.9±7.18‡ § #	32.3±7.16‡	55.3±9.91† ‖ #
Heart Weight (gm)	1.72±0.09	2.16±0.33*	2.64±0.41‡ ‖ #	2.17±0.34*	2.42±0.32‡

*$p < 0.05$ vs Sham. †$p < 0.01$ vs Sham. ‡$p < 0.001$ vs Sham. §$p < 0.05$ vs heart failure. ‖$p < 0.01$ vs heart failure. #$p < 0.05$ vs metoprolol.

[a] % Δ weight represents the percentage change in pre-treatment to post-treatment weights.

Clen+Meto, clenbuterol and metoprolol.

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Table 3. Quantitative Immunohistochemistry Staining of Apoptosis, DNA Damage, and DNA Repair Markers

	Sham	Heart Failure	Clenbuterol	Metoprolol	Clen+Meto
TUNEL (%)	0.2±0.1	21±4.1†	11±2.6† ‡	12±3.6† ‡	8.2±1.9* ‡
8-oxoG (%)	0.5±0.1	18±3.9†	10±2.1† ‡	12±2.6† ‡	5±1.6* ‡ ‖
human MutY homologue (score)[a]	0.6±0.2	1.4±0.5	2.8±0.5† §	2.6±0.4† §	3.2±0.6† ‡
8-oxoG glycosylase (%)	0.2±0.1	0.5±0.1	8.2±2.2† ‡	6.4±1.5† §	13±3.6† ‡ ‖

*$p < 0.01$ vs Sham. †$p < 0.001$ vs Sham. ‡$p < 0.001$ vs heart failure. §$p < 0.01$ vs heart failure. ‖$p < 0.01$ vs clenbuterol and metoprolol.

[a] Scoring system for human MutY homologue nuclear and cytoplasmic staining: 0 = 0%; 1 = 0-15%; 2 = 15-30%; 3 = 30-50%; and 4 = >50% positive.

Clen+Meto, clenbuterol and metoprolol; TUNEL, terminal deoxynucleotidyltransferase end labeling; 8-oxoG, 8-oxo-7,8-dihydrodeoxyguanine.

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Figure 1.

