ORIGINAL PAPER

Effects of timed administration of doxycycline or methylprednisolone on post-myocardial infarction inflammation and left ventricular remodeling in the rat heart

Ricardo A. Garcia · Katrina V. Go · Francisco J. Villarreal

Abstract The development of strategies to ameliorate post-myocardial infarction (MI) remodeling and improve function continues to be an area of clinical importance. Use of steroids for this purpose is controversial since the effects of timed treatment on relevant inflammatory, biochemical and structure/function endpoints are unclear. In a previous report, we demonstrated that use of doxycycline pre-treatment improves post-MI remodeling and passive left ventricular (LV) function. However, the effects of timed doxycyline post-MI treatment are unknown. To examine these issues, we performed a study using a rat MI model. Animals were administered one of the following: doxycycline (DOX), the corticosteroid methylprednisolone (MP), or aqueous vehicle. Treatment was given early, short-term (at time of MI to 24 h post-MI) or late, long term (2–7 days post-MI). Animals were sacrificed at 3, 7 or 42 days post-surgery. We assessed LV hemodynamics, pressure–volume, and pressure–scar strains, histomorphometry, inflammation via measurements of myeloperoxidase activity, and matrix metalloproteinase (MMP) activity. Late MP treatment yielded a robust right-shifted pressure–volume curve, which was accompanied by increased scar strains. Late DOX treatment yielded reduced average heart weight and size and preserved scar thickness. DOX treatment did not suppress inflammation, which contrasts with the suppressive effects of MP. Use of early or late MP yielded increased MMP activity in infarcted and non-infarcted regions. Early and late treatment with DOX yielded infarct–associated MMP activity levels comparable to those of vehicle–treated animals. In conclusion, results indicate that late use of MP yields adverse post-MI structure/function outcomes that correlate with suppression of inflammation and increased MMP activity. These observations contrast with those of DOX, in particular, late treatment where improved outcomes were observed in LV structure and were accompanied by the lack of suppression of inflammation.

R. A. Garcia · K. V. Go · F. J. Villarreal
Department of Medicine, University of California San Diego, La Jolla, CA, USA

F. J. Villarreal (✉)
Cardiology, UCSD, 9500 Gilman Dr. BSB 2004, La Jolla, CA 92093-0613J, USA
e-mail: fvillarr@ucsd.edu

Introduction

The development of adverse left ventricular (LV) remodeling following a myocardial infarction (MI) is known to negatively affect the clinical evolution of patients [1]. Indeed, adverse LV remodeling can lead to the development of heart failure, which is the most common cause of hospitalization in the USA in medicare patients [2]. At present, pharmacological options to limit the development of adverse remodeling include the use of agents that interrupt or block the action of angiotensin II, catecholamines and aldosterone [2]. Despite the availability of these agents, a significant number of patients continue to progress towards heart failure and either succumb to the disease or receive a heart transplant. Thus, the development of novel treatment strategies to reduce adverse LV remodeling following MI is warranted.

For many years, modulation of post-MI inflammation has been considered a target of interest for therapy [3]. In this regard, two opposing views exist. One view notes that the suppression of inflammation is associated with adverse outcomes and thus, this strategy should be avoided [3]. Support for this view is derived from studies in animal models and humans where use of glucocorticoids or non-steroidal anti-inflammatory agents are associated with worsened post-MI ventricular structure/function and increased mortality [4–7]. However, these observations are countered by studies where use of glucocorticoids has apparently led to improved outcomes [8–12]. Use of reperfusion therapy post-MI (in particular, late reperfusion (24 h and beyond) where myocardial salvage is unlikely) and its demonstrated benefits lends credence to the counter view that facilitation of an inflammatory infiltrate speeds up healing/scarring and ultimately yields improved outcomes [13, 14]. The ability of tissues to heal and scar is closely associated with the inflammatory cascade. Post-MI inflammation is characterized by two major phases. One, the acute phase associated with neutrophil infiltration and a second, partially overlapping chronic phase associated with macrophage infiltration [15]. It has been proposed, and partially examined in animals, that development of acute inflammation is not essential for proper healing/scarring to occur and that neutrophil infiltration may accentuate local damage partly by leading to increased matrix metalloproteinase (MMP) activity [16]. Increased MMP activity can damage fibrillar collagens whose integrity limits infarct expansion [15]. This hypothesis has not been systematically tested in the infarcted myocardium. Thus, the issue of timing of steroid treatment is potentially critical since the development of proper infarct scarring post-MI may rely on the ability of these compounds to preferentially exert their actions during specific phases of the inflammatory process while not adversely affecting the overall capacity of the myocardium to heal.

