Long-Term Effects of Sildenafil in a Rat Model of Chronic Mitral Regurgitation: Benefits of Ventricular Remodeling and Exercise Capacity

Running title: Kim et al.; Effect of sildenafil on mitral regurgitation

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Abstract:

**Background** - We tested the hypothesis that chronic treatment with sildenafil attenuates left ventricular (LV) remodeling and prevents exercise intolerance in chronic mitral regurgitation (MR).

**Methods and Results** - MR was created in SD rats by making a hole on the mitral leaflet. Two weeks after MR creation, MR and LV dilatation were confirmed by echocardiography and rats were randomly assigned to sildenafil (SIL) treatment (MR+SIL group, 50mg/kg p.o. bid, N=16) or normal saline only group (MR group, N=16) and continued for 4 months. Sixteen sham rats were compared to MR rats. After 4 months, LV size was smaller in MR+SIL compared to MR (LV end-systolic dimension, 4.7±0.3 for sham vs 5.9±0.3 for MR+SIL vs 7.4 ± 0.5 mm for MR p<0.05; LV end-diastolic dimension, 8.3±0.4 vs 10.5±0.2 vs 11.7±0.61 mm, p<0.05). LV ejection fraction was greater in MR+SIL than MR. (70.2%±2.2 for sham vs 67.0±4.2 for MR+SIL vs 58.9±2.5 for MR, p=0.01). Serial treadmill test revealed that exercise capacity was reduced in MR but not in MR+SIL. Transcriptional profiling of cardiac apical tissues revealed that gene sets related with inflammatory response, DNA damage response, cell cycle checkpoint, and cellular signaling pathways were significantly enriched by genes with reciprocal changes. Pathological analysis showed perivascular fibrosis was more prominent in MR than MR+SIL and the percentage of TUNEL-positive cells was two-fold greater in MR compared to MR+SIL.

**Conclusions** - Sildenafil significantly attenuates LV remodeling and prevents exercise intolerance in a rat model of chronic MR. This benefit might be associated with anti-apoptotic, anti-inflammatory effects of sildenafil.

**Key words:** mitral regurgitation, sildenafil, left ventricular remodeling, exercise capacity, microarrays

**Abbreviations:** MR=mitral regurgitation; ACE=angiotension-converting enzyme; PDE5=phosphodiesterase-5; NO=nitric oxide; cGMP=cyclic guanosine monophosphate; LV=left ventricle; EF=ejection fraction; IHC=immunohistochemistry
Introduction

Mitral regurgitation (MR) is a growing clinical problem as the mean age of the population advances. Currently, effective medical therapy is not available to prevent left ventricular (LV) remodeling and deterioration of exercise capacity, and thus delay the need for surgery. Although vasodilator is effective in acute MR, its benefit is not established in chronic MR.

Sildenafil citrate (SIL) is a selective inhibitor of phosphodiesterase-5 (PDE5) that catalyzes the breakdown of cGMP, one of the primary factors causing smooth muscle relaxation. By its potent action of enhancing NO-driven cGMP accumulation and ensuing vasodilation, sildenafil is widely used for the treatment of erectile dysfunction. However, growing evidence supports that inhibition of PDE5 may have important antihypertrophic, anti-apoptotic, and ischemic preconditioning effects that may limit myocardial remodeling in response to stress and attenuate the substrate for heart failure development. In murine models of myocardial pressure overload, inhibition of PDE5 was associated with inhibition of myocyte hypertrophy and interstitial fibrosis, preservation of cardiac function, and deactivation of various hypertrophy signaling cascades. PDE5 inhibition also promotes opening of mitochondrial KATP channels, a putative target of reactive oxygen species generated in response to both ischemia/reperfusion and doxorubicin treatment, and reduces doxorubicin or ischemia/reperfusion-induced apoptosis. Additionally, clinical studies of sildenafil in heart failure patients have reported improved exercise capacity coupled to reduced pulmonary vascular resistance and better endothelial function.

Accordingly, we hypothesized that sildenafil may have a beneficial effect in chronic MR because the process of LV remodeling shares some of aforementioned molecular mechanisms in chronic MR. In the present study, we evaluated long term effect of sildenafil on survival, myocardial function, remodeling and exercise capacity using a rat model of...
chronic MR. Additionally, we investigated potential mechanisms for the beneficial effects of sildenafil using cDNA microarray technology.

Methods

Animal handling

Male Sprague Dawley (SD) rats (n=52) with an initial weight of 350-400g were used. The rats were given free access to tap water and standard rat chow and housed in a room with a 12:12-h light cycle, a temperature of 21°C, and a humidity of 55%. The experimental protocols were approved by Institutional Animal Care and Use Committee of Seoul National University Hospital.

Creation of MR model and study design

Surgical creation of MR was performed under the guidance of transesophageal echocardiography in 11-week-old rats. We used intracardiac echocardiographic catheters (Acuson/Siemens Corp, Mountain View, CA) for transesophageal studies. After the insertion of echocardiographic catheter into the esophagus, left thoracotomy was performed through the fourth or fifth intercostals space. A needle (0.5mm external diameter) was inserted into the LV via apical puncture. After we found the needle tip under the echocardiographic image, we advanced the needle toward the anterior mitral valve to puncture the leaflets and create MR (detailed methodology in online supplement). MR was considered significant if a regurgitant jet area larger than 45% of the left atrium. Sham animals underwent the same procedures except for mitral leaflet injury.

Two weeks after the surgery, we confirmed the development of MR with LV dilation and the rats were randomly assigned to sildenafil (SIL) treatment (MR+SIL group, 50mg/kg p.o. bid, N=16) or normal saline only group (MR group, N=16) (Table 1). An oral dose of 100mg/kg/day sildenafil (Viagra, Pfizer) were administered orally by gavage twice a day.
based on the previous studies showing the pleiotropic vascular effect in a heart failure model\textsuperscript{15-17}. Sixteen sham rats were compared to MR rats.

We started the treatment 2 weeks after MR creation and continued for 14 weeks according to the results of our pilot study\textsuperscript{13}; (1) ejection fraction (EF) remained in the normal range despite progressive increase of LV size and (2) no additional death occurred during this period (see online supplement for the details). Cardiac function and exercise capacity was monitored every 2 or 4 weeks. At the end of the 14-week treatment, pressure-volume analysis was performed and the hearts and lungs were harvested for tissue determinations. The detailed experimental protocol is shown in Figure 1.

Survival Analysis

We examined the effects of sildenafil on the survival of MR rats. The day of oral administration of sildenafil was defined as day 0. This survival analysis covered the entire experimental period to day 100.

Physiologic study

Echocardiography

Transthoracic echocardiography was performed 1 week before and 2, 4, 6, 9, 12 and 16 weeks after the creation of MR on spontaneously breathing rats placed on their dorsal recumbency position. Rats were lightly sedated with inhalation of the lowest possible dose of isoflurane (initially 4%, then approximately 2-3%) mixed with oxygen. Images were acquired with a 9 MHz transducer connected to a Toshiba echocardiography machine (Nemio, Toshiba Co., Tokyo, Japan). LV septal and posterior wall thickness (SWT and PWT) and LV end diastolic/systolic dimension (EDD/ESD) were measured using M-mode echocardiography at the papillary muscle level. LV ejection fraction (EF) was calculated as \((\text{LVEDD}^2-\text{LVESD}^2)/\text{LVEDD}^2\). LV mass was estimated by a formula validated in small animals\textsuperscript{18} and adjusted for body weight: \(\text{LV-mass}=(\text{SWT+PWT+LVEDD})^3-(\text{LVEDD})^3\times1.04\).
Color Doppler mapping of MR jets was used to semiquantitatively assess the severity of MR. MR jet areas and LA areas were measured in the apical long-axis view and the ratio of MR jet area to the LA areas was calculated \((\text{Supplemental Figure 1D})\). Early diastolic transmitial flow (E velocity) was measured in apical 4-chamber view with a 1.5mm–sized sample volume placed at the tips of the mitral leaflets. All parameters were evaluated on an average of 5 consecutive beats. A single echocardiographer experienced in rat echocardiography over 500 cases performed all data acquisition.

**Exercise Test and BP Monitoring**

Maximal exercise capacity was evaluated with Rota Rod Treadmill (Ugo Basile, Comerio, Italy). Rats run on a knurled drum as the drum rotates in order to avoid falling off. Animals were trained twice before the test to adjust the treadmill more familiarly. Treadmill speed was gradually increased from 3 rpm (revolutions per minute) to 15 rpm every 1 minute. Exercise time was recorded. The observer blinded to the study group recorded episodes of the immobility response due to exhaustion.

Systolic and diastolic blood pressures were measured in conscious rats via the tail-cuff method (Biopac System Inc) at 1 week before and 2, 4, 9, 15 weeks after the creation of MR. At least 1 day interval was given for the BP measurements after echocardiographic examination or exercise test to minimize the stress on the animals.

**Invasive Hemodynamic Measurements**

At 16 weeks after MR creation, invasive hemodynamic measurements were obtained using a 1.4-French high-fidelity Millar pressure catheter, as described previously (see supplemental detailed methodology)\(^{20,21}\).

**Histopathological analysis**

After hemodynamic measurements were performed, the rats were euthanized and the hearts, lungs were harvested and weighed and apical scar excluded.
RNA isolation

Fresh LV apex, excluding apical scar was immediately stored in RNAlater (Ambion/Applied Biosciences, Streetsville, ON, Canada) at –80°C until use. The hearts were homogenized in TRIzol Reagent (Invitrogen, Burlington, ON, Canada), and total RNA was isolated according to the manufacturer’s instructions. RNA was further purified to remove genomic DNA contamination and concentrated using an RNeasy Plus Mini kit (Qiagen, Mississauga, ON, Canada). Samples with optical density ratio 260/280 >1.8, 28S/18S >1.6 using a Bioanalyzer 2100 (Agilent, Santa Clara, CA) were selected for microarray processing.

Microarray Analysis

Apical heart RNA excluding scar tissue was labeled either with Cyanine 3-CTP (Cy3) or Cyanine 5-CTP (Cy5) (PerkinElmer, Boston, MA, USA) by Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) and hybridized onto Agilent whole rat genome array (G4131A). RNAs from three rats in each group were pooled and hybridized onto one chip. The arrays were scanned at two different intensities, and the images were analyzed for background correction. Both BMP4 and FGF2 samples were co-hybridized with RNA from the starting point and a dye-swap was performed. The arrays were normalized and the differential gene expression was analyzed using the R and bioconductor-based method LIMMA (detailed in supplemental methodology).