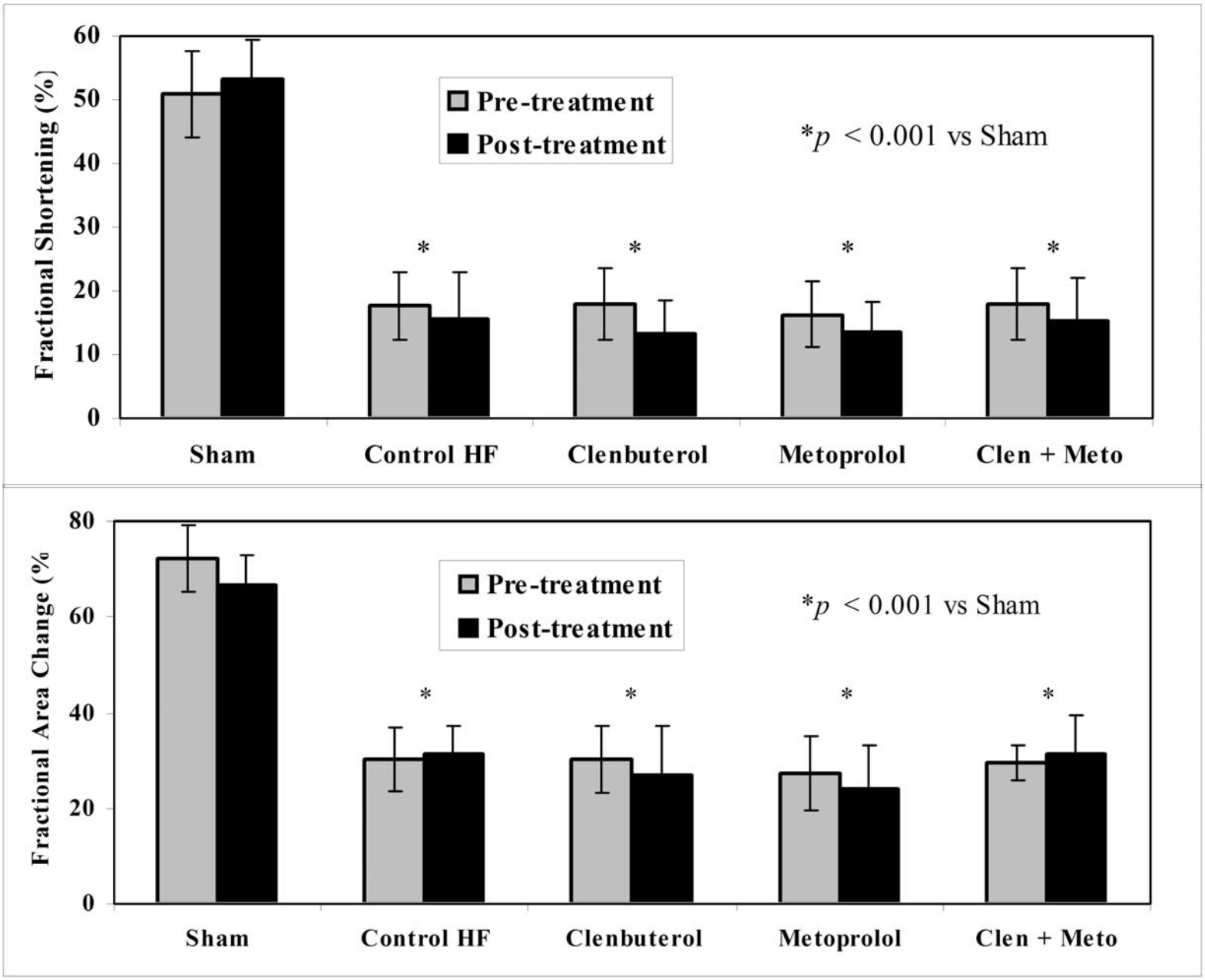

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Figure 2.

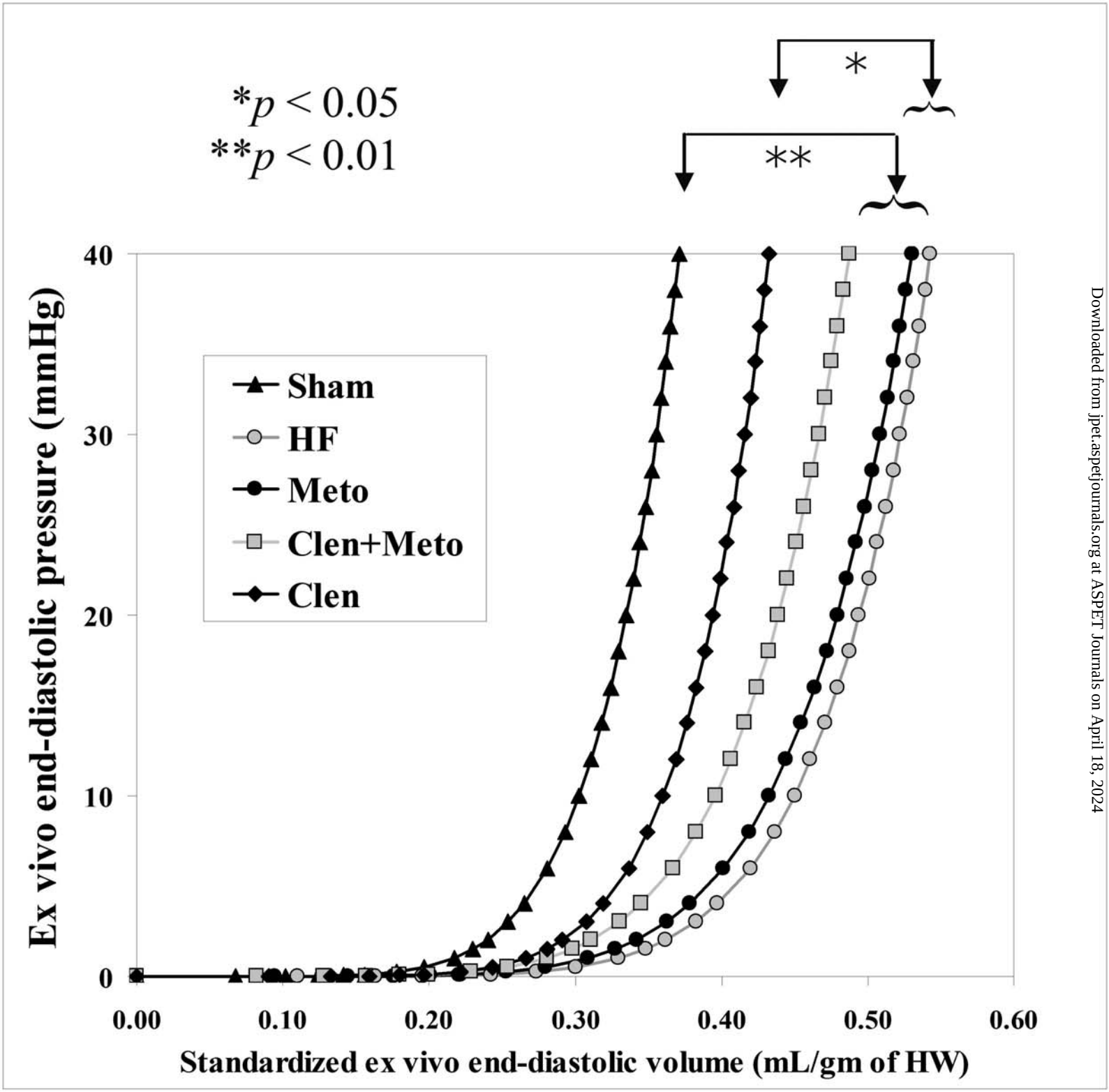

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Figure 3A.