Doxycycline (DOX) is a member of the tetracycline family of antibiotics. Interestingly, an emerging body of literature has revealed a number of nonantibiotic properties of DOX that have highlighted the therapeutic utility of this agent (for review, see [17]). Indeed, DOX has been approved for treatment of periodontal disease, a nonantibiotic indication, via a mechanism of action linked to the inhibition of MMP activity [18]. Of particular interest is the observation that DOX can function as an anti-inflammatory agent, which has been inferred from studies that demonstrated reduction in pathological levels of nitric oxide (NO) (a mediator and marker of inflammation) by

DOX [19, 20]. Overproduction of NO is implicated in the pathogenesis of chronic inflammatory diseases such as arthritis, chronic bronchitis and asthma, and ulcerative colitis [21–23].

Tetracyclines have been reported as cardioprotective in short-term models of ischemia-reperfusion injury [24–27]. We have reported improved outcomes in ventricular structure/function in hearts subjected to a permanent coronary occlusion (MI) for 4 weeks in animals were treated with DOX [28]. Treatment was provided 24 h pre MI to 48 h post-MI. In addition, the use of mass spectrometry has allowed us to better understand DOX-MMP inhibitory properties [29]. However, use of DOX to improve healing post-MI in the infarcted heart is still seen with skepticism in light of its anti-inflammatory properties. Furthermore, the applicability of our previous observations is handicapped by the fact that DOX was provided 24 h prior to coronary occlusion. Currently, nothing is known about how DOX treatment provided either at the time of MI or after it may alter the course of LV structure/function. Furthermore, nothing is known about the capacity of DOX to modify the inflammatory response that follows MI. The major objectives of this study were to: 1) study the effects of early and late treatment of DOX and methylprednisolone (MP) on post-MI global LV and scar region structure/function and; 2) examine the effects of DOX and MP treatment on inflammation and MMP activity. Early- and late-treatment strategies were designed to overlap peak phases of acute (24 h) or chronic inflammation (5–7 days) in rat myocardium following coronary artery occlusion [30].

Materials and methods

Materials

Doxycyline hydrochloride was purchased from Sigma-Aldrich. Methylprednisolone was from Pharmacia & Upjohn (Kalamazoo, MI).

Surgical preparation and drug administration

Animal studies were performed according to guidelines by the American Association for Accreditation of Laboratory Animal Care, and protocols were approved by the University of California San Diego Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (225–300 g) were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection, intubated, and venti-

lated with room air. The heart was exposed via a left thoracotomy, the pericardium opened and the left anterior descending coronary artery occluded. The chest was closed and rats were allowed to recover. Animals were randomly assigned to seven groups: sham, vehicle treated early, vehicle treated late, DOX treated early, DOX treated late, MP treated early, and MP treated late. Rats were treated according to the schedule shown in Fig. 1. Time = 0, represents the time of coronary occlusion. DOX (30 mg/kg/day) was dissolved in de-ionized water and administered by gavage every 12 h [28]. The initial dose of DOX in the early-treatment group was given immediately prior to administration of anesthesia. The dose of DOX used in this study was based on previous experiments performed by our group using small and large mammals (rats and pigs) where DOX led to improved post-MI outcomes [28, 31]. MP in saline (50 mg/kg/day) was given as a bolus intraperitoneal injection once per day [12]. The early treatment MP group received the drug immediately after the chest was closed. Vehicle–treated animals were infarcted and given water by gavage every 12 h according to the DOX treatment schedule. Shams were subjected to the surgical procedure described above and the suture placed without occluding the coronary artery.

LV sampling and morphology studies

Designated rats were sacrificed at either 3, 7 or 42 days (6 weeks) after thoracotomy. Hearts were exposed via medial sternotomy and arrested with an apical injection of ice-cold arrest solution (NaCl, 4 g/L; KCl, 4.48 g/L; NaHCO$_3$, 1 g/L; glucose, 2 g/L; 2,3-butanedione monoximine, 3 g/L; heparin, 2000 U/L) into the LV. Animals sacrificed at 3 and 7 days were processed as follows: the aorta, atria, and right ventricle were trimmed away from the LV and the dissected components weighed. Left ventricular tissue was allocated for sampling of the infarct and the non-infarcted LV septum for MPO and MMP activity. Tissue samples were
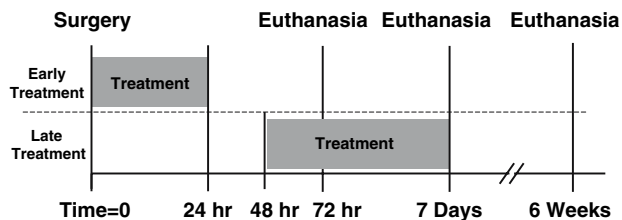