We performed the Gene Set Enrichment Analysis to find molecular pathways or Gene Ontologies that are made up of differentially expressed genes in each biological condition. Difference in mean log transformed intensity for each experimental group was used as a metric for sorting probes in GSEA. After sorting genes according to the metric, Enrichment Scores were assigned to 2,394 different gene sets curated in the mSigDB database. (http://www.broadinstitute.org/gsea/msigdb/) Gene sets with high absolute values of enrichment score are molecular pathways or Gene Ontologies that are made up of up- or
down-regulated genes and are differentially regulated pathways. P-values for the Enrichment Scores were calculated after permuting class labels of each experimental condition and gene sets with p-values less than 0.05 were extracted. Gene sets with absolute Enrichment Score more than 0.35 and p-value less than 0.05 were visualized as a network with nodes representing gene sets and edges connecting two nodes if hypergeometric p-value for testing the significance of gene overlap is within one percentile of the whole p-values.

**Verification of Gene Expression With RT-PCR**

Total RNA was isolated using RNeasyPlus Mini kit (Qiagen) and cDNA was synthesized using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara) with 1 μg of each total RNA sample according to the manufacturer’s instruction. cDNA (1 μl) was amplified by PCR using TaKaRa Ex Taq™ kit (Takara) with specific primers (Table 2). GAPDH was chosen as an endogenous control. Quantification of band intensity was analyzed using TINA 2.0 (RayTest) and normalized to the intensity of GAPDH.

**Immunohistochemical Analysis (see online supplement for the details)**

Mid ventricle were removed for histopathology and were preserved in 4% paraformaldehyde and embedded in paraffin. The tissue was sectioned into 4 μm sections, stained with Masson’s trichrome for evaluation of the degree of fibrosis and with picrosirius red for measurement of collagen deposition. Apoptosis was assessed using the Terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling (TUNEL) technique (DNA fragmentation, Oncor, Gaithersburg, MD). The detailed protocol was previously published.

**Statistical analysis**

Data are presented as mean±S.E.M. The normality of all parameters was tested using the Kolmogorov-Smirnov test. The differences among groups were compared by the unpaired t-test or one-way analysis of variance, followed by the Bonferroni test. In cases in which normality was excluded, the non-parametric Kruskal-Wallis H test was performed, and the
Mann-Whitney U test was used for post hoc analysis. Repeated measures ANOVA was performed to analyze the changes of several variables over time such as LV ejection fraction, LV dimension, LV mass index and exercise capacity. Survival data was evaluated by the Kaplan-Meier method with pairwise comparison conducted using the log-rank test. All statistical analyses were performed using SPSS 17.0 version (SPSS Inc., Chicago, US) and p-values of < 0.05 were considered statistically significant.

Results

Survival Analysis

A total of 52 rats were used in this study. Upon surgery, two rats died of bleeding and two rats died in acute stage within 2 weeks after MR operation due to pulmonary edema and unknown cause. Three of 16 rats in MR group died during the study period. However, no deaths were noted in MR+SIL and sham group. Kaplan-Meier survival curve showed the comparison between MR + SIL rats and MR rats. (p=0.07, Figure 2A)

Hemodynamic study

LV Remodeling after MR Creation

Mean MR jet area was 15±3 mm² and the mean ratio of MR jet area to the LA area was 56±8 % at 2 weeks after operation. There was no MR in the sham group. Mitral E velocity increased at 2 week (65.2±7.1 vs 105.1±15.1 cm/s, p <0.05) in MR groups whereas it did not change in the sham group (66.4±6.4 vs 64.5±5.5 cm/s, p = 0.76). Figure 3 demonstrates the time-dependent changes in LV diameter, EF and LV mass index. Baseline examinations showed no difference of LV diameters among the three groups (LVESD; 4.0±0.1 vs 4.1±0.1 vs 4.1±0.1 mm, LVEDD; 7.4±0.1 vs 7.5±0.1 vs 7.5±0.1 mm for sham, MR and MR+SIL groups, p=0.82). The LV started to dilate immediately after MR creation and showed progressive dilation until the 16th week. There was significant difference in LVESD between MR and MR+SIL from the 6th week, whereas LVEDD did not differ significantly
until the 12th week. Consequently LVEF started to decrease from the 6th week in MR group whereas it was preserved until the 16th week in MR+SIL group. With increasing LVESD, death started to occur as shown in the Kaplan-Meier curve (Figure 2A). Weight gain tended to lag behind the sham rats in MR and MR+SIL rats without statistical significance (Supplemental Figure 2). The LV mass index increased significantly in the MR group compared with the sham group (1.15±0.04 vs 1.71±0.05 mg/g, p <0.05). Sildenafil treatment attenuated the increase of LV mass index (11.7% reduction, p =0.01). There were no significant changes in blood pressure and heart rate among the groups (Figure 2B).

**Exercise Capacity**

There was no difference in exercise duration among the groups until the 6th week after MR when it became shorter in MR group than sham group (720±15 for sham vs 588±15 for MR, 617±21 seconds for MR+SIL, p <0.05 for difference from sham vs MR, Figure 4). Thereafter, exercise capacity was impaired progressively in MR group compared with sham group (at the 15th week, 705± 18 for sham vs 462±21 seconds for MR, p <0.05). However exercise duration was maintained in the sildenafil treated MR rats (641±14 seconds for MR+SIL, p<0.05 for difference from MR).

**Invasive hemodynamic measurement**

At the 16th week, rats underwent invasive hemodynamic assessment and were sacrificed for the pathological analysis (Table 3, Figure 5). Heart rate, LV end-diastolic pressure, -dP/dt, and the end-diastolic pressure-volume relation were not different among the groups. However, end-systolic volume, end-diastolic volume and stroke volume were greater in MR group compared with sham group. The end-systolic pressure-volume relation (ESPVR), +dp/dt, preload-recruitable stroke work (PRSW) and EF were significantly decreased in MR group, indicating contractile dysfunction. Sildenafil treatment prevented systolic functional impairment in the MR+SIL group compared with the MR group; EF
(+28%, p <0.05), ESPVR (+116%, p<0.05), PRSW(+30%, p< 0.05).

**Microarray analysis**

A total of 448 genes were differentially expressed by at least 1.5-fold in MR rats (P<0.05), including 197 up-regulated and 251 down-regulated genes compared with sham or MR+SIL. The heat map in **Supplemental Figure 3** demonstrates a consistent pattern of change of these genes in each condition. **Supplemental Table 1** lists genes well established in the pathophysiology of cardiovascular disease that were up-regulated>1.5-fold in MR and down-regulated in MR+SIL.

For biological interpretation, we performed Gene Set Enrichment Analysis (see methods section for details) to explore the transcriptional changes at the level of molecular pathways or Gene Ontologies. **Figure 6 (A,B)** is the network representation of gene sets that are enriched with genes up-regulated in MR and reciprocally down-regulated after treatment with sildenafil and presumed to be primarily associated with the pharmacological effect of sildenafil in MR. It is noticeable that gene sets form several tightly interconnected structures or clusters which we can annotate with inflammatory response, DNA damage response and cell cycle checkpoint, and cellular signaling pathways associated with LV remodeling.

To confirm the changes in transcription level, we selected 5 representative genes implicated in DNA damage response, inflammatory response, endothelial function and vascular remodeling, CDKN2A, IL-6, IL-18, eNOS, and iNOS26, 27. RT-PCR demonstrated the validity of DNA microarray (**Figure 6 C, D**).

With the transcriptional profiling study, it is suggested that the chronic hemodynamic stress induced by mitral regurgitation might activate stress response pathways like inflammatory pathways, DNA damage response and cell cycle checkpoint pathways, leading to cell fate decisions like apoptosis and LV remodeling. With the attenuation of the hemodynamic stress by treatment with sildenafil, the activated stress response pathways are
moderated, suggesting that the three clusters of pathways provide the basis for the molecular and cellular mechanism of the pathological changes in heart undergoing chronic volume and pressure overload from mitral regurgitation.

**Histopathological analysis with cardiac fibrosis and apoptosis**

Gross pathological examination showed that the heart was significantly enlarged with eccentric hypertrophy in MR group at the 16th week (Figure 7A). However the hypertrophy was reduced in sildenafil treated MR rats. We found no difference in interstitial fibrosis among the groups. However, the extent of perivascular fibrosis was significantly larger in MR group compared to MR+SIL group (Figure 7C, D, E, F). TUNEL staining showed a significant increase of cardiac apoptosis in MR group (0.24±0.3%, p < 0.05, Figure 8). In contrast, a significant inhibition of apoptosis was evident in MR+SIL (0.05±0.02%), which was comparable to sham group (0.03±0.04%).

**Discussion**

The purpose of this study was to evaluate the effect of chronic administration of sildenafil on cardiac remodeling, function, and exercise capacity in rats with chronic MR. The major finding of the present study was that sildenafil prevented LV remodeling and exercise intolerance caused by chronic experimental MR. To our knowledge this is the first study that shows sildenafil is efficacious in the treatment of MR-induced LV remodeling and cardiac dysfunction. Additionally we proposed the potential mechanisms related with the effect of sildenafil; inhibition of inflammation and apoptosis.

**Medical management in chronic MR**

MR induces chronic LV volume overload and leads LV contractile dysfunction, heart failure and, finally, death. Although surgical correction of MR, the only definitive way to cure, carries reasonably low mortality and morbidity, medical therapeutics would have a role
in many clinical situations such as in patient population with greater surgical risk. It becomes more relevant as the main etiology of MR has been changed from rheumatic to degenerative valve disease \(^{25}\) and the prevalence of MR increases with age because operative mortality for elderly patients is still high\(^{26}\). However, there is currently no recommended pharmacological therapy for chronic MR. Despite previous efforts, medical therapies for chronic MR have produced disappointing and conflicting results.