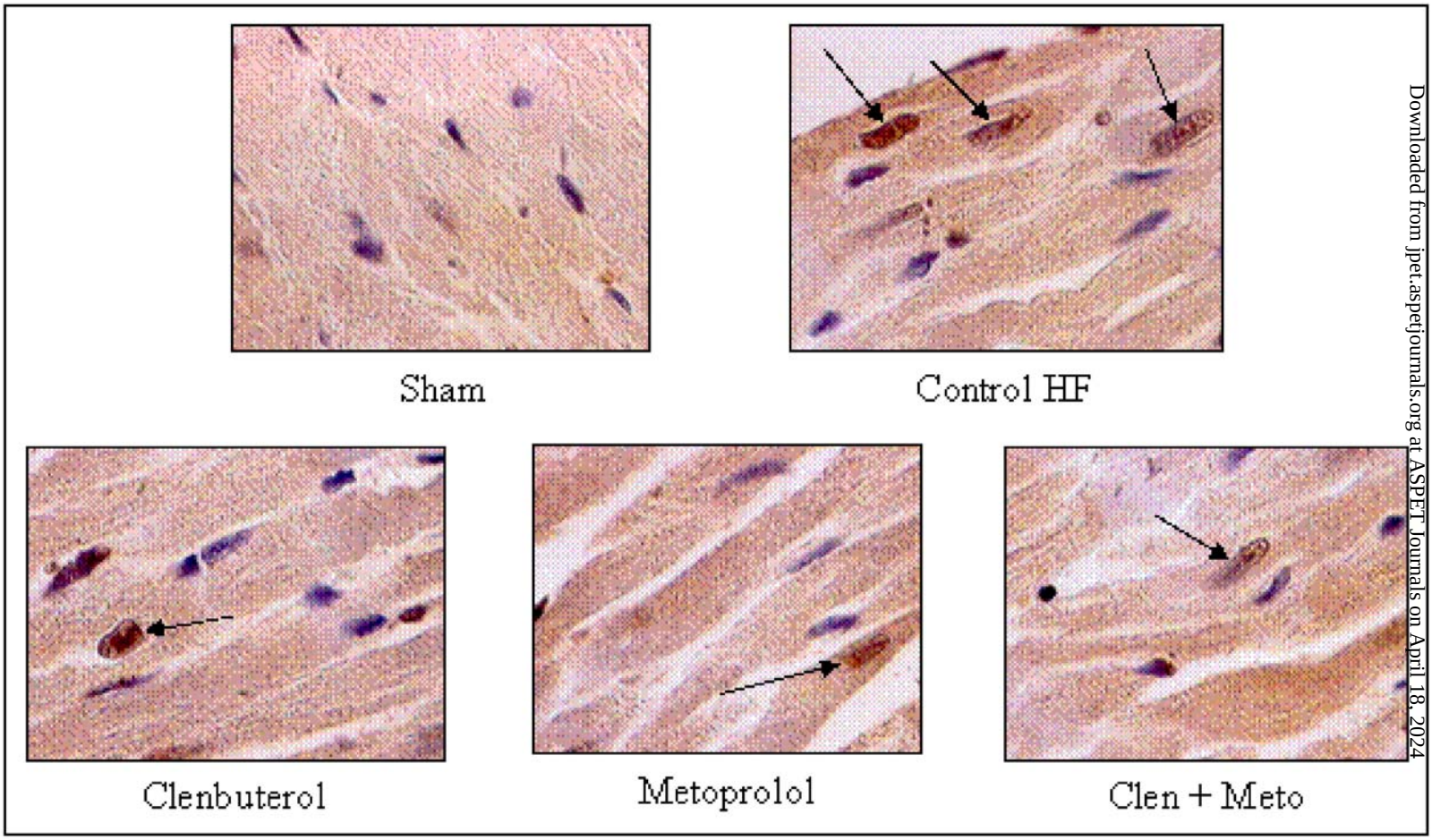

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Figure 3B.