Fig. 1 Schematic depicting the treatment protocols used, times of euthanasia and duration of the study. Time = 0, represents the time of coronary occlusion

frozen on dry ice. The 6 week animals hearts were used for pressure–volume/pressure–strain studies (as described in subsequent section). Following analyses, hearts were trimmed of the right ventricle and a 2 mm ring section within the injury region of infarcted subjects was taken from the middle of the LV. The 2 mm ring was stained for ~5 min in 2,3,5-triphenyl-2H-tetrazolium chloride (TTC; 1.5% w/v in arrest solution) and photographed for determination of infarct size (Fig. 2). Diameters were derived from inner LV circumferences. Infarct and septal wall thickness were also measured from the ring section. Spherical LV volumes were computed from the measured internal LV diameters.

Fluorescence analyses for MMP activity

MMP activity was measured using a fluorogenic substrate amenable to cleavage by multiple MMPs (P126, BIOMOL Research Inc.) as previously described [32]. Three and 7 day tissue samples were homogenized with a hand-held tissue homogenizer in ice-cold buffer (50 mM Tris, 150 mM NaCl, 5 mM CaCl$_2$). Homogenates were centrifuged to remove insoluble particulates and supernatants were isolated. Homogenates samples with 50 μg protein and 10 μM of substrate were mixed in buffer. As in a prior study in our lab, selected samples were supplemented with the MMP inhibitor phenanthroline (1 mM), or the serine protease inhibitor aprotinin (50 kallikrein inhibitory units/ml) to demonstrate that cleavage of substrate was mediated by MMPs (data not shown) [32]. Kinetic fluorescence measurements were performed using a microplate reader (excitation 340 nm/emission 405 nm). Substrate cleavage rates were determined from the linear regions of the kinetic curves.

Myeloperoxidase (MPO) Assays

MPO assays were used as an indicator of tissue inflammation with samples collected 3 and 7 days post-MI as previously described [32]. Approximately 10 mg of tissue was homogenized in ice-cold buffer (50 mM KH$_2$PO$_4$, 0.5% hexadecyltrimethylammonium bromide, pH 6.0). Homogenates were incubated on ice for 30 min, centrifuged in a microfuge at top speed for 5 min at 4° C and the supernatant was removed. Substrate solution (2 mg/ml tetramethylbenzidine dissolved in dimethyl sulfoxide) was diluted 1:10 in reaction buffer (50 mM KH$_2$PO$_4$, pH 6.0, supplemented with 4 μl of 30 % hydrogen peroxide per 10 ml). Homogenates were diluted 1:10 in reaction buffer (100 μl final volume) and placed into a 96-well

VEHICLE　　　**MP**　　　**DOXCYLINE**

Fig. 2 Representative ring sections obtained from rat hearts subjected to a 6 week MI with vehicle, methylprednisolone or doxycyline treatment. Sections were stained using TTC. Viable myocardium is seen as darker halftone, with scar tissue in light. Note that the thickness of the infarcted wall of the DOX heart are preserved compared with either vehicle–treated or MP–treated hearts

microplate. Substrate solution (100 μl) was added to the wells and kinetic absorbance measurements of MPO activity were immediately monitored at 655 nm (readings every 40 s for 20 min). Data were normalized to protein concentrations determined using the BCA Protein Assay Kit (Pierce Inc., Rockford, IL).

Hemodynamics

Six-week subjects were anesthetized with 5% isoflurane and maintained with 1–2% isoflurane. LV and aortic pressures were measured with a catheter–pressure transducer (2 French, 140 cm; Millar Instruments Inc. Houston, TX) introduced via the carotid artery. Pressures and electrical activity were monitored using WINDAQ software (version 2.15, DATAQ Instruments Inc.).

Pressure–volume and pressure-strain studies

Passive mechanics of the LV and mature scar were analyzed in 6 week subjects as previously described [28]. Immediately following hemodynamics measurements, the heart was exposed via medial sternotomy and arrested as described above. The heart was excised, rinsed and the atria removed. The right ventricle was vented via a longitudinal stab through the free wall and the LV was pierced through the apex with a sharp cannula to evacuate residual volume. A purse-string suture was placed around the mitral annulus, a deflated balloon attached to a cannula was inserted into the LV and the suture tightened. The balloon was made by shaping a sheet of plasticized polyvinyl chloride over a cast of the LV cavity. To measure two-dimensional epicardial scar strains with changing LV pressure, a triangle of white titanium dots (about 3–4 mm separation between

dots) was painted on the surface of the LV within the scar. A digital video camera was set up to record the position of the dots during balloon inflation. Water was infused into the balloon to reach zero pressure. Internal balloon pressure and infused volume were simultaneously measured as the balloon was slowly inflated at a rate of ~1 ml/min to a peak pressure of 30 mm Hg. Data were acquired after two to three cycles of inflation and deflation of the balloon. For pressure–strain analyses, video frames at defined pressures were digitized using Scion Image (Scion Corp., Fredrick, MD). Two-dimensional epicardial scar strains were calculated with respect to the cardiac coordinate system yielding circumferential (E_{11}), longitudinal (E_{22}), and in-plane shear (E_{12}) strains as previously described [28]. Upon completion of these studies, hearts were processed for morphometry as described above.