**Small animal model of chronic MR**

Little is known about molecular and cellular mechanisms of LV remodeling induced by volume overload partly due to the absence of small animal model. In their pioneering work, Pu et al. developed an MR rat model for the first time\(^{14}\). We successfully established a small animal model of chronic MR and verify the pathophysiologic features of this model. We described the detailed time course of LV remodeling and the relationship between progressive LV dilatation and LV dysfunction. Immediately after MR creation, we observed slight increase of LV diastolic dimension without change of systolic dimension and thus hyperdynamic LV. In this stage, LV wall thinning occurred as LV began to dilate. Chronic compensated stage could be defined from the 3\(^{rd}\) week to around the 16\(^{th}\) week. In this stage, LV diastolic and systolic volume increased progressively. Though remained in the normal range, LVEF became significantly lower in MR group from the 12\(^{th}\) week. At the 16\(^{th}\) week, load-independent contractile indices were significantly lower in MR group whereas diastolic parameters were not different. These results are consonant with several previous studies that demonstrated that MR is one of the few cardiac diseases in which diastolic function is supernormal\(^{27}\). Chronic adaptation to volume overload tends to decrease LV chamber stiffness and increase diastolic filling rates and dimension lengthening irrespective of systolic function in chronic MR\(^{28}\). Additionally, we did not find any increase of the LV interstitial fibrosis in this stage of MR. Interstitial fibrosis is closely related with LV diastolic function.
Mechanism for LV remodeling in MR

MR causes progressive LV dilation, wall thinning, and cardiomyocyte elongation, which recapitulates eccentric remodeling with inflammatory cell infiltration and extracellular matrix degradation. It was suggested that inflammation and energy metabolism initiate cardiac remodeling. Nemoto et al. showed that administration of the PPAR-γ agonist rosiglitazone ameliorates MR-induced LV dysfunction accompanied by a decline in lipid content. Similarly in volume overload model of aortocaval fistula, there was upregulation of genes related to inflammation, the extracellular matrix, the cell cycle, and apoptosis. There was a 40-fold increase in the matricellular protein periostin, which inhibits connections between collagen and cells, thereby potentially mediating a side-to-side slippage of cardiomyocytes and LV dilatation. These findings are consistent with our results that inflammation, the cell cycle and DNA damage pathways were upregulated in MR that were prevented by sildenafil treatment.

Increased matrix metalloproteinase activity is associated with progressive LV dilatation and extracellular matrix degradation, contractile dysfunction, and neurohormonal activation in volume overload animal models. Zheng J. et al. recently reported that eccentric LV remodeling in isolated MR was associated with increased matrix metalloproteinase activity using a gene array analysis. However, we could not find a significant difference in matrix metalloproteinase families among the groups. This may imply that the relative contributions of each of these mechanisms change at the different stage of the remodeling. Extracellular matrix loss, inflammation or bioenergetics dysfunction has been implicated at different time points in the pathophysiology of volume overload.

Sildenafil for treatment of MR

Emerging data indicate that PDE5 is present within cardiac myocytes and may play a role in modulating cGMP activity in various cardiac diseases. Several observations from
preclinical and short-term clinical studies support a role of sildenafil in the treatment of heart failure. In the present study, anti-inflammatory and anti-apoptotic effect of sildenafil seemed to play a key role to prevent LV remodeling.

The endocardial perivascular area may represent an early fibrotic change in MR model, probably due to relative endocardial ischemia due to the prolonged elevation of LV diastolic filling pressures. However the extent of perivascular fibrosis was significantly smaller in MR +SIL group compared to MR group. In recent years, there has been considerable interest in studying the effect of sildenafil on endothelial cell protection that may trigger a signaling cascade (through the action of kinases including protein kinase C and other mitogen-activated protein kinases) and generation of nitric oxide (NO) by phosphorylation of endothelial NO synthase (eNOS). An important property of sildenafil is its ability to increase eNOS and inducible NOS proteins in the heart and this has a direct cause and effect relationship in protection against myocardial infarction, as well as apoptosis in cardiomyocytes. Because of the ability of sildenafil to augment NOS and cGMP levels, it is logical to hypothesize that cardiovascular dysfunction induced by chronic NOS inhibition would be alleviated by concomitant treatment with sildenafil.

Clinical and experimental data support a link between endothelial dysfunction and inflammation. Proinflammatory cytokines are increased in the myocardium as a response to stretch in MR patients and correlated with the extent of LV dilatation. Inflammatory response and cytokine elaboration are integral components of the host response to tissue injury and play an active role for myocardial remodeling and thus inflammatory mediators play a crucial role in the development of heart failure. Accordingly, several strategies to counterbalance the inflammatory response have been suggested. Possible targets involve pro- and anti-inflammatory cytokines and their receptors, adhesion molecules, NO and NOS, reactive oxygen species, and different types of leucocytes. In this study, we demonstrated that
inflammatory response was decreased in the sildenafil treated group compared with MR rats by microarray analysis.

“Cell loss” is another unifying feature found in nearly all forms of cardiomyopathies. Currently, the best understood form of cell loss or cell death in heart failure is apoptosis. DNA fragmentation and cellular and nuclear shrinkage are one of the biochemical and morphological changes that classically characterize apoptosis. Barouch et al. demonstrated cardiomyocyte apoptosis is associated with increased DNA damage and decreased survival in murine model of obesity.\textsuperscript{38} In this study, we showed that DNA damage pathway was upregulated in MR rats compared with sham and MR+SIL rats and apoptotic cells were more prevalent in MR rats.

Nagayama et al. demonstrated that sildenafil treatment prevents further cardiac and myocyte dysfunction and progressive remodeling in animal model of established hypertrophy caused by pressure-overload. In the present study, we found similar beneficial effect of sildenafil in animal model of volume-overload. The therapeutic effect of sildenafil has also been demonstrated in other animal models including ischemia-reperfusion model and anthracycline cardiomyopathy. These findings suggest that there is substantial overlap of molecular pathways in the development of different types of cardiac remodeling induced by various stimuli. In addition, they also suggest the multiple effects of sildenafil and cGMP.

**Limitation of the study**

MR was created by making a hole on the mitral leaflet and this MR model may not represent MR in humans, especially ischemic MR. This is an inherent limitation of animal model of valve disease. However, our model is still useful to evaluate the effect of medical treatment on LV remodeling induced by MR. In this regard, the beneficial effect of sildenafil on volume overload-induced LV remodeling might have clinical implication. Further
research is needed to delineate the exact mechanisms involved to allow for translation into the clinical setting.

**Conclusion**

Sildenafil attenuates LV remodeling and prevents exercise intolerance in a rat model of chronic MR. This benefit might be associated with anti-apoptotic, anti-inflammatory effects of sildenafil. Further research is needed to delineate the exact mechanisms involved and demonstrate the beneficial effects in patients with chronic MR.

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**Conflict of Interest Disclosures:** None

**References:**


Angiotensin ii receptor blockade does not improve left ventricular function and remodeling in subacute mitral regurgitation in the dog. *Journal of the American College of Cardiology*. 2002;39:1374.


16. Hassan M, Kett A. Sildenafil citrate increases myocardial cgm content in rat heart, decreases its hypertrophic response to isoproterenol and decreases myocardial leak of


Table 1. General and Echocardiographic Characteristics 2 Weeks after Mitral Regurgitation (13 weeks of age)

<table>
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<th>Variables</th>
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<th>MR+SIL group (n=16)</th>
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</tbody>
</table>

Data are means±S.E.M. MR, mitral regurgitation; SIL, sildenafil; HR, heart rate; EF, ejection fraction; SWT, end-diastolic wall thickness of interventricular septum; PWT, end diastolic wall thickness of posterior wall; EDD, end diastolic dimension; ESD, end systolic dimension; E velocity, mitral inflow E velocity cm/s

*Heart rate and LV mass were estimated in vivo by echocardiography.
†p<0.05 for difference from control.

Table 2. Primer Sequences for Validating Microarray by Real-Time PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Genebank ID</th>
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<tr>
<td>IL6</td>
<td>TCTCGAGCCCAACAGGAAAGAAA</td>
<td>GTAGGGAAAGGCAGTGCTGTCA</td>
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<tr>
<td>IL18</td>
<td>ATCCTAGCTGCCTGCTCCAGCTG</td>
<td>CTGGTCTGGGATTCGTTGGCTGT</td>
<td>AY077842</td>
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<tr>
<td>CDKN2a</td>
<td>CTCTCCGACCCTGGCATCAGCA</td>
<td>TAGGCACCTGGGCGTGCTTG</td>
<td>NM_031550</td>
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<tr>
<td>NOS2</td>
<td>ACTCCATCGACCCGCCGCCACA</td>
<td>GCAGTGCTACAGCTCCGCCG</td>
<td>NM_012611</td>
</tr>
<tr>
<td>NOS3</td>
<td>TGCCCTTGGCTCGAGCGGTG</td>
<td>CTCCACTAGGCGCGCGGT</td>
<td>NM_012589</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CAAATTCGGTGTCATTACCAG</td>
<td>CGTGGAAAGGACTCATGAC</td>
<td>NM_017008.3</td>
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</tbody>
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Table 3. Hemodynamic Parameters 16 Weeks after Mitral Regurgitation (27 weeks of age)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Sham Group (n= 16)</th>
<th>MR group (n=13)</th>
<th>MR+Sil group (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, mg</td>
<td>614.0±10.0</td>
<td>587.1±10.2</td>
<td>594.2±5.7</td>
</tr>
<tr>
<td>Lung (g)</td>
<td>1.08±0.15</td>
<td>1.46±0.20†</td>
<td>1.23±0.17†‡</td>
</tr>
<tr>
<td>Lung/BW (mg/g)</td>
<td>1.75±0.24</td>
<td>2.48±0.31†</td>
<td>2.07±0.27†‡</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>321.5±20.1</td>
<td>322.7±24.7</td>
<td>325.4±22.8</td>
</tr>
<tr>
<td>ESV, μl</td>
<td>127.2±5.9</td>
<td>443.5±25.4†</td>
<td>321.2±37.4†‡</td>
</tr>
<tr>
<td>EDV, μl</td>
<td>430.9±27.6</td>
<td>1113.6±61.9†</td>
<td>998.7±54.7†‡</td>
</tr>
<tr>
<td>EF, %</td>
<td>70.2±1.4</td>
<td>60.7±3.5†</td>
<td>67.2±3.7</td>
</tr>
<tr>
<td>SV, μl</td>
<td>303.1±21.0</td>
<td>670.4±35.8†</td>
<td>677.5±48.5†</td>
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<tr>
<td>LV ESP, mmHg</td>
<td>101.3±7.5</td>
<td>105.4±6.3</td>
<td>106.4±4.0</td>
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<tr>
<td>LV EDP, mmHg</td>
<td>10.5±3.5</td>
<td>12.3±2.3</td>
<td>11.2±2.1</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>5124±228</td>
<td>4367±315†</td>
<td>4527±204</td>
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<tr>
<td>-dP/dt, mmHg/s</td>
<td>-3781±366</td>
<td>-3543±312</td>
<td>-3431±311</td>
</tr>
<tr>
<td>ESPVR, mmHg/μl</td>
<td>0.324±0.102</td>
<td>0.121±0.010†</td>
<td>0.262±0.031‡</td>
</tr>
<tr>
<td>EDPVR, mmHg/μl</td>
<td>0.010±0.002</td>
<td>0.013±0.001</td>
<td>0.011±0.003</td>
</tr>
<tr>
<td>PRSW, mm Hg</td>
<td>111.1±24.5</td>
<td>68.7±14.2†</td>
<td>89.3±10.1†</td>
</tr>
</tbody>
</table>