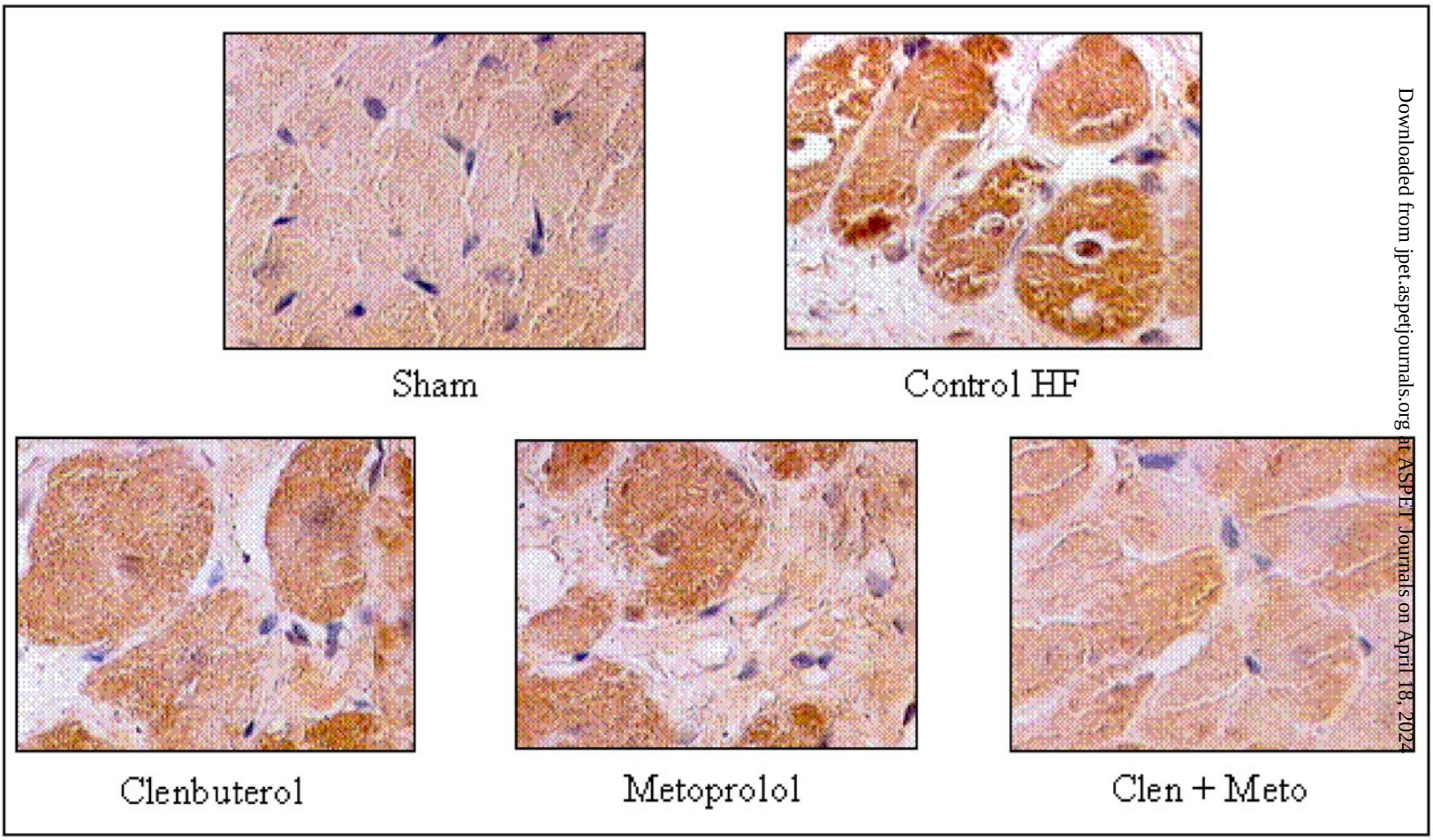

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Figure 3C.

