Sepiapterin Decreases Acute Rejection and Apoptosis in Cardiac Transplants
Independent of Changes in Nitric Oxide and iNOS Dimerization

Galen M. Pieper, Irina A. Ionova, Brian C. Cooley, Raymond Q. Migrino, Ashwani K. Khanna, Jennifer Whitsett, Jeannette Vasquez-Vivar

Transplant Surgery (G.M.P., I.A.I), Orthopaedic Surgery (B.C.C.), Division of Cardiovascular Medicine (R.Q.M.), Department of Biophysics (J.W., J.V.V.), the Cardiovascular Research Center (G.M.P.) and the Free Radical Research Center (G.M.P., J.V.V.), Medical College of Wisconsin, Milwaukee, Wisconsin; Division of Cardiology (A.K.K.), University of Maryland School of Medicine, Baltimore, Maryland
Running title: Sepiapterin, Cardiac Allograft Rejection and Apoptosis

Correspondence to:
Dr. Galen M. Pieper
Division of Transplant Surgery
Medical College of Wisconsin
9200 West Wisconsin Avenue
Milwaukee, WI 53226 USA
Phone: 414-456-5899; Fax: 414-456-6222; e-mail: gmpieper@mcw.edu

Number of text pages (including tables): 32
Number of tables: 3
Number of figures: 12
Number of references: 38
Word count (abstract): 249
Word count (introduction): 414
Word count (discussion): 1496

Abbreviations: BH₄, tetrahydrobiopterin; BH₂, dihydrobiopterin; GTPCH, GTP cyclohydrolase 1; 4-HNE, 4-hydroxy-2-nonenal; iNOS, inducible nitric oxide synthase; LV, left ventricular; NO, nitric oxide; TNFα, tumor necrosis factor α; IFNγ, interferon-γ; IL-2, interleukin-2
Abstract
Tetrahydrobiopterin (BH₄), a co-factor of iNOS, is an important post-translational regulator of NO bioactivity. We examined whether treatment of cardiac allograft recipients with sepiapterin, a precursor of BH₄, inhibited acute rejection and apoptosis in cardiac transplants. Heterotopic cardiac transplantation was performed in Wistar-Furth donor to Lewis recipient strain rats. Recipients were treated daily following transplantation with 10 mg/kg sepiapterin, S(-)-2-amino-7,8-dihydro-6-(2-hydroxy-1-oxopropyl)-4-(1H)-pteridinone. Grafts were harvested on post-transplant day 6 for analysis of BH₄ (HPLC), expression of inflammatory cytokines (RT- and real-time PCR), iNOS (Western blots) and NO (Griess reaction and NO analyzer). Histological rejection grade was scored and graft function was determined by echocardiography. Apoptosis, protein nitration and oxidative stress were determined by immunohistochemistry. Treatment of allografts with sepiapterin increased cardiac BH₄ levels by 3-fold without changing protein levels of GTP cyclohydrolase, the enzyme that regulates de novo BH₄ synthesis. Sepiapterin decreased inflammatory cell infiltrate and significantly inhibited histological rejection scores and apoptosis similar in magnitude to cyclosporine. Sepiapterin also decreased nitrative and oxidative stress. Sepiapterin caused a smaller increase in left ventricular (LV) mass vs. untreated allografts but without improving fractional shortening. Sepiapterin did not alter TNFα and IFNγ expression while it decreased IL-2 expression. Sepiapterin did not change total iNOS protein or monomer levels, or plasma and tissue NO metabolites levels. It is concluded that the mechanism(s) of anti-rejection are due, in part, to decreased apoptosis, protein nitration and oxidation of cardiomyocytes which appears mediated at the immune level by limiting inflammatory cell infiltration via decreased IL-2-mediated T-lymphocyte expansion.
Introduction

Inducible nitric oxide synthase (iNOS) is up-regulated in response to various inflammatory stimuli. In most cases, iNOS produces large amounts of NO. The production of NO by iNOS is critically influenced by the availability of the cofactor, tetrahydrobiopterin (BH₄). Using purified iNOS protein, it is well established that BH₄ regulates NO production by stabilizing iNOS protein and facilitating iNOS homodimerization, thereby, establishing its catalytic production of NO from arginine (Baek et al., 1993). The biological impact of this regulation was first demonstrated in BH₄-deficient NIH3T3 cells which were unable to support NO production after retroviral infection to over-express iNOS (Tzeng et al., 1995). It was found that increases in NO production from iNOS occurred after supplementing cells with BH₄ or its precursor, sepiapterin. This finding showed for the first time the crucial role of increased BH₄ synthesis in regulating NO production via iNOS in a cellular setting. GTP cyclohydrolase (GTPCH) is considered to be the rate-limiting step in *de novo* BH₄ synthesis. The simultaneous up-regulation of both GTPCH and iNOS is understood to co-ordinate the increased synthesis of BH₄ levels at a sufficiently high level to facilitate enzymatic NO production from iNOS protein (Gross and Levi, 1992; Geller et al., 2000).

Cardiac allograft rejection is associated with the robust increase in iNOS in the heart. Expression of iNOS in this setting has been proposed to be linked with contractile dysfunction and heart failure (see review, Pieper and Roza, 2008). In the past, there has been a primary focus on iNOS mRNA and/or protein expression in defining the role of iNOS in acute cardiac rejection. Information regarding the actual NO bioactivity deriving from iNOS in acute cardiac rejection is noticeably absent. Previous studies from our laboratory suggest that GTPCH is also up-regulated in cardiac allografts but that at later stages of rejection the synthesis of biopterin was insufficient to support NO production seen at earlier time periods (Pieper et al., 2005). This suggested that the progression to full rejection of the graft involves a BH₄-deficient state. This happened to coincide with protein nitration and onset of graft dysfunction. With this background, it is
presumed that increasing intragraft BH₄ levels might decrease rejection. In the present study, we tested the hypothesis that treatment of allograft recipients with sepiapterin as a means to increase intragraft BH₄ levels would inhibit acute cardiac rejection and apoptosis. Furthermore we examined if this effect was related to improving NO levels and iNOS homodimerization or to some other action on immune activation.