Data are means±S.E.M.; MR, mitral regurgitation group; MR+Sil, sildenafil treated MR; HR, heart rate; ESV, end-systolic volume; EDV, end-diastolic volume; SV, stroke volume; LV ESP, LV end systolic pressure; LV EDP, LV end diastolic pressure; ESPVR, end systolic pressure volume relationship; EDPVR, end diastolic pressure volume relationship; PRSW, preload recruitable stroke work. †p<0.05 for difference from control, ‡ p < 0.05 for difference from MR

Figure Legends:

Figure 1. Experimental protocol, a schematic illustration. wk; week, MR; mitral regurgitation, Echo; Echocardiography, BP; blood pressure, Bwt; body weight, PV; pressure-volume

Figure 2. (A) Survival analysis. Kaplan-Meier survival curve showed the comparison between MR+ SIL rats and MR rats. (p=0.07) (B) Serial change in blood pressure and heart rate
Figure 3. (A) Serial changes in echocardiographic measurements. (B) Representative M-mode echocardiograms from the three groups. LV; left ventricle, MR; mitral regurgitation, B; baseline, wk; weeks after MR operation, SIL; sildenafil. *p < 0.05 for difference from sham group. † p <0.05 for difference from MR group

Figure 4. Comparison of exercise duration among the three groups. * p < 0.05 for difference from sham group. † p <0.05 for difference from MR group. B; baseline, MR; mitral regurgitation, SIL; sildenafil.

Figure 5. Representative pressure-volume loops from the three groups. MR+SIL group showed smaller left ventricular volume and greater end-systolic pressure volume relation (ESPVR, dotted line) compared with MR group.

Figure 6. (A and B) A network of gene sets with significant Enrichment Scores (P<0.05), made up of genes that are up-regulated in MR rat model. Nodes represent gene sets and edges connect two nodes if the two gene sets share a significant number of genes in common by hypergeometric test.(see methods) Isolated nodes are omitted. Yellow nodes are Gene Ontology gene sets (C5) and green nodes are curated gene sets from public pathway databases (C2) and pink nodes represent computational gene sets (C4) B: a network of gene sets with significant Enrichment Scores (P<0.05), made up of genes that are down-regulated after treatment with sildenafil in MR rat model. (C) mRNA regulation in MR with sildenafil group. RT-PCR of iNOS, eNOS, IL-6, IL-18 and CDKN2A (n=3 each). mRNA of factors for vascular remodeling, inflammation and DNA damage were increased in mitral regurgitation (MR) group but down-regulated in MR+sildenafil (MR+SIL) group. However,
eNOS was down-regulated in MR group but increased in MR+sildenafil group. (D)
Quantitative graph (n=3). *p<0.05 for difference from MR+SIL group.

**Figure 7.** Comparison of the pathologic results (A) Eccentric hypertrophy was developed in MR group. (B) The hole created in the anterior leaflet of mitral valve. (C, D, E, G) Masson trichrome and picrosirius red staining showed no difference of interstitial fibrosis (F,H) The extent of perivascular fibrosis was significantly greater in MR group compared to MR+SIL group († p <0.05 for difference from MR group)

**Figure 8.** Representative apoptotic cells by TUNEL analysis and quantitative apoptotic indices († p <0.05 for difference from MR group). Arrows indicate the apoptotic cells
Daily monitoring: pain, appetite/drinking, behavior and responsiveness
Weeks after operation
MR+SIL down regulation

Cell cycle regulation

Inflammatory response

LV remodeling
Relative mRNA expression (Fold)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sham</th>
<th>MR</th>
<th>MR+SIL</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- iNOS
- eNOS
- IL6
- IL18
- CDKN2A
- GAPDH

### (C)

### (D)

- **iNOS**
  - Sham: 1.0 ± 0.2
  - MR: 3.5 ± 0.5
  - MR+SIL: 2.0 ± 0.3
- **eNOS**
  - Sham: 1.2 ± 0.1
  - MR: 2.5 ± 0.4
  - MR+SIL: 1.8 ± 0.3
- **IL6**
  - Sham: 1.5 ± 0.2
  - MR: 4.0 ± 0.5
  - MR+SIL: 2.5 ± 0.4
- **IL18**
  - Sham: 1.0 ± 0.2
  - MR: 3.5 ± 0.5
  - MR+SIL: 2.0 ± 0.3
- **CDKN2A**
  - Sham: 1.2 ± 0.1
  - MR: 2.5 ± 0.4
  - MR+SIL: 1.8 ± 0.3
- **GAPDH**
  - Sham: 1.2 ± 0.1
  - MR: 2.5 ± 0.4
  - MR+SIL: 1.8 ± 0.3

* p < 0.05
Long-Term Effects of Sildenafil in a Rat Model of Chronic Mitral Regurgitation: Benefits of Ventricular Remodeling and Exercise Capacity
Kyung-Hee Kim, Yong-Jin Kim, Jung-Hun Ohn, Jimin Yang, Sang-Eun Lee, Sae-Won Lee, Hyung-Kwan Kim, Jeong-Wook Seo and Dae-Won Sohn

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Long-Term Effects of Sildenafil in a Rat Model
with Chronic Mitral Regurgitation;
Benefits on ventricular remodeling and exercise capacity

Kyung-Hee Kim, MD¹; Yong-Jin Kim, MD¹; Jung-Hun Ohn, MD²; Sang-Eun Lee, MD¹;
Jimin Yang, MA¹; Sae-Won Lee, PhD¹; Hyung-Kwan Kim, MD¹;
Jeong-Wook Seo³, MD; Dae-Won Sohn, MD¹

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Running Title: Effect of Sildenafil on Mitral Regurgitation

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Detailed Methodology

Surgical procedure and some concerns for mitral regurgitation creation

The rats were anesthetized with 3% isoflurane at a rate of 4L/min oxygen and then they were intubated with a blunt 16-gauge needle, followed by mechanical ventilation with the M-683 (Harvard Apparatus Inc., MA, U.S.A) under 2-3% isoflurane in a mixture of 50% O2. Body temperature was maintained at 37°C by placing the rats on a heating pad during the procedure. After successful anesthesia, intracardiac echocardiographic catheter (AcuNav™, Acuson/Siemens Corp, Mountain View, CA) was inserted into the esophagus for transesophageal echocardiography. A lateral thoracotomy was performed and the pericardium was opened. To locate the insertion site of the puncture needle accurately, the LV was gently pressed with a clean cotton swap. A needle (23 gauge, 0.5 mm external diameter) was inserted into the LV via apical puncture and was advanced toward the mitral valve to make a hole in the leaflet and create MR under the guidance of transesophageal echocardiography (supplementary Fig. 1). MR was considered significant if a regurgitant jet area occupied more than 45% of the left atrium (LA). The sham group underwent the same procedure but without damaging the mitral valve. All rats were given 2 ml subcutaneous injection of isotonic saline, meloxicam (Boehringer Ingelheim, Germany 0.4mg/g s.c.) postoperatively and also, cefazolin (50mcg/g i.m.) for preventing infection 2 times a day for 2 days.

In the establishment of our animal model, we focused on several aspects to make it useful for future experiments. Our first concern was the heart size and gender that have been neglected in some of the previous experiments. In our pilot study, we found that rats showed a rapid growth of the heart between 8 and 10 weeks of age (LVESD; 3.40±0.15mm vs 4.02±0.27mm, LVEDD; 6.41±0.34mm vs 7.31±0.34mm for 8 vs 10 weeks of age, p < 0.01) and thus this period was not optimal for the evaluation of LV remodeling. Therefore, in this study,
we used only male rats at the age of 10 weeks. Second, we had to determine adequate severity of MR to induce substantial LV remodeling without excessive mortality in acute phase. We found that 23G needle with external diameter of 0.5 mm was the best for the purpose. At the postmortem study, the mean area of the hole was 0.79 ± 0.34 mm². Obviously, a “learning curve” was noted in our experience. In our first pilot study, the MR operation was successful only three out of nine (33%) rats. It took 4 months for the consistent technique for MR formation in this study. Interestingly, the hole of mitral leaflet spontaneously regressed in some of the animals of our pilot study. Thus, serial echocardiographic confirmation of MR and direct visualization of the hole at the harvest time were important.

In our animal model of MR, acute stage was defined from MR creation to the 2nd week after MR. We observed slight increase of LV diastolic dimension without change of systolic dimension and thus hyperdynamic LV systolic function. In this stage, LV wall thinning occurred as LV began to dilate. Chronic compensated stage could be defined from the 3rd week to around the 14th week. In this stage, LV diastolic and systolic volume increased constantly. Though remained in the normal range, LVEF became significantly lower in MR group from the 12th week with systolic dimension dilatation. The decompensation seemed to begin after the 14th week and became evident between the 20th and 26th week when the mortality dramatically increased. So we decided to analyse microarray before decompensation phase.