Collagen–area fraction analyses

Trichrome-stained LV cross-sections (10 μM thickness) were imaged on Nikon Optiphot-2 light microscope and digital photographs obtained at $10 \times$ magnification. Images were obtained from the middle of the infarct zone. Images were analyzed using Image J (version 1.36b). Collagen-area fraction was calculated for each LV cross section as previously described [28].

Data and statistical analysis

All data are reported as mean ± standard error of the mean. A minimum of five animals per group were used for statistical comparison. Statistical analyses were performed using a Student's unpaired t-test, one-way or two-way ANOVA and Tukey's post-hoc test, as appropriate. Results were considered statistically significant at $p \le 0.05$.

Results

A total of 162 rats were used in this study. Post-MI mortality was ~34% and no significant differences were noted between groups. At 6 weeks following surgery, animals were catheterized to assess LV contractile function. Results are shown in Table 1. No significant changes were observed in heart rate, LV end-diastolic or systolic pressure, or mean aortic pressure between groups. Fig. 3A and 3B show mean LV pressure–volume (P–V) curves obtained from early- and late-treatment groups and compared to those of shams. Early treatment with MP yielded a right-shifted P–V curve (Fig. 3A) relative to vehicle and DOX treatments ($p < 0.001$). Pressure–volume slopes for early treatment (Fig. 3C) yielded significant differences between DOX and vehicle ($p < 0.005$) and between DOX and MP ($p < 0.002$) at 5 mmHg. DOX values at 5 mm Hg were comparable to those of shams. No significant differences were detected between groups at 20 mm Hg. The interactive effect of treatment and pressure for P–V slopes data was significant ($p = 0.005$). Cross comparisons between early vehicle- and MP-treatment groups at 5 and 20 mm Hg revealed statistical differences ($p < 0.001$). Late treatment with MP yielded a robust rightward shift in P–V curve relative to DOX treatment ($p < 0.001$). Analysis of P–V slopes (Fig. 3D), however, did not yield significant differences between treatments compared at 5 or 20 mm Hg. No significant interaction between treatment and pressure was detected.

Figure 4 shows mean LV pressure scar strains obtained in early- (Fig. 4A,B) and late- (Fig. 4C,D) treatment groups. Irrespective of drug used, with early or late treatment, scar tissue is more compliant in the E_{11} direction (compare E_{11} strains with E_{22} strains). With early treatment, two-way ANOVA revealed significant effects of treatment ($p < 0.001$) on compliance in the E_{11} direction. In particular, DOX treatment resulted in a stiffer scar in the E_{11} direction relative to vehicle ($p = 0.05$). Notable differences in E_{11} were also detected between early DOX- and early MP-treated groups ($p < 0.001$). On average, early treatment E_{22} strains were small for all groups indicating a stiff scar in the longitudinal direction (Fig. 4B). Although late treatment did not yield significant increases in E_{11} strains, a trend was evident with MP treatment suggesting that MP increases scar compliance (Fig. 4C). With late MP treatment, mean E_{11} values exceeded those observed for uninfarcted tissue (i.e., shams). As observed with the early treatment groups, irrespective of treatment, all E_{22} strains were small (i.e., stiff) with late treatments. No interaction between treatment and pressure was detected in any of the analyses. In-plane shear (E_{12}) was small and not different between groups (data not shown).

Upon sacrifice, hearts were excised and histomorphometric values assessed. Fig. 2 illustrates representative cross sections of TTC-stained vehicle, DOX, and MP late (7 day) treatment hearts. As can be observed, whereas DOX treated hearts were smaller versus vehicle, MP treatment tended to yield larger hearts. These data are summarized in Table 2. On the basis of scar size, all groups had comparable infarct sizes ranging from an average of 27–36%. With late DOX treatment, a significant reduction in HW/BW ratios versus vehicle- or MP-treated animals was observed. A significant preservation of infarcted wall thickness was observed in the early and late DOX versus vehicle groups. No differences in septal wall thickness or scar region collagen area fraction were observed between groups. A significant reduction in LV diameter and volume was observed in the late treatment DOX versus MP groups.