**Methods**

**Transplantation.** All animal procedures were approved by the local institutional animal care and use committee. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” and the “Guide for the Care and Use of Laboratory Animals”. Lewis (Lew:RT1¹) and Wistar-Furth (WF:RT1¹) rat strains (Harlan Labs, Indianapolis, IN) were chosen for genetic disparity at both the major and minor histocompatibility loci for donor-to-recipient combination of Lewis → Lewis (for isografts) or Wistar-Furth → Lewis (for allografts) rats. Isogeneic or allogeneic heterotopic cardiac transplantation was performed aseptically in pentobarbital (50 mg/kg, i.p.) anesthetized animals as described (Pieper et al., 2000). Donor hearts were arrested in cold University of Wisconsin preservation solution and transplanted into anesthetized (50 mg/kg pentobarbital i.p.) recipient animals. To increase intragraft BH₄ levels, allograft recipients were treated with sepiapterin (10 mg/kg/day, i.p.). Drug treatments were given to recipient animals starting on the day of transplantation at 1 hr before donor heart transplantation and continued daily until harvesting of grafts on post-operative day 6.

Grafts were arrested in pentobarbital-anesthetized animals at post-operative day 6 by flushing with cold University of Wisconsin preservation solution, and portions were frozen in liquid N₂ for various analysis. Other portions of the graft were taken and evaluated for histological rejection as described below. In some cases, plasma was also taken to document changes in plasma and cardiac NO metabolite levels using a Griess reaction assay and/or by chemiluminescence using a NO analyzer.
**Histological rejection scoring.** Tissue was fixed in 10% phosphate-buffered formalin. Paraffin-embedded sections were stained with hematoxylin and eosin. Histological rejection was scored blinded using criteria established by the International Society for Heart and Lung Transplantation as modified to a linear score system to allow statistical analysis as described in detail previously (Pieper et al., 2002). Initial scoring was determined blinded with the average scoring of two individuals used for analysis.

**Immunohistochemistry.** Apoptosis was measured by the TUNEL assay using ApopTag® technology (Chemicon International, Temecula, CA) according to manufacturer’s instructions and as previously established [Pieper et al., 2008]. Sections were counterstained for visualization of apoptotic nuclei. Apoptotic nuclei were counted from at least 4 section fields for each graft sample, averaged and the mean for each experimental group determined.

Nitration of tyrosine residues on graft protein was determined by immunohistochemistry with and without counterstaining similar to that previously described (Pieper et al., 2004). Sections of hearts were incubated with 1:50 dilution of anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY). To determine oxidative stress at the protein level, we examined 4-hydroxy-2-nonenal (4-HNE) protein adduct formation. Tissue sections were incubated with 1:100 anti-4-HNE (Calbiochem, San Diego, CA). After antibody exposure sections were incubated with horseradish peroxidase-conjugated secondary antibody. Reactivity was revealed using 3,3’-diaminobenzidine tetrahydrochloride and sections were counterstained with either hematoxylin alone or hematoxylin with eosin for examination by light microscopy. Nitrotyrosine and 4-HNE levels were scored as follows: 0, complete absence of immunoreactivity; 1, a single foci or weak areas of staining; 2, moderate staining; 3, intense staining in 1/3rd or more myocytes; 4, intense staining and/or multifocal intense staining. Individual sections from individual grafts were scored independently by two individuals and the values averaged.

**Echocardiography.** Echocardiography was performed using a Vivid 7 electrocardiograph (General Electric, Waukesha WI) and 11 MHz M12L linear
array transducer. The parasternal short-axis mid-left ventricular view was used with the papillary muscles as anatomic landmarks. Image depth was 2-2.5 cm, frame rates of 234-256 frames per second and second harmonic imaging was used. The images were sent to a separate workstation for analysis using EchoPAC software with Q analysis (General Electric, Waukesha WI).

LV size (internal diameter in diastole, LVIDd and systole, LVIDs) and thickness (anterior and inferior diastolic thickness, At and It respectively) were measured using anatomical M-mode of the 2-dimensional B-mode images through the plane of the anterior and inferior segments. Fractional shortening (FS) was calculated using the formula FS = (LVIDd-LVIDs)/LVIDd x 100%. LV ejection fraction was calculated using the Teicholz method (Teicholz et al., 1976). LV mass was derived using the formula: 0.8[1.04{(It+LVID+At)3-LVID3}]+0.6 [Devereux et al., 1986]. Mid-left ventricular myocardial area was derived by subtracting the endocardial area by the epicardial area at mid-left ventricle during diastole. Diastolic measurements were performed at the R wave of electrocardiographic gating that corresponded to the donor heart rhythm in cases where two distinct R waves were seen (i.e., from the native and heterotopically-transplanted donor heart). In cases where the donor heart rhythm could not be distinguished from the native heart on electrocardiography, diastolic measurements were done at the largest diameter of the cardiac cycle.