**Surgical Procedure for Hemodynamic Monitoring**

The rats were anesthetized with isoflurane inhalation and placed in the supine position on a heat pad with a rectal probe connected to a thermoregulator and ventilated. The right internal jugular vein was cannulated for fluid administration. After anterior thoracotomy, the microtip PV catheter (SPR-838, Millar Instruments, US) was introduced into the LV cavity via the
apical approach along the longitudinal axis of the heart until stable PV loops were obtained\(^1\). PV loops were acquired with the ventilator off for 5-10 seconds after 10 minutes of stabilization. The sampling rate was 1,000/s using the ARIA PV conductance system (Millar Instruments) coupled to a PowerLab/4SP A/D converter (AD Instruments; Mountain View, CA, USA) and a personal computer. To get Vo, additional loops were acquired during injection of 500µl of 15% hypertonic saline. Ten to twenty successive cardiac cycles were obtained. True volumes were calculated with correction based on the EF of echocardiography and saline calibrations as described previously \(^3\). Analyses of the loops were performed using a commercially available cardiac PV analysis program, PVAN 3.5 (Millar Instruments). Heart rate, maximal LV systolic pressure, LV end-diastolic pressure (EDP), mean arterial pressure, maximal slope of systolic pressure increment (+dP/dt) and diastolic pressure decrement (-dP/dt), time constant of LV pressure decay (τ), stroke volume (SV) and end-systolic/diastolic volume (ESV/EDV) were calculated. Additionally, end-systolic pressure volume relation (ESPVR), SW-EDV relation [preload recruitable stroke work (PRSW)], dP/dtmax-EDV relation, and end-diastolic PV relation (EDPVR) were measured by transient occlusion of the inferior vena cava with a silk snare suture.

**Immunohistochemical Analysis**

Mid ventricle were removed for histopathology and were preserved in 4% paraformaldehyde and embedded in paraffin. The tissue was sectioned into 4 µm sections, stained with Masson’s trichrome for evaluation of the degree of fibrosis and with picrosirius red for measurement of collagen deposition. Quantitative measurement of the area of perivascular fibrosis was calculated as the ratio of the area of fibrosis surrounding the vessel wall to the total vessel area using NIH image analysis software (Image J ver. 1.38, National Institutes of Health) \(^5\). At least 10 arterial cross sections were examined per field. Apoptosis
was assessed using the Terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling (TUNEL) technique (DNA fragmentation, Oncor, Gaithersburg, MD). The detailed protocol was previously published. To quantify apoptosis, TUNEL-positive cells in 10 fields for each section was quantitatively evaluated as a percent of that of total cells at a magnification of 400X in a blind manner.

**Microarray analysis**

For control and test RNAs, the synthesis of target cRNA probes and hybridization were performed using Agilent’s Low RNA Input Linear Amplification kit (Agilent Technology, USA) according to the manufacturer’s instructions. The hybridized images were scanned using Agilent’s DNA microarray scanner and quantified with Feature Extraction Software (Agilent Technology, Palo Alto, CA). All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3 (Agilent Technology, USA). The averages of normalized ratios were calculated by dividing the average of normalized signal channel intensity by the average of normalized control channel intensity.

References


Supplementary figure legends

Supplementary figure 1.

Surgical procedures of making mitral regurgitation (MR)

A. Lateral thoracotomy was performed on the left side of the sternum through the 3rd or 4th intercostal space and a needle was inserted.

B. Advancing a needle toward mitral valve leaflet; Red arrow indicates the needle.

C. Severe MR was detected by color Doppler after MR procedure

D. The MR jet and LA area were measured 1 week after MR development by transthoracic echocardiography.

Supplementary figure 2.

Mean body weight among three groups. The mean weight gain of the MR and MR+SIL rats during the 16 weeks period tended to lag behind the sham rats without statistical significance.

Supplementary figure 3

Heat map of the 448 genes were expressed by at least 1.5-fold in MR rats including 197 upregulated (A) and 251 downregulated (B) genes compared with sham and MR+SIL. Red indicates upregulation; black, no change; green, downregulation vs baseline with scale of color corresponding to fold change.
Supplementary figure 2.

The graph shows the body weight in mg over weeks after operation for different groups: Sham, MR, and MR+SIL. The x-axis represents weeks after operation, ranging from B (baseline) to 16wk. The y-axis represents body weight in mg, ranging from 300 to 700.
**Supplementary table 1.**

The List of Gene Sets with Significant Enrichment Scores (ES, P<0.01).

<table>
<thead>
<tr>
<th>Gene Set</th>
<th>Class</th>
<th>Genes (No)</th>
<th>Sham vs. MR ES</th>
<th>p value</th>
<th>MR vs. MR+SIL Size</th>
<th>ES</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GPCRPATHWAY</strong></td>
<td>C2</td>
<td>23</td>
<td>-0.68255293</td>
<td>0</td>
<td>23</td>
<td>0.52691597</td>
<td>0</td>
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<tr>
<td><strong>UVB_NHEK4_6HRS_UP</strong></td>
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<td>16</td>
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<tr>
<td><strong>CROONQUIST_IL6_STROMA_UP</strong></td>
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<td>18</td>
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<tr>
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<tr>
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<td>0</td>
<td>20</td>
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<td>21</td>
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<tr>
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<td>17</td>
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만성 승모판 폐쇄부전증의 Rat Model에서 Sildenafil은 좌심실 확장을 완화한다

강 덕 현 교수 서울대학교병원 순환기내과

Summary

배경
만성 승모판 폐쇄부전증(mitral regurgitation, MR)에서 sildenafil 치료가 좌심실 재형성(remodeling)을 완화하고 운동 능력을 감소하는지 검증하고자 하였다.

방법 및 결과
승모판엽에 바늘로 구멍을 내어 백서(rat)에서 MR을 만들었고, 2주 경과하여 심초음파로 MR과 좌심실 확장을 확인한 후 백서들을 sildenafil 치료군(n=16)과 위약(생리식염수) 투여군(n=16)으로 무작위 배정하여 4개월간 투여하였다. 승모판 손상을 제외하고 같은 처치가 시행된 백서들(sham rats, n=16)을 대조군으로 하여 MR군과 비교하였다.

4개월 경과 후 좌심실 수축기말 내경/확장기말 내경이 각각 대조군 4.7±0.3/8.3±0.4 mm, sildenafil 치료군 5.9±0.3/10.5±0.2 mm, 위약 투여군 7.4±0.5/11.7±0.6 mm로 좌심실 크기는 sildenafil 치료군에서 위약 투여군에 비해 유의하게 작았고(P<0.05), 좌심실 구혈률은 sildenafil 치료군에서 위약 투여군에 비해 유의하게 컸다(대조군, 70.2±2.2% vs. sildenafil 치료군, 67.0±4.2% vs. 위약 투여군 58.9±2.5%; P=0.01). 답차(treadmill) 운동검사에서 운동 능력은 위약 투여군에서 감소하였지만 sildenafil 치료군에서는 감소하지 않았다. 4개월 경과 후 침습적 혈역학 검사가 시행되었고, 검사 후 백서들을 안락사시켰다. 심청부 조직을 이용한 미세배열 분석에서 염증 반응 및 DNA 손상 반응, cellular signaling pathway와 연관된 유전자들의 발현이 위약 투여군에서 sildenafil 치료군에 비해 농축되었다. 조직병리학적 분석에서도 위약 투여군에서 sildenafil 치료군에 비해 혈관주변 섬유화가 현저하였고, 자멸세포의 빈도도 2배 이상 높았다.

결론
Sildenafil은 만성 MR의 백서 모델에서 좌심실 재형성을 유의하게 완화하고 운동 능력을 감소하는 억제 효과가 이러한 치료 효과와 연관될 수 있다.

다음 단계의 연구로는 본 동물 실험 결과를 어떠한 환자에서 적용할 수 있을지 결정하는 임상연구가 필요할 것으로 판단되는데 반드시 고려해야 할 문제들이 있다. 연구자들은 수술 위험이 높은 퇴행성 또는 기질성(organic) MR 환자에서 sildenafil 치료가 도움이 될 것 으로 기대하였는데, 이러한 환자군에서는 항후에도 MR을 교정하는 수술적 치료법 또는 MR에 의한 volume overload 자체를 감소시키는 치료법이 선호될 것이 다. 수술적 치료의 효과가 입증되지 못한 분야, 즉 심부전 환자에서 좌심실이 확장되면서 이차적으로 기능성(functional) MR이 동반된 경우에 본 연구 결과를 활용할 수 있는 지가 임상적으로 보다 중요하다. 심부전 환자에 기능성 MR이 발생하면 MR에 의한 volume overload에 의해 좌심실이 더욱 확장되면서 심부전이 악화되고 MR도 더욱 심해지므로 이런 악순환을 끊는데 sildenafil이 작용할 수 있기를 기대한다. 또한, 기능성 MR에 의해 폐동맥 고혈압이 발생한 환자에서는 sildenafil이 폐동맥 고혈압을 낮추는 데에도 기여할 수 있으므로, 이러한 환자들에서는 sildenafil 치료가 더욱 바람직하다. 그러나 동물실험 결과를 실제 환자에 적용하는 데에는 일반적으로 심각한 제한점이 있는데, 첫째, 본 실험에서 투여되었던 약물의 용량(sildenafil 50mg/kg PO twice a day)이 매우 많았는데, sildenafil이 인체에서 허가된 용량에서도 좌심실의 확장을 예방할지 불명확하다. 둘째, organic MR 환자들에 비해 functional MR 환자들은 좌심실 기능이 현저히 저하되어 있는데, 본 실험모델은 organic MR model이고 좌심실 기능이 정상인 백서를 대상으로 하였으므로 실제 functional MR이 동반된 심부전 환자에서는 저혈압, 심근허혈, 급사 등의 합병증을 오히려 증가시킬 위험이 있다. Sildenafil의 작용기전에 대한 결과 및 설명이 길어서 연구의 핵심 논점이 희석된 점이 있지만, 다양한 실험 연구기를 시행해보려는 연구자들의 의욕의 산물로 이해되며 한국에서는 드물게 충분한 연구비와 연구자의 열정이 아우러진 좋은 연구이다. 판막질환의 연구에 유용한 동물실험 모델을 만들려는 연구자들의 노력이 앞으로도 좋은 결실을 맺기를 기대한다.
Long-Term Effects of Sildenafil in a Rat Model of Chronic Mitral Regurgitation

Benefits of Ventricular Remodeling and Exercise Capacity

Kyung-Hee Kim, MD; Yong-Jin Kim, MD; Jung-Hun Ohn, MD; Jimin Yang, MA; Sang-Eun Lee, MD; Sae-Won Lee, PhD; Hyung-Kwan Kim, MD; Jeong-Wook Seo, MD; Dae-Won Sohn, MD

Background—We tested the hypothesis that chronic treatment with sildenafil attenuates left ventricular (LV) remodeling and prevents exercise intolerance in chronic mitral regurgitation (MR).