Figure 5 illustrates changes observed in MPO activity for early-treatment groups at 3 days (Fig. 5A) and 7 days (Fig. 5B) in the septal non-infarcted (LVS) region and in the infarcted (LVI) wall. The use of DOX did not lead to decreases in MPO activity at 3 or 7 days after MI. In fact, significant increases were observed at the septum at 3 (Fig. 5A) and 7 days (Fig. 5B) and in the infarcted wall at 7 days versus

Table 1 Hemodynamic data of 6 week studies

	Sham	Vehicle MI	MP–E	MP–L	DOX–E	DOX–L
Group size	5	10	6	5	5	5
HR (bpm)	329 ± 9	313 ± 8	312 ± 20	309 ± 15	289 ± 7	286 ± 9
LVSP (mm Hg)	110 ± 4	101 ± 2	101 ± 3	103 ± 3	98 ± 1	100 ± 2
LVEDP (mm Hg)	5 ± 1	7 ± 1	8 ± 1	6 ± 1	8 ± 2	7 ± 1
MAP (mmHg)	96 ± 4	88 ± 3	90 ± 3	93 ± 3	85 ± 1	88 ± 3

MP = methylprednisolone, DOX = doxycycline, E = early, L = late

HR = heart rate, LVSP = left ventricular systolic pressure, LVEDP = left ventricular end diastolic pressure, MAP = mean aortic pressure
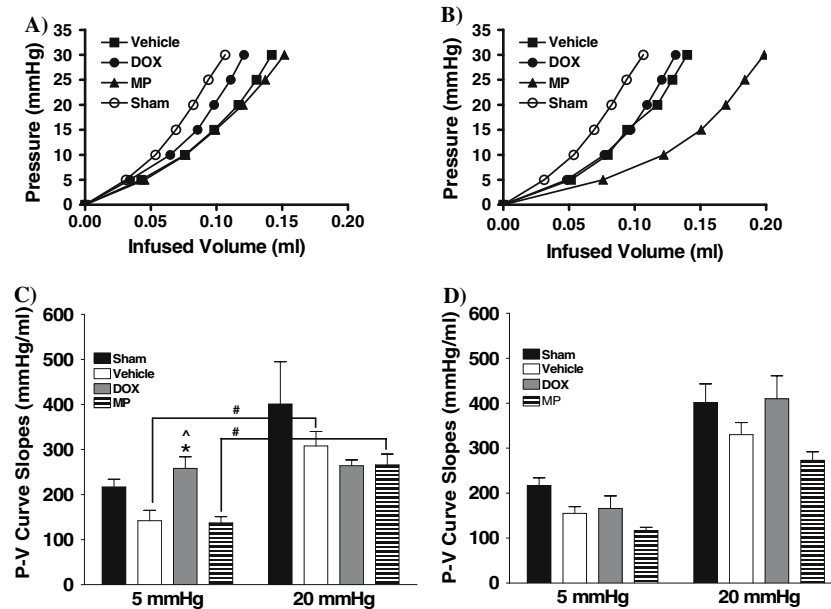
Fig. 3 A (early treatment) and **B (late treatment)**, average passive left ventricular (LV) pressure volume (P–V) relations for sham ($n = 7$; open circles), vehicle infarcted ($n = 5$; squares), doxycycline (DOX) treated infarcted ($n = 7$; closed circles) and methylprednisolone (MP) treated ($n = 5$; triangles) hearts after 6 weeks. (**A**) Early treatment P–V curves: Two-way ANOVA followed by Tukey's test revealed significant differences between MP and vehicle ($p < 0.001$) and MP and DOX ($p < 0.001$). (**B**) Late-treatment P–V curves: Two-way ANOVA followed by Tukey's test revealed significant differences between MP and

DOX ($p < 0.001$). No interactive effect between treatment and pressure detected for early or late P–V curves. **C (early treatment)** and **D (late treatment)**, average ± SE slopes of LV P–V relationships for sham, vehicle, DOX and MP infarcted hearts at 5 and 20 mm Hg. (**C**) Early-treatment P–V slopes: * DOX versus vehicle ($p < 0.005$); ^ DOX versus MP ($p < 0.002$); # ($p < 0.001$). Two-way ANOVA of early treatment P–V slopes revealed an interactive effect between treatment and pressure ($p = 0.005$). (**D**) Late-treatment P–V slopes