Radial strain and circumferential strain were measured as previously published (Migrino et al., 2007). In brief, the mid-left ventricle was divided into 6 segments as defined by the American Society of Echocardiography (Lang et al., 2005). The beginning of the cardiac cycle was defined as the R wave of the donor heart if electrocardiographic gating could distinguish native and donor heart rhythm, or the time of the cardiac cycle with the greatest LV diameter followed on successive frames by reduction in size, in cases where donor heart rhythm could not be distinguished. The endocardial border was traced and the outer border was adjusted to fit the epicardial contour. The software automatically selected acoustic objects within the myocardium to track and computed radial strain in the segments of the mid-ventricle throughout the
cardiac cycle. End systolic strain was obtained for each segment and the average used to compute global radial or circumferential strain. Using the same software, average peak systolic circumferential strain rate from the six segments was calculated and represents the highest systolic change in circumferential strain by time. This parameter is more closely related to myocardial contractility than ejection fraction (Stoylen et al., 1999).

**Western blot.** The distribution of iNOS monomers and dimers was analyzed by Western blots under reducing conditions as previously described (Vasquez-Vivar et al., 2008). Briefly samples were harvested and freshly homogenized in lysis buffer in the presence of protease inhibitors. β-Mercaptoethanol was omitted from the Laemmli sample buffer. Total protein concentration for each sample was determined using Bio-Rad DC protein assay. Proteins were resolved on 7.5% SDS PAGE and transferred to nitrocellulose membranes. A dilution of 1:1000 of iNOS antibody (Cayman Chemical, Ann Arbor, MI and Santa Cruz Biotechnology, Santa Cruz, CA) was used overnight at 4°C. Immunoblots were developed with SuperSignal West Femto Maximum Sensitivity Substrate kit (Pierce, Rockford, IL). Other Western blots were performed under reducing conditions (i.e., boiled in the presence of β-mercaptoethanol) and probed at 4°C overnight with antibodies to GTPCH (1:5000 dilution) or GAPDH (1:1000 dilution). Immunoreactivity for iNOS protein was effectively blocked in both reducing and non-reducing samples in the presence of blocking peptide (not shown).

**PCR analysis.** Total cellular RNA was isolated from graft biopsies by using 1 ml of TRIzol® reagent (Invitrogen, Carlsbad, CA) per 100 µg of heart tissue according to the manufacturer’s protocol. Genomic DNA was digested by treatment with RNase-free DNase (Ambion, Austin, TX) and RNA concentration was determined spectrophotometrically. Complementary DNA was synthesized from 1 µg of total RNA and random hexamer primers by using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer’s directions. Primers and conditions used for these analyses are summarized in Table 1. PCR reactions were performed in a 25-µl volume
containing 1 µl of cDNA, 25 pmol of sequence-specific primers and 22.5 µl of Platinum® PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA). A 10 µl aliquot of the PCR product was resolved by 1% TAE-agarose gel electrophoresis and densitometric analysis of specific bands was performed using Alpha Imager (Alpha Innotech Corp, San Leandro, CA). Additional studies on cytokine gene expression were performed using real-time quantitative PCR. Primer sequences and conditions are described in Table 2. Relative mRNA level was determined as 2[(Ct/β-actin – CT/gene of interest)]. The results are presented as fold expression normalized to β-actin.

**Tetrahydrobiopterin and 7,8-dihydrobiopterin analysis.** Quantification of BH₄ and 7,8-dihydrobiopterin (BH₂) was performed by HPLC with electrochemical detection. Tissue samples were homogenized in 300 µl of 50 mM phosphate buffer (pH 2.6) containing 0.1 mM diethylenetriaminepentaacetic acid and freshly added 1 mM dithiothreitol. Samples were centrifuged at 12,500 rpm for 10 min at 4°C, and supernatants were loaded onto Centricon filters (10,000 molecular weight cut-off). Filtrates were analyzed on an HPLC system (ESA Biosciences CoulArray® system, Model 582 and 542) using an analytical Polar-RP column eluted with argon saturated 50 mM phosphate buffer, pH 2.6 as described [Whitsett et al., 2007]. Calibration curves were made by summing up the peak areas collected at 0 and 150 mV for BH₄ and 280 and 365 mV for BH₂. Intracellular concentrations were calculated using authentic BH₄ and BH₂ (10-100 nM) standards and concentrations normalized to respective protein content of test samples.

**Plasma and tissue NOₓ analysis.** Blood was collected in EDTA and plasma separated by centrifugation. Plasma samples were deproteinized by filtering through 10,000 MW cut-off filters. To measure NO metabolites in heart, approximately 40 mg of heart tissue was homogenized in DPBS (pH 7.4). Samples were centrifuged at 14,000 rpm at 4°C for 30 min. Supernatants were collected and filtered through pre-wet 10,000 MW cut-off Microcon filters (Millipore Corporation, Bedford, MA). A 20 µL aliquot of each sample or standard
was used to inject into a NO analyzer (Sievers Instruments, Inc., Boulder, CO) and NO$_x$ levels were normalized to total protein. In addition to the NO analyzer, some samples were also processed for NO metabolites using the Griess reaction assay (Cayman Chemical, Ann Arbor, MI).

**Data Analysis.** All values are expressed as mean ± S.E.M. Statistical analysis was performed by one-way analysis of variance with Student-Newman-Keuls test for multiple comparisons of multiple group means or with Student’s $t$ test for comparisons between two group means. Statistical significance was set at $P<0.05$.