Methods and Results—MR was created in Sprague-Dawley rats by making a hole on the mitral leaflet. Two weeks after MR creation, MR and LV dilatation were confirmed by echocardiography, and rats were randomly assigned to sildenafil treatment (MR+sildenafil group; 50 mg/kg PO twice a day; n=16) or normal saline only (MR group; n=16) and continued for 4 months. Sixteen sham rats were compared with MR rats. After 4 months, LV size was smaller in the MR+sildenafil compared with the MR group (LV end-systolic dimension, 4.7±0.3 for sham versus 5.9±0.3 for MR+sildenafil versus 7.4±0.5 mm for MR; P<0.05; LV end-diastolic dimension, 8.3±0.4 versus 10.5±0.2 versus 11.7±0.61 mm, respectively; P<0.05). LV ejection fraction was greater in the MR+sildenafil group than in the MR group (70.2±2.2% for sham versus 67.0±4.2% for MR+sildenafil versus 58.9±2.5% for MR; P=0.01). Serial treadmill test revealed that exercise capacity was reduced in the MR but not in the MR+sildenafil group. Transcriptional profiling of cardiac apical tissues revealed that gene sets related to inflammatory response, DNA damage response, cell cycle checkpoint, and cellular signaling pathways were significantly enriched by genes with reciprocal changes. Pathological analysis showed that perivascular fibrosis was more prominent in the MR than in the MR+sildenafil group and that the percentage of terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling–positive cells was 2-fold greater in the MR compared with the MR+sildenafil group.

Conclusions—Sildenafil significantly attenuates LV remodeling and prevents exercise intolerance in a rat model of chronic MR. This benefit may be associated with the antiapoptotic, anti-inflammatory effects of sildenafil. (Circulation. 2012;125:1390-1401.)

Key Words: exercise capacity ■ left ventricular remodeling ■ microarrays ■ mitral regurgitation ■ sildenafil

Mitral regurgitation (MR) is a growing clinical problem as the mean age of the population advances.1,2 Currently, effective medical therapy is not available to prevent left ventricular (LV) remodeling and deterioration of exercise capacity and thus delay the need for surgery. Although vasodilator therapy is effective in acute MR,3 it is beneficial is not established in chronic MR.4,5

Clinical Perspective on p 116

Sildenafil citrate is a selective inhibitor of phosphodiesterase-5, which catalyzes the breakdown of cGMP, one of the primary factors causing smooth muscle relaxation. By its potent action of enhancing NO-driven cGMP accumulation and ensuing vasodilation, sildenafil is widely used for the treatment of erectile dysfunction. However, growing evidence supports that inhibition of phosphodiesterase-5 may have important antihypertrophic, antiapoptotic, and ischemic preconditioning effects that may limit myocardial remodeling in response to stress and attenuate the substrate for heart failure development. In murine models of myocardial pressure overload, inhibition of phosphodiesterase-5 was associated with inhibition of myocyte hypertrophy and interstitial fibrosis, preservation of cardiac function, and deactivation of various hypertrophy signaling cascades.6-8 Phosphodiesterase-5 inhibition also promotes opening of mitochondrial K-ATP channels, a
putative target of reactive oxygen species generated in response to both ischemia/reperfusion and doxorubicin treatment, and reduces doxorubicin or ischemia/reperfusion-induced apoptosis. Additionally, clinical studies of sildenafil in patients with heart failure have reported improved exercise capacity coupled with reduced pulmonary vascular resistance and better endothelial function.

Accordingly, we hypothesized that sildenafil may have a beneficial effect in chronic MR because the process of LV remodeling shares some of the aforementioned molecular mechanisms in chronic MR. In the present study, we evaluated the long-term effect of sildenafil on survival, myocardial function, remodeling, and exercise capacity using a rat model of chronic MR. Additionally, we investigated potential mechanisms for the beneficial effects of sildenafil using cDNA microarray technology.

**Methods**

**Animal Handling**

Male Sprague-Dawley rats (n=52) with an initial weight of 350 to 400 g were used. The rats were given free access to tap water and standard rat chow and housed in a room with a 12/12-hour light cycle, a temperature of 21°C, and a humidity of 55%. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital.

**Creation of MR Model and Study Design**

Surgical creation of MR was performed under the guidance of transeosophageal echocardiography in 11-week-old rats. We used intracardiac echocardiographic catheters (Acuson/Siemens Corp, Mountain View, CA) for transeosophageal studies. After the insertion of an echocardiographic catheter into the esophagus, left thoracotomy was performed through the fourth or fifth intercostal space. A needle (0.5-mm external diameter) was inserted into the LV via apical puncture. After we found the needle tip under the echocardiographic image, we advanced the needle toward the anterior mitral apical puncture. After we found the needle tip under the echocardiographic image, we advanced the needle (0.5-mm external diameter) was inserted into the LV via apical puncture. After we found the needle tip under the echocardiographic image, we advanced the needle toward the anterior mitral valve to puncture the leaflets and create MR (detailed methodology is described in the online-only Data Supplement). MR was considered significant if a regurgitant jet area was >45% of the left atrium. Sham animals underwent the same procedures except for mitral leaflet injury.

Two weeks after the surgery, we confirmed the development of MR with LV dilation, and the rats were randomly assigned to sildenafil treatment (MR+sildenafil group; 50 mg/kg PO twice a day; n=16) or normal saline only (MR group; n=16) (Table 1). An oral dose of 100 mg/kg per day of sildenafil (Viagra, Pfizer) was administered orally by gavage twice a day on the basis of previous studies showing a pleotropic vascular effect in a heart failure model. Sixteen sham rats were compared with MR rats.

We started the treatment 2 weeks after MR creation and continued for 14 weeks according to the results of our pilot study, as follows: (1) Ejection fraction (EF) remained in the normal range despite progressive increase of LV size; (2) no additional death occurred during this period (see the online-only Data Supplement for details). Cardiac function and exercise capacity were monitored every 2 or 4 weeks. At the end of the 14-week treatment, pressure-volume analysis was performed, and the hearts and lungs were harvested for tissue determinations. The detailed experimental protocol is shown in Figure 1.

**Survival Analysis**

We examined the effects of sildenafil on the survival of MR rats. The day of oral administration of sildenafil was defined as day 0. This survival analysis covered the entire experimental period to day 100.

**Table 1. General and Echocardiographic Characteristics 2 Weeks After Mitral Regurgitation (13 Weeks of Age)**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Sham Group</th>
<th>MR Group</th>
<th>MR+Sildenafil</th>
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<tr>
<td>(n=16)</td>
<td>(n=16)</td>
<td>(n=16)</td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>437.0±8.8</td>
<td>422.8±8.8</td>
<td>423.8±10.4</td>
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<tr>
<td>Systolic BP, mm Hg</td>
<td>130.5±12.9</td>
<td>133.2±6.5</td>
<td>128.4±8.2</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>93.6±15.1</td>
<td>95.7±14.4</td>
<td>90.1±10.1</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>350±18.1</td>
<td>354±14.9</td>
<td>347±22.4</td>
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<tr>
<td>HR, bpm*</td>
<td>312.8±17.3</td>
<td>318.3±15.7</td>
<td>315.3±18.7</td>
</tr>
<tr>
<td>EF, %</td>
<td>70.4±0.94</td>
<td>78.5±1.01</td>
<td>78.7±1.54</td>
</tr>
<tr>
<td>EDD, mm</td>
<td>7.76±0.2</td>
<td>9.01±0.44</td>
<td>9.01±0.48†</td>
</tr>
<tr>
<td>ESD, mm</td>
<td>4.21±0.19</td>
<td>4.15±0.19</td>
<td>4.11±0.22</td>
</tr>
<tr>
<td>SWT, mm</td>
<td>1.32±0.04</td>
<td>1.1±0.05†</td>
<td>1.09±0.06†</td>
</tr>
<tr>
<td>PWT, mm</td>
<td>1.35±0.01</td>
<td>1.07±0.06</td>
<td>1.08±0.07†</td>
</tr>
<tr>
<td>LV mass, g</td>
<td>683±14.44</td>
<td>695±15.7</td>
<td>697.7±14.3</td>
</tr>
<tr>
<td>LV mass index, mg/g*</td>
<td>1.60±0.04</td>
<td>1.65±0.06</td>
<td>1.62±0.05</td>
</tr>
</tbody>
</table>

Data are mean±SEM. MR indicates mitral regurgitation; BP, blood pressure; HR, heart rate; EF, ejection fraction; EDD, end-diastolic dimension; ESD, end-systolic dimension; SWT, wall thickness of intersventricular septum; PWT, end-diastolic wall thickness of posterior wall; E velocity, mitral inflow E velocity (cm/s); and, LV, left ventricular.

*Heart rate and LV mass were estimated in vivo by echocardiography. †P<0.05 for difference from control.

**Physiological Study**

**Echocardiography**

Transtracheal echocardiography was performed 1 week before and 2, 4, 6, 9, 12, and 16 weeks after the creation of MR on spontaneously breathing rats placed in the dorsal recumbent position. Rats were lightly sedated with inhalation of the lowest possible dose of isofurane (initially 4%, then 2% to 3%) mixed with oxygen. Images were acquired with a 9-MHz transducer connected to a Toshiba echocardiography machine (Nemio, Toshiba Co, Tokyo, Japan). LV septal and posterior wall thickness (SWT and PWT) and LV end-diastolic/systolic dimension (EDD/ESD) were measured with M-mode echocardiography at the papillary muscle level. LVEF was calculated as (LVEDD3−LVESD3)/LVEDD3. LV mass was estimated by a formula validated in small animals and adjusted for body weight: LV mass=(SWT+PWT+LVEDD3)+1/2. Color Doppler mapping of MR jets was used to semiquantitatively assess the severity of MR. MR jet areas and LA areas were measured in the apical long-axis view, and the ratio of MR jet area to LA areas was calculated (Figure 1D in the online-only Data Supplement). Early diastolic transmitial flow (E velocity) was measured in the apical 4-chamber view with a 1.5-mm–sized sample volume placed at the tips of the mitral leaflets. All parameters were evaluated on an average of 5 consecutive beats. A single echocardiographer experienced >500 cases of rat echocardiography performed all data acquisition.

**Exercise Test and Blood Pressure Monitoring**

Maximal exercise capacity was evaluated with a Rota Rod treadmill (Ugo Basile, Comerio, Italy), in which rats run on a knurled drum as the drum rotates to avoid falling off. Animals were trained twice before the test to adjust the treadmill more familiarly. Treadmill speed was gradually increased from 3 to 15 rpm every 1 minute. Exercise time was recorded. The observer blinded to the study group recorded episodes of the immobility response as a result of exhaustion.