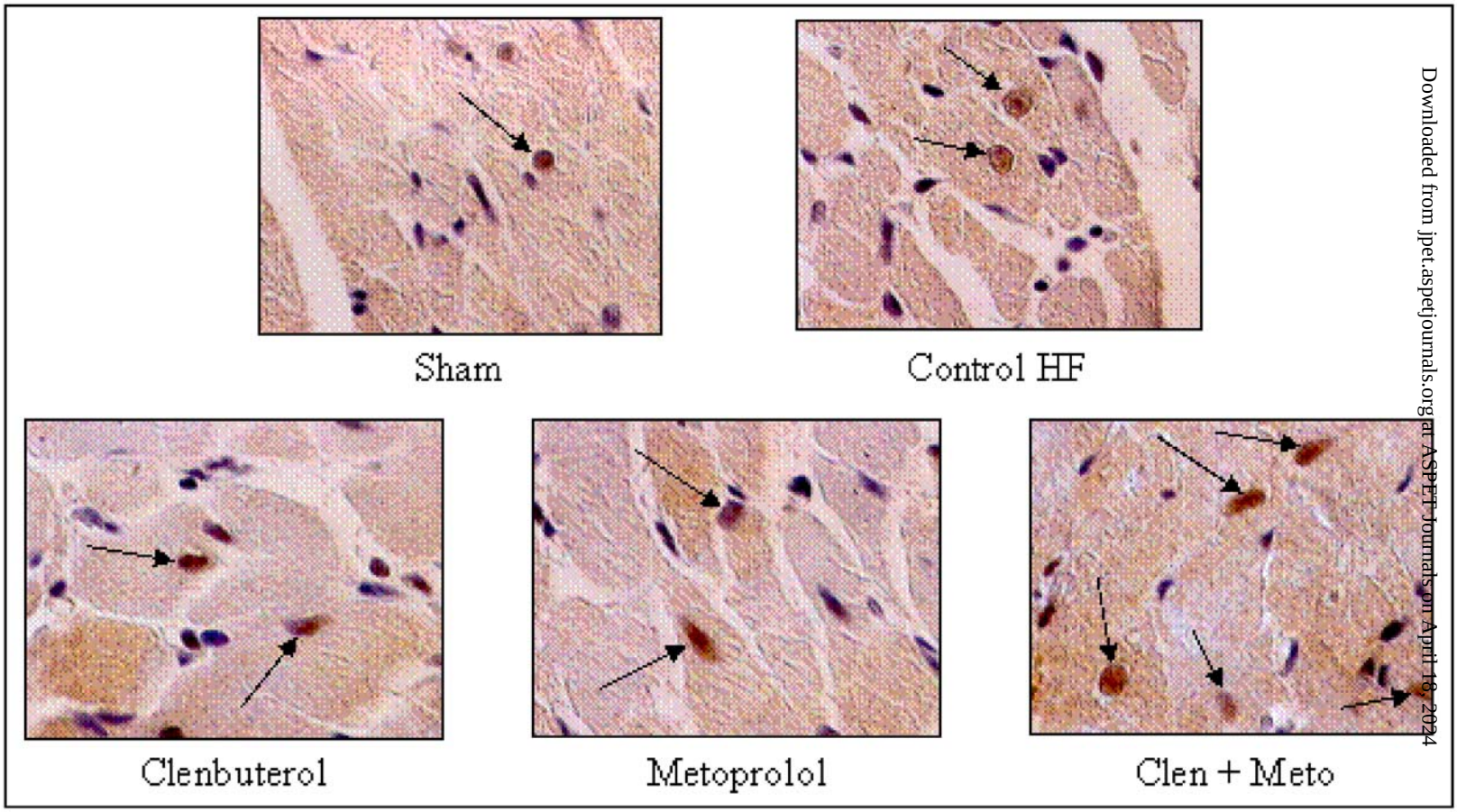

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Figure 3D.