**Results**

Histological rejection was not evident in isografts (Fig. 1A). In contrast in untreated allografts, there were areas of inflammatory cell infiltrate, necrosis, vasculitis, hemorrhage and interstitial edema which were decreased in allografts treated with sepiapterin (Fig. 1A). Histological rejection scores were significantly ($P<0.001$) increased in allografts vs. isograft controls (Fig. 1B). Treatment of allograft recipients with sepiapterin significantly ($P<0.001$) decreased histological rejection scores compared to that observed in untreated allografts. For comparison, the level of protection achieved by treatment with sepiapterin was similar to that achieved by treatment with cyclosporine at a dose previously determined to prolong graft survival to at least 111 days when given continuously (Khanna et al., 2004). Acute rejection of untreated allografts was also associated with enhanced apoptosis vs. isograft controls (Fig. 2A). The number of TUNEL-positive, apoptotic cells was significantly ($P<0.01$) decreased in recipients treated with sepiapterin (Fig. 2B). The degree of decrease in apoptosis was equivalent to that seen in recipients treated with cyclosporine (Fig. 2).

Echocardiography revealed an increase in LV mass of 58.1% in allografts compared to isograft controls. The increase in LV mass was 40.4% in sepiapterin-treated allografts vs. isograft controls. This represents a significant ($P<0.05$) reversal of 30% in sepiapterin-treated allografts from untreated allografts (Fig. 3). Both untreated and sepiapterin-treated allografts displayed
similar levels of systolic dysfunction (i.e. low ejection fraction, fractional
shortening and global radial and circumferential strain) (see Table 3).

To determine whether treatment with sepiapterin altered intragraft
bioppterin levels, we performed HPLC on graft samples at post-operative day 6 for
BH₄ and BH₂. Comparison of values in samples frozen in liquid N₂ and stored vs.
analysis of freshly harvested, unfrozen samples yielded similar values (data not
shown). The levels of BH₄ were enhanced by 3-fold in recipients treated with the
BH₄-precursor, sepiapterin compared to untreated allografts (Fig. 4). Treatment
with sepiapterin also significantly increased intragraft levels of BH₂ (Fig. 4).

Treatment with sepiapterin did not alter the levels of GTPCH protein indicating
that the increase in BH₄ due to sepiapterin cannot be due to up-regulation in
GTPCH expression (Fig. 5).

We next examined functional effects of treatment with sepiapterin on iNOS
and NO levels. Plasma levels of NO metabolites measured by the Griess assay,
revealed increased levels of NO production in allografts vs. isografts which were
not altered by treatment of recipients with sepiapterin (Fig. 6A). Treatment with
sepiapterin did not alter the cardiac content of NO in allografts determined by
chemiluminescence using a NO analyzer (Fig. 6B). The lack of further increase in
intracardiac NO levels by treatment with sepiapterin was additionally confirmed
using a Griess reaction assay (not shown). These findings were consistent with
the finding that sepiapterin did not alter iNOS protein levels (Fig. 5).

As the level of arginase expression may indirectly alter the detection of
NO levels and might be differentially expressed under treatment conditions, we
next examined arginase 1 expression in isografts, untreated allografts and
allografts treated with sepiapterin. Arginase 1 expression was weakly expressed
in isografts but significantly (P<0.01) up-regulated in allograft recipients (Fig. 7A).
Treatment of allograft recipients with sepiapterin did not significantly alter
arginase 1 expression compared to expression in untreated allografts (Fig. 7B).

The allo-immune-induced increases in the protein levels of iNOS in
allografts vs. isograft controls were not altered by treatment with sepiapterin (Fig.
6). Using tissue samples that were frozen and stored in liquid N₂ and processed
under reducing conditions, iNOS was detected only in allografts, but not in isografts, and only as iNOS monomers (i.e., at 130 kDa) (Fig. 8A). Under non-reducing conditions, both iNOS monomers and higher molecular weight species, herein termed ‘iNOS dimers’, were detected and the pattern was similar using two different commercial anti-iNOS antibodies (Fig. 8A). In these and other tests, the intensity of the monomer band was always equal to or larger than the upper ‘iNOS dimer’ band. To determine if freeze-thawing might influence dissociation of iNOS dimer, we also performed similar studies from freshly-harvested samples (Fig. 8B). In this case, both iNOS monomers and dimers were present in cardiac allograft samples but the proportion attributed to monomers was lower than that observed using frozen allograft samples. To avoid this complication, subsequent blots were performed only on freshly-harvested, but unfrozen, samples from recipient grafts. Here, both iNOS monomers and dimers were also present in sepiapterin treatment; however compared to untreated allografts, sepiapterin did not change the levels of iNOS monomers (Fig. 8B and C).

Nitration of proteins develops during acute cardiac allograft rejection. In the samples examined in this study, we observed immunohistochemical evidence for increased formation of nitrotyrosine during rejection in untreated allografts vs. isograft controls (Fig. 9A). Staining in allografts was prominent throughout many cardiomyocytes and occasionally in blood vessels and infiltrating cells. In general, treatment with sepiapterin decreased both the proportion of cardiomyocytes staining for nitrotyrosine as well as the intensity of focal areas of staining for nitrotyrosine. Semi-quantitative analysis indicated that treatment with sepiapterin significantly (P<0.001) decreased nitrotyrosine levels relative to untreated allografts (Fig.9B). This decrease in sepiapterin-treated allografts while significant was still elevated above isograft controls in contrast to the ablation in nitration using cyclosporine shown previously (Pieper et al., 2004) and in the present study using cyclosporine (Fig. 9B).

To characterize changes in oxidative stress due to treatment with sepiapterin, we also examined 4-HNE adducts in cardiac grafts by immunohistochemistry. In general, staining for 4-HNE was not observed in
isografts while there was prominent staining in a many cardiomyocytes of untreated allografts (Fig. 10). Most of the staining was confined to strong staining in cardiomyocytes. Occasionally, staining for 4-HNE could be seen in blood vessels but this was not always the case. Treatment with sepiapterin and cyclosporine decreased both the intensity and frequency of 4-HNE staining in cardiomyocytes of allograft recipients. The greatest decrease in 4-HNE was seen in the cyclosporine-treated recipients.