Fig. 4 Average two-dimensional circumferential (E_{11}) and longitudinal (E_{22}) LV epicardial strains in the infarcted (scar) area as functions of inflation pressure for early- (**A** and **B**) or late- (**C** and **D**) treatment groups at 6 weeks. Two-way ANOVA followed by Tukey's test indicates that early DOX treatment yields a stiffer scar in the E_{11} direction relative to vehicle ($p = 0.05$). E_{11} strains for early DOX versus MP were statistically different ($p < 0.001$). No interactive effects between treatment and pressure were detected with early or late treatment algorithms
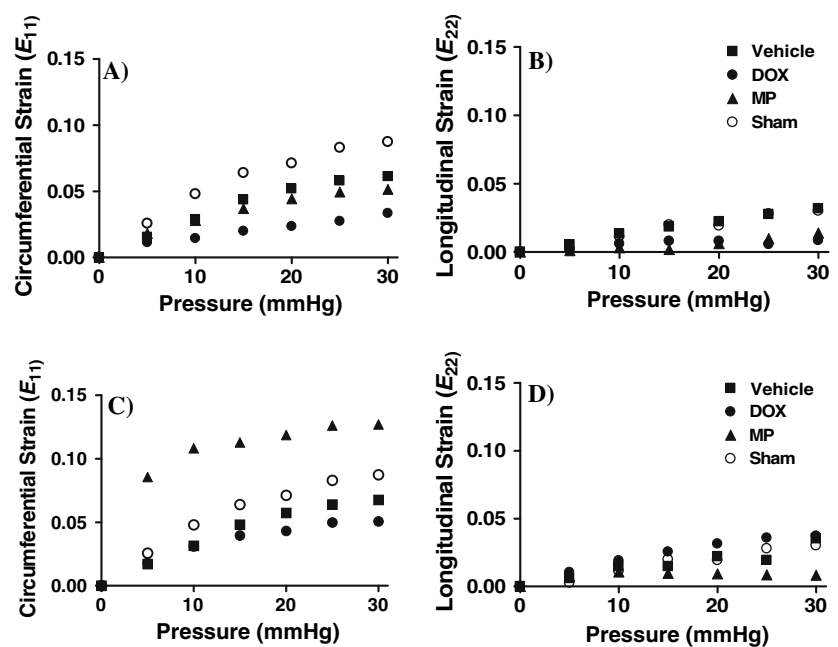
vehicle. Use of MP led to significant decreases in MPO activity versus vehicle in both walls at 3 days and versus DOX at 7 days. Figure 6 shows changes in MPO

activity in the late-treatment groups at 3 days (Fig. 6A) and 7 days (Fig. 6B). Use of DOX led to a modest but significant decrease in infarcted wall MPO

Table 2 Morphometric data of 6 week studies

	Sham	Vehicle MI	MP–E	MP–L	DOX–E	DOX–L
Group size	5	10	6	5	5	5
HW/BW ratio	3.9 ± 0.3	4.7 ± 0.1	5.2 ± 0.4	4.8 ± 0.3	4.1 ± 0.4	4.1 ± 0.1[ab]
Scar size % of LV area	NA	32 ± 2	36 ± 2	27 ± 2	32 ± 5	28 ± 4
IW Thick. (mm)	3.6 ± 0.1	1.4 ± 0.1	1.7 ± 0.1	1.8 ± 0.3	1.8 ± 0.2[a]	2.0 ± 0.2[a]
SW Thick. (mm)	2.9 ± 0.1	3.2 ± 0.1	3.1 ± 0.2	3.2 ± 0.1	3.0 ± 0.3	3.2 ± 0.2
CAF (%)	0.2 ± 0.2	76 ± 1	77 ± 5	71 ± 3	84 ± 4	75 ± 1
LV diam. (mm)	4.6 ± 0.5	5.8 ± 0.3	6.0 ± 0.2	6.8 ± 0.2	6.5 ± 0.4	5.1 ± 0.5[b]
LV Volume (mm^3)	60 ± 22	112 ± 18	116 ± 12	165 ± 12	153 ± 23	78 ± 22[b]

MP = methylprednisolone, DOX = doxycycline, E = early, L = late

CAF = collagen area fraction, HW/BW = heart weight/body weight, IW = infarcted wall, SW = septal wall, LV = left ventricle