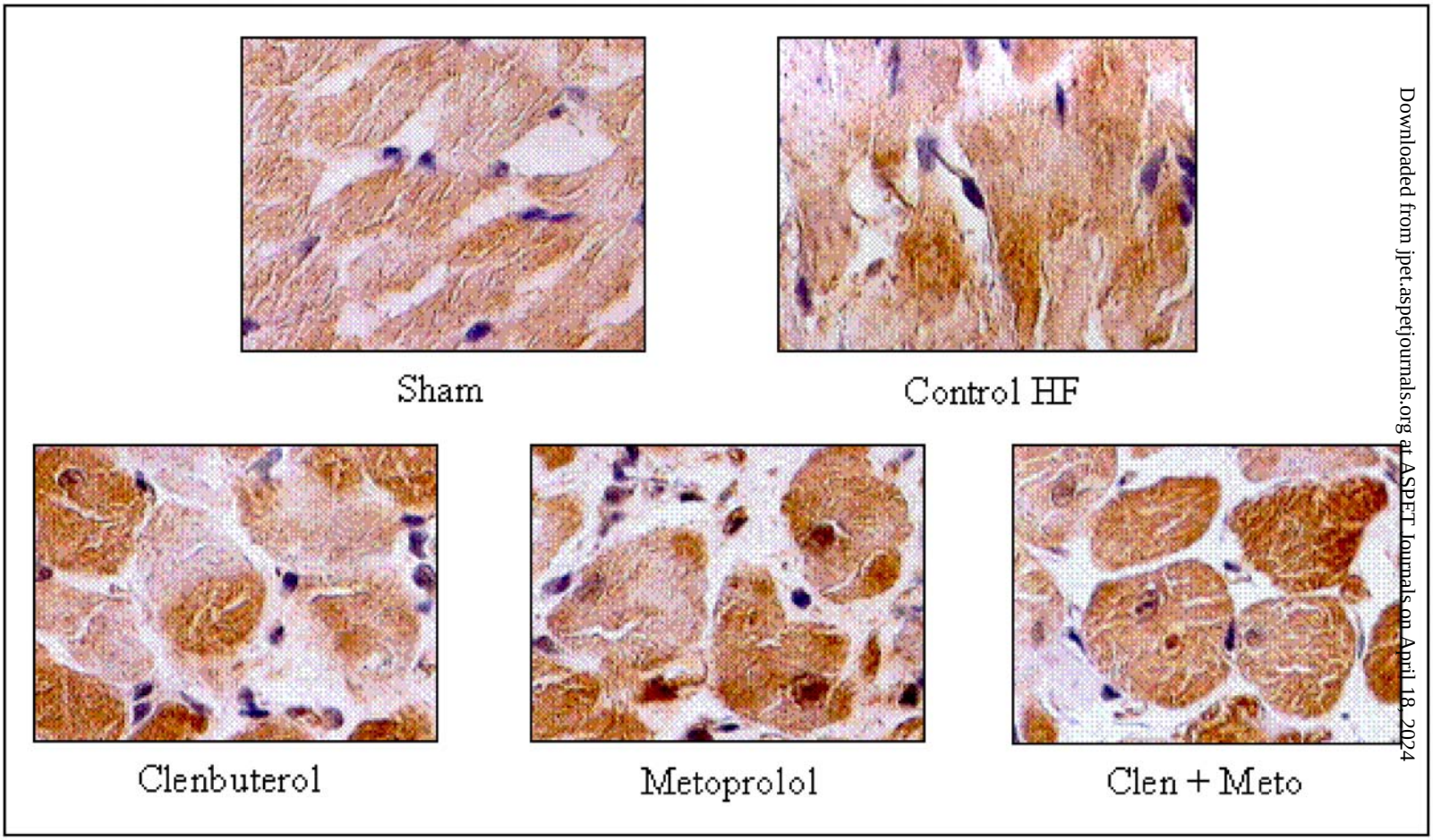

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Figure 3E.