Systolic and diastolic blood pressures were measured in conscious rats via the tail-cuff method (Biopac System Inc) at 1 week before...
and 2, 4, 9, and 15 weeks after the creation of MR. At least a 1-day interval was given for the blood pressure measurements after the echocardiographic examination or exercise test to minimize stress on the animals.

**Invasive Hemodynamic Measurements**

At 16 weeks after MR creation, invasive hemodynamic measurements were obtained with the use of a 1.4F high-fidelity Millar pressure catheter, as described previously (see detailed Methods in the online-only Data Supplement).

**Histopathological Analysis**

After hemodynamic measurements were performed, the rats were euthanized, the hearts and lungs were harvested and weighed, and the apical scar was excluded.

**RNA Isolation**

Fresh LV apex, excluding the apical scar, was immediately stored in RNAlater (Ambion/Applied Biosciences, Streetsville, Ontario, Canada) at −80°C until use. The hearts were homogenized in TRIzol reagent (Invitrogen, Burlington, Ontario, Canada), and total RNA was isolated according to the manufacturer’s instructions. RNA was further purified to remove genomic DNA contamination and was concentrated with the use of an RNeasy Plus Mini kit (Qiagen, Mississauga, Ontario, Canada). Samples with optical density ratio 260/280 ≥ 1.8, 28S/18S ≥ 1.6 with the use of a Bioanalyzer 2100 (Agilent, Santa Clara, CA) were selected for microarray processing.

**Microarray Analysis**

Apical heart RNA excluding scar tissue was labeled with either cyanine 3-CTP (Cy3) or cyanine 5-CTP (Cy5) (PerkinElmer, Boston, MA) with the use of the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) and hybridized onto an Agilent whole rat genome array (G4131A). RNA samples from 3 rats in each group were pooled and hybridized onto 1 chip. The arrays were scanned at 2 different intensities, and the images were analyzed for background correction. Both BMP4 and FGF2 samples were cohybridized with RNA from the starting point, and a dye swap was performed. The arrays were normalized, and the differential gene expression was analyzed by the R and bioconductor-based method LIMMA (detailed in Methods in the online-only Data Supplement). We performed gene set enrichment analysis to find molecular pathways or gene ontologies that are made up of differentially expressed genes in each biological condition. Difference in mean log-transformed intensity for each biological group was used as a metric for sorting probes in gene set enrichment analysis. After genes were sorted according to the metric, enrichment scores were assigned to 2394 different gene sets curated in the mSigDB database (http://www.broadinstitute.org/gsea/msigdb/). Gene sets with high absolute values of enrichment score are molecular pathways or gene ontologies that are made up of upregulated or downregulated genes and are differentially regulated pathways. P values for the enrichment scores were calculated after permuting class labels of each experimental condition, and gene sets with P values < 0.05 were extracted. Gene sets with absolute enrichment score > 0.35 and P value < 0.05 were visualized as a network with nodes representing gene sets and edges connecting 2 nodes if hypergeometric P value for testing the significance of gene overlap is within 1 percentile of the whole P values.

**Verification of Gene Expression With Reverse Transcription Polymerase Chain Reaction**

Total RNA was isolated with the use of an RNeasyPlus Mini Kit (Qiagen), and cDNA was synthesized with the use of the PrimeScript First-Strand cDNA Synthesis Kit (Takara) with 1 μg of each total RNA sample according to the manufacturer’s instruction. cDNA (1 μL) was amplified by polymerase chain reaction with the use of the TaKaRa Ex Taq Kit (Takara) with specific primers (Table 2). GAPDH was chosen as an endogenous control. Quantification of band intensity was analyzed with the use of TINA 2.0 (RayTest) and normalized to the intensity of GAPDH.

**Immunohistochemical Analysis**

See the online-only Data Supplement for details. Mid ventricles were removed for histopathology and were preserved in 4% paraformaldehyde and embedded in paraffin. The tissue was sectioned into 4-μm sections and stained with Masson trichrome for evaluation of the degree of fibrosis and with picrosirius red for measurement of collagen deposition. Apoptosis was assessed with the use of the terminal deoxynucleotidyl transferase–mediated UTP nick-end labeling (TUNEL) technique (DNA fragmentation; Oncor, Gaithersburg, MD). The detailed protocol was published previously.
Statistical Analysis
Data are presented as mean±SEM. The normality of all parameters was tested with the Kolmogorov-Smirnov test. The differences among groups were compared by unpaired *t* test or 1-way ANOVA, followed by the Bonferroni test. In cases in which normality was excluded, the nonparametric Kruskal-Wallis H test was performed, and the Mann–Whitney *U* test was used for post hoc analysis. Repeated-measures ANOVA was performed to analyze the changes of several variables over time such as LVEF, LV dimension, LV mass index, and exercise capacity. Survival data were evaluated by the Kaplan–Meier method with pairwise comparison conducted with the log-rank test. All statistical analyses were performed with the use of SPSS 17.0 version (SPSS Inc, Chicago, IL), and *P* values <0.05 were considered statistically significant.

Results

Survival Analysis
A total of 52 rats were used in this study. After surgery, 2 rats died of bleeding, and 2 rats died in the acute stage within 2 weeks after MR operation as a result of pulmonary edema and unknown cause. Three of 16 rats in the MR group died during the study period. However, no deaths were noted in the MR+sildenafil and sham groups. A Kaplan–Meier survival curve showed the comparison between MR+sildenafil and MR rats (*P*=0.07; Figure 2A).

Hemodynamic Study

LV Remodeling After MR Creation
Mean MR jet area was 15±3 mm², and the mean ratio of MR jet area to LA area was 56±8% at 2 weeks after the operation. There was no MR in the sham group. Mitral E velocity increased at 2 weeks (65.2±7.1 versus 105.1±15.1 cm/s; *P*<0.05) in the MR groups, whereas it did not change in the sham group (66.4±6.4 versus 64.5±5.5 cm/s; *P*=0.76). Figure 3 demonstrates the time-dependent changes in LV diameter, EF, and LV mass index. Baseline examinations showed no difference in LV diameters among the 3 groups (LVESD, 4.0±0.1 versus 4.1±0.1 versus 4.1±0.1 mm;
LVEDD, 7.4±0.1 versus 7.5±0.1 mm for sham, MR, and MR+sildenafil groups, respectively; \( P=0.82 \). The LV started to dilate immediately after MR creation and showed progressive dilation until week 16. There was a significant difference in LVEDD between the MR and MR+sildenafil groups from week 6, whereas LVEDD did not differ significantly until week 12. Consequently, LVEF started to decrease from week 6 in the MR group, whereas it was preserved until week 16 in the MR+sildenafil group. With increasing LVESD, death started to occur, as shown in the Kaplan–Meier curve (Figure 2A). Weight gain tended to lag behind that of the sham rats in the MR and MR+sildenafil rats, but this was without statistical significance (Figure II in the online-only Data Supplement). The LV mass index increased significantly in the MR group compared with the sham group (1.15±0.04 versus 1.71±0.05 mg/g; \( P<0.05 \)). Sildenafil treatment attenuated the increase of LV mass index (11.7% reduction; \( P=0.01 \)). There were no significant changes in blood pressure and heart rate among the groups (Figure 2B).

**Exercise Capacity**

There was no difference in exercise duration among the groups until week 6 after MR, when it became shorter in the MR group than in the sham group (720±15 for sham versus

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**Figure 3.** A, Serial changes in echocardiographic measurements. B, Representative M-mode echocardiograms from the 3 groups. MR indicates mitral regurgitation; SIL, sildenafil; LV, left ventricle; B, baseline; and wk, weeks after MR operation. *\( P<0.05 \) for difference from sham group; †\( P<0.05 \) for difference from MR group.
588±15 for MR versus 617±21 seconds for MR+sildenafil; P<0.05 for difference from sham group; †P<0.05 for difference from MR group.

**Invasive Hemodynamic Measurement**

At week 16, rats underwent invasive hemodynamic assessment and were euthanized for pathological analysis (Table 3 and Figure 5). Heart rate, LV end-diastolic pressure, −dP/dt, and the end-diastolic pressure-volume relation were not different among the groups. However, end-systolic volume, end-diastolic volume, and stroke volume were greater in the MR group than in the sham group. The end-systolic pressure-volume relation, +dP/dt, preload recruitable stroke work, and EF were significantly decreased in the MR group, indicating contractile dysfunction. Sildenafil treatment prevented systolic functional impairment in the MR+sildenafil group compared with the MR group, as follows: EF, +28% (P<0.05); end-systolic pressure-volume relation, +116% (P<0.05); and preload recruitable stroke work, +30% (P<0.05).

**Microarray Analysis**

A total of 448 genes were differentially expressed by at least 1.5-fold in MR rats (P<0.05), including 197 upregulated and 251 downregulated genes compared with the sham or the MR+sildenafil group. The heat map in Figure III in the online-only Data Supplement demonstrates a consistent pattern of change of these genes in each condition. Table I in the online-only Data Supplement lists genes well established in the pathophysiology of cardiovascular disease that were upregulated >1.5-fold in the MR group and downregulated in the MR+sildenafil group.

For biological interpretation, we performed gene set enrichment analysis (see Methods for details) to explore the transcriptional changes at the level of molecular pathways or gene ontologies. Figure 6A and 6B is the network representation of gene sets that are enriched with genes upregulated in MR and reciprocally downregulated after treatment with sildenafil and presumed to be primarily associated with the pharmacological effect of sildenafil in MR. It is notable that gene sets form several tightly interconnected structures or clusters that we can annotate with inflammatory response, DNA damage response, cell cycle checkpoint, and cellular signaling pathways associated with LV remodeling.

To confirm the changes in transcription level, we selected 5 representative genes implicated in DNA damage response, inflammatory response, endothelial function, and vascular remodeling: CDKN2A, interleukin-6, interleukin-18, endo-
reduced in sildenafil-treated MR rats. We found no difference in interstitial fibrosis among the groups. However, the extent of perivascular fibrosis was significantly larger in the MR group than in the MR+sildenafil group (Figure 7C through 7F). TUNEL staining showed a significant increase of cardiac apoptosis in the MR group (0.24±0.3%; P<0.05; Figure 8). In contrast, a significant inhibition of apoptosis was evident in the MR+sildenafil group (0.05±0.02%), which was comparable to that in the sham group (0.03±0.04%).