Along with up-regulation of iNOS expression, allo-immune activation increases expression of several inflammatory cytokines. Thus, we first examined gene expression by RT-PCR for the inflammatory cytokines, TNFα and IFNγ, which are known to be increased in cardiac allograft rejection. TNFα and IFNγ, normalized to GAPDH were significantly (P<0.01) up-regulated in allografts compared to isograft controls (Fig. 11). Treatment with sepiapterin did not alter the increased expression of these cytokines vs. that seen in untreated allografts. Similar analysis using real-time PCR confirmed these initial findings (not shown).

To determine if sepiapterin might have some effect on T-cell function, we performed additional analysis using real-time PCR of the expression of IL-2, an important T-lymphocyte activation product and stimulator of T-cell proliferation and expansion. Expression of IL-2 was decreased by treatment with sepiapterin (Fig. 12).

**Discussion**

We showed that sepiapterin significantly decreased histological rejection. The mechanism for anti-rejection was due, in part, to decreased IL-2 expression and was associated with decreased apoptosis, nitration and oxidation of protein in cardiomyocytes. The magnitude of protection was comparable to that achieved by using low-dose cyclosporine. To our knowledge this is the first study to report the protective effects of multiple-day treatment with sepiapterin in a model of cardiac allograft rejection.

Cardiac graft rejection in humans is associated with increased LV mass (Kawauchi et al., 1992; Mondillo et al., 2008). In the present study, we showed that sepiapterin significantly attenuated the increase in LV mass.
of conventional echocardiographic measurement of LV function in the heterotopically-transplanted heart is challenging due, in part, to mechanical unloading. Thus, values for systolic function (i.e., fractional shortening or ejection fraction) are lower in isografts compared to normal hearts (Zhou et al., 2007). This may explain the inability to discriminate systolic function between isografts and untreated allografts. Measurements of strain and peak systolic strain rate are a novel application to assessing function in this model. These parameters measure deformation and are better suited for estimation of systolic function (Migrino et al., 2007 and 2008). While these better discriminate graft dysfunction in allografts vs. isografts, measurements were not sufficiently sensitive to discriminate improvements using sepiapterin. In contrast the benefits of sepiapterin on LV mass are more consistent with this as a functional measure of rejection. Our finding using sepiapterin is consistent with observations that authentic BH$_4$ did not improve contractile function assessed by subjective, graded scoring derived by external graft palpation in mouse transplants (Brandacher et al., 2006). Collectively, these findings support the notion that histological rejection and graft dysfunction do not always correlate (de Groot-Kruseman et al., 2002) and may arise from different etiologies.

BH$_4$ levels are critical to optimal production of NO from iNOS. Previously, we reported that cardiac allografts displayed early transient increases in total biopterin that regressed at later stages suggesting the development of a BH$_4$ deficit (Pieper et al., 2005). We surmised that this arose from a critical switch in iNOS expression initially from the inflammatory cell infiltrate and later in cardiomyocytes. In this regard unlike neonatal cardiomyocytes, we recently documented the low efficiency of adult cardiomyocytes to synthesize BH$_4$ in response to inflammatory stimuli (Kalivendi et al., 2005; Vasquez-Vivar et al., 2008).

For inflammatory cells, the addition of sepiapterin to macrophages transfected to over-expressing iNOS supported iNOS homodimerization from inactive iNOS monomers and increased NO production (Sakai et al., 2006). This was one of the first cell-based studies to demonstrate the biological feasibility of
augmenting intracellular BH₄ in order to reproduce the pterin-facilitated iNOS homodimerization demonstrated using purified iNOS protein. Previously, authentic BH₄ was shown not to alter plasma NO or cardiac graft iNOS mRNA levels in mouse heart transplants (Brandacher et al., 2006). However, the effects on NO levels in cardiac grafts per se were not examined. In our study, we found that sepiapterin did not increase intracardiac NO levels. In contrast, sepiapterin was shown to increase tissue NO in renal transplants (Huismann et al., 2002). The conflicting results may be explained by differences in experimental design, NO detection methods or intrinsic differences in metabolism of sepiapterin in different organs. Indeed, renal NO was determined 24 hrs after transplantation; therefore, a consequence of treatment on early post-transplant reperfusion injury. We determined cardiac graft NO levels several days after daily treatment with sepiapterin which reflects more the effects on allo-immune activation and rejection.

Under certain conditions, arginase can compete with iNOS for arginine, thereby limiting NO production (de Bono et al., 2007; Romero et al., 2008). In consideration of this possibility, we confirmed that arginase 1 is inducible in cardiac allografts. Arginase 1 expression in the heart is likely to derive from non-cardiomyocyte cells (e.g. fibroblasts, endothelial and vascular smooth muscle cells). This conclusion comes from our recent findings that arginase is absent and not induced in adult cardiomyocytes stimulated with a variety of cytokine stimuli (Ionova et al., 2008). However, this cannot explain the ineffectiveness of sepiapterin to increase intracardiac NO levels since arginase 1 expression was unaltered by sepiapterin.