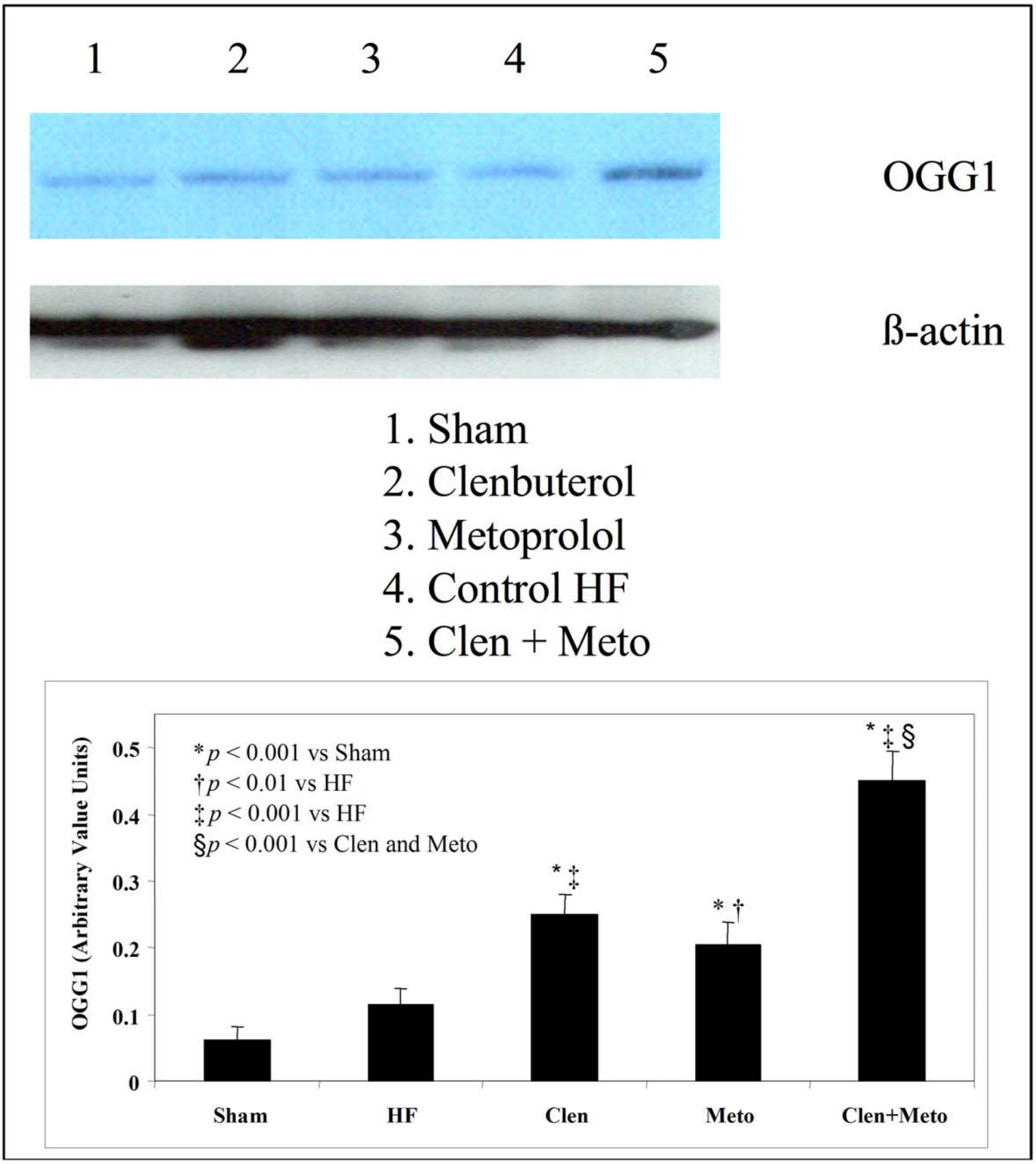

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Figure 3F.