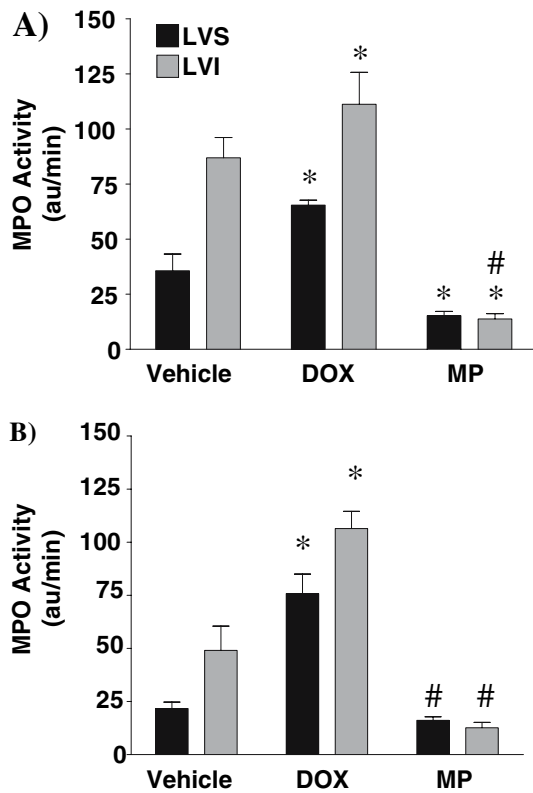[a] $p < 0.05$ versus vehicle, [b] versus methylprednisolone groups

Fig. 5 Early treatment myeloperoxidase (MPO) activity observed in septal and infarcted LV tissue 3 (**A**) or 7 days (**B**) after coronary occlusion in either untreated, MP or DOX–treated MI rats. MPO activity was assessed using a chromogenic substrate. MPO activity from sham animals not detectable under assay conditions. *Significant differences versus corresponding vehicle ($p < 0.01$). #Significant differences between corresponding MP and DOX groups ($p < 0.001$)

Fig. 6 Late treatment myeloperoxidase (MPO) activity observed in septal and infarcted LV tissue 3 (**A**) or 7 days (**B**) after coronary occlusion in either untreated, MP or DOX–treated MI rats. MPO activity was assessed using a chromogenic substrate. MPO activities from sham animals not detectable under assay conditions. *Significant differences versus corresponding vehicle ($p < 0.05$). #Significant differences between corresponding MP and DOX groups ($p < 0.01$)

activity at 3 days versus vehicle (Fig. 6A). MP led to significant decreases in MPO activity in the infarcted walls at 3 days (versus vehicle or DOX) and at 7 days (versus DOX). At 7 days, MPO activity was comparable in DOX treated versus vehicle hearts (Fig. 6B).
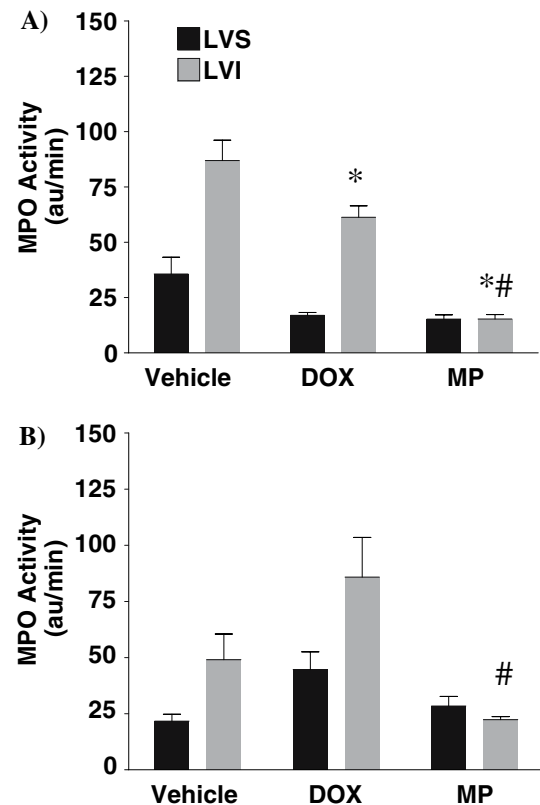
Table 3 shows MMP activity corresponding to early and late treatments at 3 and 7 days post-MI in the septum and infarcted LV wall. On average, in vehicle treated animals, MMP activity in the infarcted LV wall is higher than that observed in the LV septum at 3 and

Table 3 Matrix metalloproteinase (MMP) activity of 3 and 7 day myocardial samples

	3 days post-MI		7 days post-MI	
	LVS	LVI	LVS	LVI
Vehicle	78 ± 11	192 ± 21	76 ± 11	167 ± 27
MP-E	154 ± 21[ab]	165 ± 22	176 ± 14[ab]	209 ± 13 [ab]
MP-L	132 ± 46	193 ± 75	178 ± 12[ab]	220 ± 9[c]
DOX-E	79 ± 9	150 ± 18[c]	102 ± 12	135 ± 10
DOX-L	85 ± [a]10	116 ± 33	127 ± 7[a]	211 ± 35[c]