Despite the studies concerning BH₄ and the relationship to endothelial NOS homodimerization in diseased blood vessels and myocardium (Schmidt and Alp, 2007), published studies on homodimerization of iNOS in heart are extraordinarily rare consisting of a single publication in a model of ventricular hypertrophy (Zhang et al., 2007). Using non-reducing Western analysis, we confirmed the presence of the expected 130 kDa inactive iNOS monomers in acutely-rejecting cardiac allografts. Our unexpected finding that sepiapterin
treatment did not alter iNOS monomer levels is consistent with the data showing that cardiac NO content was also unchanged. This finding; however, is consistent with our studies showing that the addition of authentic BH₄ (Vasquez-Vivar et al., 2008) or sepiapterin (Ionova et al., 2008) to isolated rat cardiomyocytes stimulated with inflammatory cytokines failed to increase NO production from iNOS or decrease iNOS monomer levels. Thus, the findings in the in vivo cardiac model allo-immune inflammation complement and extend the findings seen in cytokine-stimulated cardiomyocytes.

Of significant importance to this understanding were our findings that adult rat cardiomyocytes lack both mRNA and protein expression for dihydrofolate reductase (Ionova et al., 2008). Dihydrofolate reductase is a critical step that is necessary for conversion back to BH₄ of the BH₂ formed following transport of both BH₄ and sepiapterin into the cell (Sawabe et al., 2004). Lack of dihydrofolate reductase would be predicted to cause intracellular build-up of BH₂ after administration of sepiapterin. Indeed, we found that there was a large increase in intracardiac BH₂ levels that might be expected to offset any benefits on increased NO production and iNOS homodimerization due to increased BH₄. The finding of increase in BH₂ in the present in vivo study is similar to that found previously by our laboratory in isolated cardiomyocytes treated with sepiapterin (Ionova et al., 2008). Our findings do not exclude the possibility that sepiapterin may be increasing NO in other cell types which express dihydrofolate reductase in vivo (e.g. endothelial cells). However, if this occurred in endothelial cells, any overall increase in intragraft content of NO due to sepiapterin may not be detected since endothelial cells contribute only a small proportion of total graft NO levels.

Although sepiapterin clearly increased intragraft BH₄ levels, the benefits to decreasing rejection and apoptosis occurred independently of any changes in allo-immune-induced levels of intracardiac iNOS protein, iNOS homodimerization or NO levels. This finding would not be expected by conventional theory derived from other tissues or cells but could be explained if a large part of the increased intracardiac BH₄ levels produced via sepiapterin occurred in non-cardiomyocyte
cells such as cardiac fibroblasts rather than in cardiomyocytes which is a prime location of increased iNOS expression in this model. This indicates a unique mechanism of action of sepiapterin \textit{in vivo} that is more complex than simply increasing NO bioactivity.

The findings in the present study of rat cardiac allografts is consistent with the anti-rejection activity of authentic BH4 or its 4-amino derivative in a murine model of acute cardiac rejection (Brandacher et al., 2001; Brandacher et al., 2004). Of interest is the fact that the 4-amino analog which inhibits catalytic activity of purified iNOS protein, unlike BH4, also had an anti-rejection effect in the mouse model. Together with the present findings using sepiapterin, the results suggest that these derivatives have anti-rejection actions via some other process independent of increased intracellular BH4 and/or NO production.

Previous studies show that genetic and pharmacologic strategies which limit nitration also improve rejection (Szabolcs et al., 2001 and 2002; Pieper et al., 2005; Nilakantan et al., 2005). In the present study our findings using sepiapterin are consistent with the notion that protein nitration and rejection are linked. Regarding oxidative stress, the present study is the first to show oxidative stress in acute cardiac allograft rejection using 4-HNE. Our findings suggest that sepiapterin decreased rejection, in part, by decreasing oxidative stress. Because of the inherit deficiencies in BH4 synthesis and NO production via iNOS in adult cardiomyocytes per se, we suggest that the decrease in nitrateive and oxidative stress due to sepiapterin treatment is more likely related to an external effect of recipient immune cells on cardiomyocytes in this model.

The mechanism of protection by sepiapterin did not involve changes in expression for the two major inflammatory cytokines, TNF\(\alpha\) and IFN\(\gamma\) in agreement with findings using authentic BH4 in mouse cardiac transplants (Brandacher et al., 2006). This does not exclude the possibility that sepiapterin improved rejection by some other redundant gene pathway. An intriguing possibility for the anti-rejection mechanism of sepiapterin is via an effect on T-cell function (Thoeni et al., 2005). In this context, we showed that sepiapterin decreased IL-2 expression, an important product of T-cell activation and a growth
factor for T-cells. Thus, the decrease in rejection by sepiapterin could be explained at the immune cell level by inhibiting IL-2-mediated T-cell proliferation and expansion.

Acknowledgements
The technical assistance of Gail Hilton and Chao-Ying Chen are acknowledged.
References


Lang RM, Bierig M, Devereux RB, Flachskampf FA, Foster E, Pellikka PA, Picard MH, Roman MJ, Seward J, Shanewise JS, Soloman SD, Spencer KT, Sutton MS, Stewart WJ; Chamber Quantification Writing Group; American Society of Echocardiography’s Guidelines and Standards Committee; and European Association of Echocardiography. (2005) Recommendations for chamber quantification: a report from the American Society of Echocardiography’s Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. *J Am Soc Echocardiogr* 18:1440-1463.