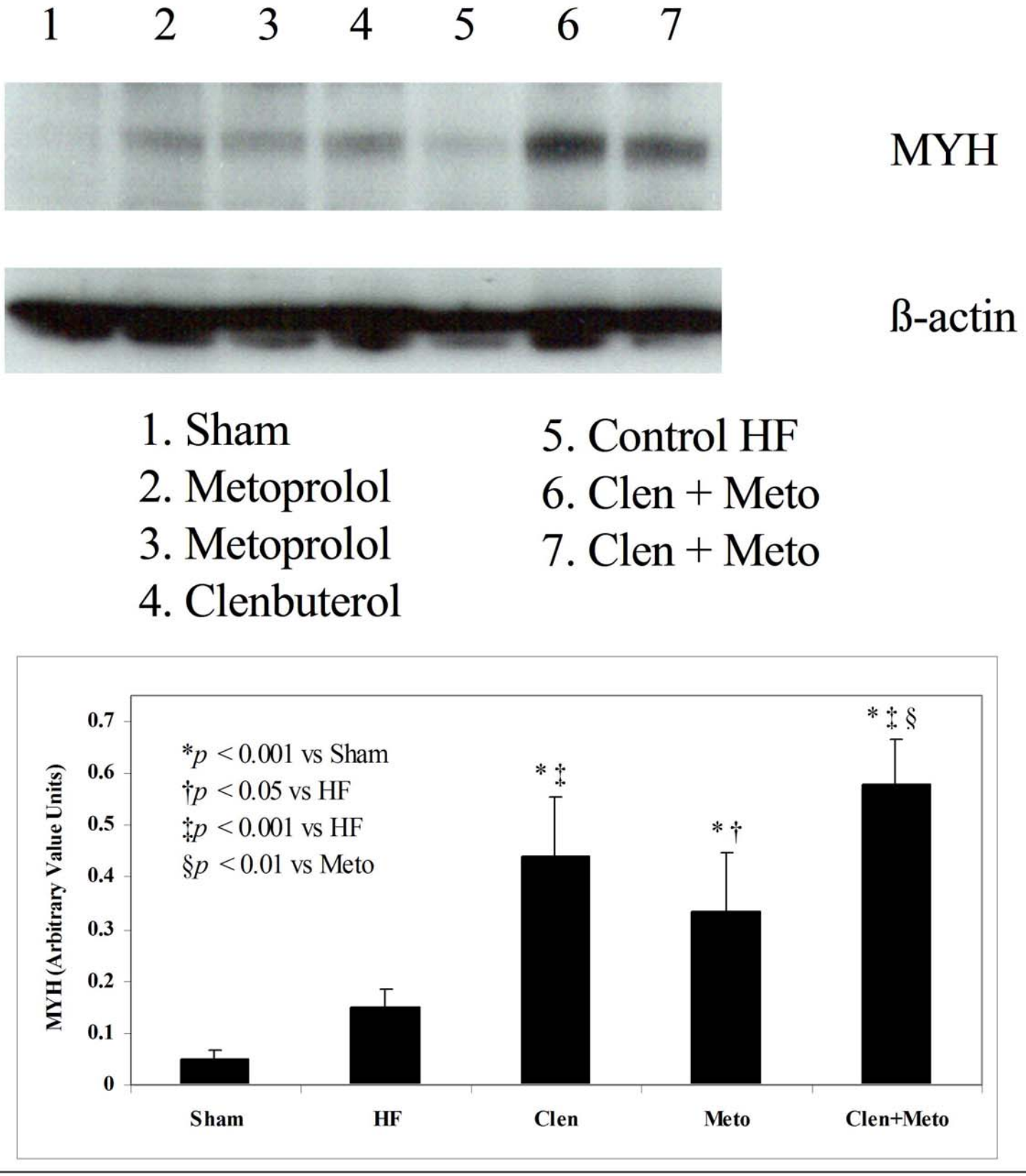

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Figure 4A.

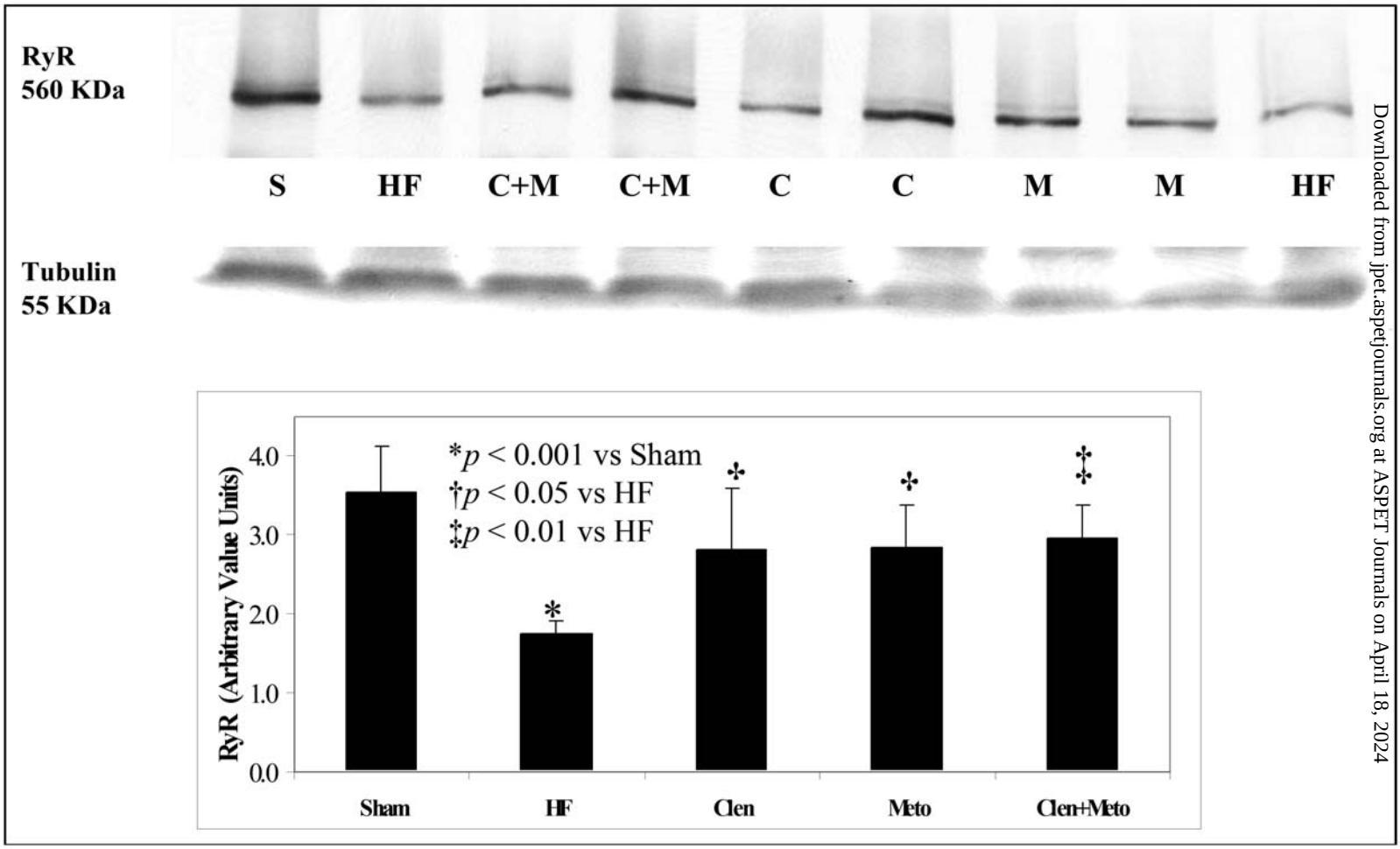

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Figure 4B.