Discussion
The purpose of this study was to evaluate the effect of chronic administration of sildenafil on cardiac remodeling, function, and exercise capacity in rats with chronic MR. The major finding of the present study was that sildenafil prevented LV remodeling and exercise intolerance caused by chronic experimental MR. To our knowledge, this is the first study that shows that sildenafil is efficacious in the treatment of MR-induced LV remodeling and cardiac dysfunction. Additionally, we proposed potential mechanisms related to the effect of sildenafil: inhibition of inflammation and apoptosis.

Medical Management in Chronic MR
MR induces chronic LV volume overload and leads to LV contractile dysfunction, heart failure, and, finally, death. Although surgical correction of MR, the only definitive cure, carries reasonably low mortality and morbidity risks, medical therapeutics have a role in many clinical situations such as in a patient population with greater surgical risk. This has become more relevant because the main etiology of MR has changed from rheumatic to degenerative valve disease,25 and the prevalence of MR increases with age because operative mortality for elderly patients is still high.26 However, there is currently no recommended pharmacological therapy for chronic MR. Despite previous efforts, medical therapies for chronic MR have produced disappointing and conflicting results.

Small-Animal Model of Chronic MR
Little is known about the molecular and cellular mechanisms of LV remodeling induced by volume overload partly because of the absence of a small-animal model. In their pioneering work, Pu et al14 developed an MR rat model for the first time. We successfully established a small-animal model of chronic MR and verified the pathophysiological features of this model. We described the detailed time course of LV remodeling and the relationship between progressive LV dilatation and LV dysfunction. Immediately after MR creation, we observed a slight increase of LV diastolic dimension without a change of systolic dimension and thus hyperdynamic LV. In this stage, LV wall thinning occurred as the LV began to dilate. A chronic compensated stage could be

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Figure 5. Representative pressure-volume loops from the 3 groups. Mitral regurgitation (MR)+sildenafil (SIL) group showed smaller left ventricular volume and greater end-systolic pressure-volume relation (ESPVR) (dotted line) compared with the MR group. EDPVR indicates end-diastolic pressure-volume relation.
Figure 6. A and B. Network of gene sets with significant enrichment scores ($P < 0.05$), made up of genes that are upregulated in the mitral regurgitation (MR) rat model. Nodes represent gene sets, and edges connect 2 nodes if the 2 gene sets share a significant
defined from week 3 to approximately week 16. In this stage, LV diastolic and systolic volume increased progressively. Although it remained in the normal range, LVEF became significantly lower in the MR group from week 12. At week 16, load-independent contractile indices were significantly lower in the MR group, whereas diastolic parameters were not different. These results are consonant with several previous studies that demonstrated that MR is one of the few cardiac diseases in which diastolic function is supernormal.27 Chronic adaptation to volume overload tends to decrease LV chamber stiffness and increase diastolic filling rates and dimension lengthening irrespective of systolic function in chronic MR.28 Additionally, we did not find any increase of LV interstitial fibrosis in this stage of MR. Interstitial fibrosis is closely related to LV diastolic function.

Mechanism for LV Remodeling in MR

MR causes progressive LV dilation, wall thinning, and cardiomyocyte elongation, which recapitulates eccentric remodeling with inflammatory cell infiltration and extracellular matrix degradation. It has been suggested that inflammation and energy metabolism initiate cardiac remodeling.29 Nemoto et al30 showed that administration of the peroxisome proliferator-activated receptor-γ agonist rosiglitazone ameliorates MR-induced LV dysfunction accompanied by a decline in lipid content. Similarly, in the volume overload model of aortocaval fistula, upregulation of genes was related to inflammation, the extracellular matrix, the cell cycle, and apoptosis.31 There was a 40-fold increase in the matricellular protein periostin, which inhibits connections between collagen and cells, thereby potentially mediating side-to-side slippage of cardiomyocytes and LV dilatation. These findings are consistent with our results that inflammation, the cell cycle, and DNA damage pathways that were upregulated in MR were prevented by sildenafil treatment.

Increased matrix metalloproteinase activity is associated with progressive LV dilatation and extracellular matrix degradation, contractile dysfunction, and neurohormonal activation in volume overload animal models. Zheng et al.,32 using a gene array analysis, reported recently that eccentric LV remodeling in isolated MR was associated with increased matrix metalloproteinase activity. However, we could not find a significant difference in matrix metalloproteinase families among the groups. This may imply that the relative contributions of each of these mechanisms change at different stages of remodeling. Extracellular matrix loss, inflammation, or bioenergetics dysfunction has been implicated at different time points in the pathophysiology of volume overload.33

Sildenafil for Treatment of MR

Emerging data indicate that phosphodiesterase-5 is present within cardiac myocytes and may play a role in modulating cGMP activity in various cardiac diseases.34 Several observations from preclinical and short-term clinical studies support a role of sildenafil in the treatment of heart failure. In the present study, the anti-inflammatory and antiapoptotic effects of sildenafil seemed to play a key role in preventing LV remodeling.
The endocardial perivascular area may represent an early fibrotic change in the MR model, probably because of relative endocardial ischemia due to the prolonged elevation of LV diastolic filling pressures. However, the extent of perivascular fibrosis was significantly smaller in the MR + sildenafil group than in the MR group. In recent years, there has been considerable interest in studying the effect of sildenafil on endothelial cell protection, which may trigger a signaling cascade (through the action of kinases including protein kinase C and other mitogen-activated protein kinases) and generation of NO by phosphorylation of endothelial NO synthase. An important property of sildenafil is its ability to increase endothelial NO synthase and inducible NO synthase proteins in the heart, and this has a direct cause-and-effect relationship in protection against myocardial infarction, as well as apoptosis in cardiomyocytes. Because of the ability of sildenafil to augment NO synthase and cGMP levels, it is logical to hypothesize that cardiovascular dys-

**Figure 7.** Comparison of pathological results. **A**, Eccentric hypertrophy was developed in the mitral regurgitation (MR) group. SIL indicates sildenafil. **B**, The hole created in the anterior leaflet of the mitral valve. **C, D, E, G**, Masson trichrome and picrosirius red staining showed no difference in interstitial fibrosis (**F and H**). The extent of perivascular fibrosis was significantly greater in the MR group than in the MR + SIL group († P < 0.05 for difference from the MR group).

**Figure 8.** Representative apoptotic cells by terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling analysis and quantitative apoptotic indices († P < 0.05 for difference from the mitral regurgitation [MR] group). Arrows indicate apoptotic cells. SIL indicates sildenafil.
function induced by chronic NO synthase inhibition would be alleviated by concomitant treatment with sildenafil.

Clinical and experimental data support a link between endothelial dysfunction and inflammation. Proinflammatory cytokines are increased in the myocardium as a response to stretch in MR patients and correlate with the extent of LV dilatation. Inflammatory response and cytokine elaboration are integral components of the host response to tissue injury and play an active role in myocardial remodeling, and thus inflammatory mediators play a crucial role in the development of heart failure. Accordingly, several strategies to counterbalance the inflammatory response have been suggested. Possible targets involve proinflammatory and anti-inflammatory cytokines and their receptors, adhesion molecules, NO and NO synthase, reactive oxygen species, and different types of leukocytes. In this study, we demonstrated by microarray analysis that the inflammatory response was decreased in the sildenafil-treated group compared with MR rats.

Cell loss is another unifying feature found in nearly all forms of cardiomyopathies. Currently, the best-understood form of cell loss or cell death in heart failure is apoptosis. DNA fragmentation and cellular and nuclear shrinkage are of the biochemical and morphological changes that classically characterize apoptosis. Barouch et al demonstrated that cardiomyocyte apoptosis is associated with increased DNA damage and decreased survival in a murine model of obesity. In this study, we showed that the DNA damage pathway was upregulated in MR rats compared with sham and MR+sildenafil rats and that apoptotic cells were more prevalent in MR rats.

Nagayama et al demonstrated that sildenafil treatment prevents further cardiac and myocyte dysfunction and progressive remodeling in an animal model of established hypertrophy caused by pressure overload. In the present study, we found a similar beneficial effect of sildenafil in an animal model of volume overload. The therapeutic effect of sildenafil has also been demonstrated in other animal models including an ischemia/reperfusion model and in anthracycline cardiomyopathy. These findings suggest that there is substantial overlap of molecular pathways in the development of different types of cardiac remodeling induced by various stimuli. In addition, these findings also suggest the multiple effects of sildenafil and cGMP.

Limitation of the Study
MR was created by making a hole in the mitral leaflet, and this MR model may not represent MR in humans, especially ischemic MR. This is an inherent limitation of the animal model of valve disease. However, our model is still useful to evaluate the effect of medical treatment on LV remodeling induced by MR. In this regard, the beneficial effect of sildenafil on volume overload–induced LV remodeling may have clinical implications. Further research is needed to delineate the exact mechanisms involved to allow for translation to the clinical setting.

Conclusion
Sildenafil attenuates LV remodeling and prevents exercise intolerance in a rat model of chronic MR. This benefit may be associated with the antiapoptotic, anti-inflammatory effects of sildenafil. Further research is needed to delineate the exact mechanisms involved and demonstrate the beneficial effects in patients with chronic MR.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Mitrual regurgitation (MR) induces chronic left ventricular volume overload and leads to left ventricular contractile dysfunction, heart failure, and, finally, death. Although surgical correction of MR, the only definitive cure, carries reasonably low mortality and morbidity risks, medical therapeutics have a role in many clinical situations such as in a patient population with greater surgical risk. However, there is currently no recommended pharmacological therapy for chronic MR. Despite previous efforts, medical therapies for chronic MR have produced disappointing and conflicting results. Since the early 2000s, sildenafil, part of a class of selective inhibitors of phosphodiesterase type 5, has been under intense study in various areas. Multiple lines of preclinical and clinical evidence support a therapeutic role for phosphodiesterase type 5 inhibition with sildenafil in the management of heart failure. Accordingly, we hypothesized that sildenafil may have a beneficial effect in chronic MR. The major findings of the present study are that sildenafil prevented left ventricular remodeling and exercise intolerance caused by chronic experimental MR. To our knowledge, this is the first study that shows that sildenafil is efficacious in the presence of MR. Additionally, we proposed potential mechanisms related to the effect of sildenafil: inhibition of inflammation and apoptosis. With consideration of the beneficial effects of sildenafil in MR rats, we can expect a therapeutic potential for sildenafil in patients with MR. Future clinical studies are needed.