FOOTNOTES: This work was supported by the National Institutes of Health [Grant #HL078937 to G.M.P., Grant #HL067244 to J.V.V.].
Legends for Figures

Figure 1. (A) Histological evidence of rejection in untreated allografts (allo) vs. isografts (iso) and sepiapterin-treated allografts (sepi) or cyclosporine (CsA)-treated allograft recipients. Rejection is characterized by inflammatory cell infiltrate (blue arrow), necrosis (green arrow), interstitial edema (black arrow), vasculitis (white arrow) and hemorrhage (yellow arrow).  (B) Decreased histological rejections scores by treatment with sepiapterin or CsA. ***P<0.001 vs iso; (*** ) P<0.001 vs.untreated allo

Figure 2. (A) Examples of TUNEL staining showing increased apoptosis in untreated allografts (allo) vs. isograft (iso) controls and decreased apoptosis in sepiapterin-treated allografts (sepi) vs. untreated allografts (allo).  (B) Treatment with sepiapterin decreased the number of apoptotic cells similar to that achieved by treatment with CsA for comparison. **P<0.01 vs iso; (**) P<0.01 vs allo

Figure 3. Increased LV mass as shown by echocardiography in allograft recipients vs. isograft controls and attenuation by treatment with sepiapterin (n=5-7 each group). ***P<0.001 allo vs iso; **P<0.01 sepi vs. iso; (*)P<0.05 allo vs. sepi

Figure 4. HPLC analysis with electrochemical detection showing that treatment of allograft recipients with sepiapterin (sepi) increased cardiac allograft levels of BH₄ (open bars) and BH₂ (solid bars). * P<0.05 vs. untreated allografts (allo)

Figure 5. (A) Western blot and (B) densitometry showing that treatment with sepiapterin (sepi) did not alter protein expression for GTP cyclohydrolase (GTPCH) or inducible nitric oxide synthase (iNOS) normalized to GAPDH.

Figure 6. (A) Plasma (n=5-9 each) and (B) myocardial NOₓ levels (n=5-6 each) in isografts (iso) vs. untreated allografts (allo) and allograft recipients receiving
treatment with sepiapterin (sepi). Results show that treatment with sepiapterin did not alter NO levels. **P<0.01 allo or sepi vs. iso

Figure 7. (A) Increase in arginase 1 mRNA expression normalized to GAPDH in allografts (allo) compared to isograft (iso). (B) Arginase 1 mRNA is not altered by treatment of recipients with sepiapterin (sepi). ** P<0.01 vs. isograft

Figure 8. (A) Individual Western blots of iNOS monomers and dimers in allografts (allo) but not isografts (iso) in samples taken from frozen, stored cardiac graft samples but processed under non-reducing and reducing conditions. Results show similar findings using two different commercial anti-iNOS antibodies. (B) Example showing Western blot analysis using Santa Cruz anti-iNOS antibody of freshly-harvested graft samples from rat cardiac allografts run under reducing and non-reducing conditions and effects of sepiapterin (sepi) treatment. (C) Densitometry of three different Western blots for iNOS monomers in rat cardiac allograft recipients without and with treatment with sepiapterin (n=3-4 each group).

Figure 9. (A) Immunohistochemistry showing staining for nitrotyrosine in allografts (allo) vs. isograft (iso) controls (x 400). (B and C) Decreased nitrotyrosine staining in cyclosporine (CsA)-treated (x 600) and sepiapterin (sepi)-treated allografts (x 400) vs. untreated allografts. (D) Increase in nitrotyrosine intensity score in allografts (allo) vs. isograft (iso) controls by sepiapterin (sepi) or cyclosporine (CsA) (n=3 for iso; n=6-7 for each allograft group). * P<0.05 vs. iso; *** P<0.001 vs iso; (***) P<0.001 vs allo

Figure 10. (A) Immunoreactivity for 4-hydroxynonenal (4-HNE) indicative of oxidative stress is increased in cardiac allografts (allo) vs. isograft (iso) controls (x 400) and is decreased by treatment with sepiapterin (sepi) or cyclosporine (CsA). (B) Increase in 4-HNE intensity score was decreased by treatment by
both treatments (n=3 iso; n=5-6 each for all other groups). ***P<0.001 vs. iso, *** P<0.01 vs. iso, (***P<0.001 vs. allo

Figure 11. (A) RT-PCR and (B) densitometry normalized to GAPDH housekeeping gene showing that treatment with sepiapterin (sepi) did not alter the allo-immune-induced up-regulation of cytokine gene expression for TNFα and IFNγ in cardiac allografts (allo) vs. isografts (iso). ** P<0.01 vs. iso

Figure 12. Intragraft IL-2 expression in allografts (allo) is decreased in allograft recipients treated with sepiapterin (sepi). *P<0.05 vs allo; allo (n=3), sepi (n=4)
Table 1. Primers and conditions used for RT–PCR analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (forward/reverse)</th>
<th>Size (bp)</th>
<th>Annealing Temp # cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTPCHI</td>
<td>GGATACCAGGAGACCATCTCA TAGCATGGTGCTAGTGACAGT</td>
<td>148</td>
<td>57°C/27</td>
</tr>
<tr>
<td></td>
<td>TAGCATGGTGCTAGTGACAGT</td>
<td>372</td>
<td>60°C/40</td>
</tr>
<tr>
<td>iNOS</td>
<td>CACCTTGGAGTTCCACCAGGTGTTTGTAGCGCTG</td>
<td>328</td>
<td>60°C/30</td>
</tr>
<tr>
<td></td>
<td>CAGGTGAGGCTGCTGACGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARG1</td>
<td>CCAAGCCAAAGCCCATAGAGATTA CCCGTGCAGATTCCCAGAGC</td>
<td>419</td>
<td>60°C/30</td>
</tr>
<tr>
<td>TNFα</td>
<td>TCAGCCTCTTCTCATTCTGTCTGC TTGGTGTTTGTACGACGTG</td>
<td>203</td>
<td>58°C/32</td>
</tr>
<tr>
<td>IFNγ</td>
<td>CGCCCGCTCTTGGTTTTG CGACTCCTTTTCCCCTTCTTAG</td>
<td>452</td>
<td>58°C/30</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AGTTCAACGGCAGCAGATCAAG GTGGTAAGACGCCAGTGA</td>
<td>148</td>
<td>57°C/27</td>
</tr>
</tbody>
</table>

GTPCHI, GTP cyclohydrolase; iNOS, inducible nitric oxide synthase; ARG1, arginase 1; TNFα, tumor necrosis factor α; IFNγ, interferon gamma; GAPDH, glyceraldehyde phosphate dehydrogenase
Table 2. Primers and conditions used for real time PCR analyses of inflammatory cytokines

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (forward/reverse)</th>
<th>Size (bp)</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>GCCCAGACCCTCACACTC CCACTCCAGCTGCTCCTCT</td>
<td>99</td>
<td>60°C</td>
</tr>
<tr>
<td>IFNγ</td>
<td>TTTTGCAGCTCTGCCTCAT AGCATCCATGCTACTTGAGTTAAA</td>
<td>109</td>
<td>60°C</td>
</tr>
<tr>
<td>IL-2</td>
<td>CTGCAAAAGCAAAACACGCAG TGGGGAGTTTCAGATTCTTGTAAT</td>
<td>96</td>
<td>59°C</td>
</tr>
<tr>
<td>β-actin</td>
<td>CCCGCGAGTACAACCTTCT CGTCATCCATGGCGAICT</td>
<td>97</td>
<td>60°C</td>
</tr>
</tbody>
</table>

TNFα, tumor necrosis factor α; IFNγ, interferon gamma; IL-2, interleukin-2
Table 3. Echocardiography of untreated and sepiapterin-treated rat cardiac allografts

<table>
<thead>
<tr>
<th></th>
<th>Iso graft (n=5)</th>
<th>Untreated Allograft (n=7)</th>
<th>Sepiapterin Treated Allograft (n=7)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSd (cm)</td>
<td>0.172 ±0.022</td>
<td>0.429 ±0.038</td>
<td>0.453 ±0.025</td>
<td>P&lt;0.001 iso vs allo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.001 iso vs sepi</td>
</tr>
<tr>
<td>LVIDd (cm)</td>
<td>0.552 ±0.050</td>
<td>0.333 ±0.082</td>
<td>0.164 ±0.025</td>
<td>P&lt;0.05 iso vs allo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.01 iso vs sepi</td>
</tr>
<tr>
<td>LVPWd (cm)</td>
<td>0.240 ±0.035</td>
<td>0.409 ±0.050</td>
<td>0.481 ±0.023</td>
<td>P&lt;0.01 iso vs allo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.01 iso vs sepi</td>
</tr>
<tr>
<td>LVIDs (cm)</td>
<td>0.468 ±0.038</td>
<td>0.304 ±0.081</td>
<td>0.126 ±0.020</td>
<td>P&lt;0.01 iso vs sepi</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.01 allo vs sepi</td>
</tr>
<tr>
<td>EDV (cm)</td>
<td>0.436 ±0.099</td>
<td>0.191 ±0.093</td>
<td>0.017 ±0.007</td>
<td>P&lt;0.05 iso vs sepi</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.05 iso vs allo</td>
</tr>
<tr>
<td>ESV (cm)</td>
<td>0.266 ±0.055</td>
<td>0.159 ±0.085</td>
<td>0.007 ±0.004</td>
<td>P&lt;0.05 iso vs sepi</td>
</tr>
<tr>
<td>EF (%)</td>
<td>36.19 ±5.31</td>
<td>29.51 ±5.77</td>
<td>44.76 ±10.68</td>
<td>n.s.</td>
</tr>
<tr>
<td>FS (%)</td>
<td>14.98 ±2.61</td>
<td>11.74 ±2.47</td>
<td>20.55 ±5.66</td>
<td>n.s.</td>
</tr>
<tr>
<td>Radial Strain (%)</td>
<td>13.642 ±7.048</td>
<td>1.183 ±0.301</td>
<td>1.199 ±0.247</td>
<td>P&lt;0.05 iso vs allo</td>
</tr>
<tr>
<td>Circumferential Strain (%)</td>
<td>-4.169 ±2.504</td>
<td>-1.224 ±0.341</td>
<td>-1.217 ±0.297</td>
<td>n.s.</td>
</tr>
<tr>
<td>Peak Circumferential Strain Rate (1/s)</td>
<td>-1.55 ±0.59</td>
<td>-0.38 ±0.10</td>
<td>-0.40 ±0.10</td>
<td>P&lt;0.05 iso vs allo</td>
</tr>
</tbody>
</table>

IVSd, interventricular septal thickness in diastole; LVIDd, left ventricular internal diameter in diastole; LVPWd, left ventricular posterior wall diameter in diastole; LVIDs, left ventricular internal diameter in systole; EDV, end-diastolic volume; ESV, end-systolic volume; EF, left ventricular ejection fraction; FS, fractional shortening; n.s. (not significant)
**Fig 1**

A

iso

allo

sepi

CsA

B

**Histological Rejection Score**

<table>
<thead>
<tr>
<th></th>
<th>iso</th>
<th>allo</th>
<th>sepi</th>
<th>CsA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

*(*** — statistically significant difference)*
Figure 3
Figure 4
Figure 5
Figure 7

A

Arginase 1
GAPDH

-ctrl  iso  allo  

Arginase-1 mRNA [normalized to GAPDH]

iso  allo

**

B

Arginase 1
GAPDH

-ctrl  allo  sepi  

Arginase-1 mRNA [normalized to GAPDH]

allo  sepi
Figure 8
Fig. 9
Fig. 10
Figure 11
Figure 12

IL-2 expression [normalized fold]

100-

10-

1-

allo

sepi

*

This article has not been copyedited and formatted. The final version may differ from this version.