Vehicle: $n = 11$. DOX–E and DOX–L: $n = 5$. MP–E and MP–L 3 day group: $n = 6$. MP–E and MP–L 7 day group: $n = 5$.
MP = methylprednisolone, DOX = doxycycline, E = early, L = late

Data are reported as relative fluorescence units/min

[a] $p < 0.05$ versus vehicle, [b] $p < 0.05$ versus DOX, [c] $p < 0.05$ septum versus infarct. No differences were noted between early versus late groups

7 days post-MI. The overall increase in MMP activity at the infarcted LV wall versus the septum reflects changes known to occur post-MI at the site of injury. MMP activity with MP early treatment, revealed significant increases versus vehicle at 3 and 7 days at the septum and at 7 days in the infarcted wall. Significant increases with late MP treatment were noted at the septum 7 days after MI. With the exception of DOX-treated septal tissue taken 7 days post-MI, which showed higher MMP activity than the corresponding vehicle–treated septal tissue, early or late DOX MMP activities in the infarct and septal regions were comparable to vehicle. In addition, significant differences in MP versus DOX were observed in the septum 7 days post-MI. With same drug treatments, no significant differences were detected between early and late groups.

Discussion

The major objectives of this study were to examine the effects of early and late DOX and MP treatment on post-MI global LV and scar region structure/function, as well as inflammation and MMP activity. Results indicate that late MP treatment induces a right shift in the LV pressure–volume curve accompanied by increased scar strains. Suppression of inflammation and enhancement of MMP activity accompanied these observations. These results contrast with those of DOX, in particular, late treatment where improved outcomes are observed in LV structure. Furthermore, this study makes the unique observation that DOX, a drug with known pleiotropic properties, failed to suppress inflammation in this model.

Left ventricular function and scar biomechanics were assessed at six weeks, a point beyond the normal time required for post-MI wound healing and scar

maturation in the rat heart [30]. Hemodynamics measurements were used to identify possible changes in contractile function. Results in Table 1 indicate that irrespective of the timing of treatment, no significant differences in LV contractile function were noted versus vehicle. DOX hemodynamics results are comparable to data obtained in a previous report where animals were pre-treated with DOX [28]. Hemodynamics results derived from all groups were not unexpected given the moderate MI size obtained in all groups (~30%). Nonetheless, results observed in pressure–volume and pressure–strain curves suggest that further evolution of late MP treated hearts could eventually translate into loss of contractile function.

Pressure–volume curves derived for all post-MI groups, irrespective of treatment, yielded a right shift in P–V curve versus shams. Early MP yielded modest right-shifted P–V curves relative to vehicle and DOX treatments. Interestingly, with early DOX treatment, the average P–V curve slope at 5 mm Hg was comparable to that of shams, implying that DOX-treatment preserves passive structure/function post-MI. Thus, early, short-term treatment of post-MI rats with MP leads to modest changes in global passive LV function. By contrast, late MP treatment resulted in more extensive right-shifted P–V curves. These data are indicative of worsened post-MI LV remodeling with MP treatment and agree with previously published results on MP dosing in rats [7]. Late DOX treatment did not yield significant changes in global LV passive function relative to vehicle treatment, but did improve outcome relative to MP treatment. These results contrast with our previous findings derived from the use of DOX pre-treatment given 48 h pre- to 72 h post-MI [28], where significant leftward shifts were observed in P–V curves relative to vehicle treatment, indicating improved outcome. Taken together, these results imply that continuous use of DOX for the first 7 days post-

MI may yield improved outcome at the level of global LV structure/function. This conjecture is worthy of future investigation.

Assessments of scar region mechanics indicate that only late MP treatment adversely compromised local mechanics. Late MP treatment yielded increased circumferential strains with increasing pressure (Fig. 4C). These results suggest that this treatment regimen (i.e., late MP) compromises scar material properties via enhancement of local compliance, thus promoting scar expansion. Indeed, Vivaldi et al. reported that use of MP in rats resulted in reduction of mature collagen crosslinks 4 weeks post-MI, which resulted in changes in the material properties of the scar [33]. Pressure–volume and scar-area strain results derived from late MP treatment are in apparent agreement with experiments performed in small and large animal models and observations in patients where use of steroid treatment yielded adverse outcomes [1, 4, 5, 7, 8]. In a study reported by Hammermann et al., it was demonstrated in dogs that the use of high doses [4 injections @ 50 mg/kg) of MP administered from 15 min–48 h post-MI yielded marked scar thinning associated with the loss of regional function [34]. Interestingly, similar observations regarding the induction of post-MI scar thinning or loss of function were reported with the use of ibuprofen, indomethacin, or more recently with aspirin [6, 35–37]. In a study performed in MI rats it was also suggested that development of infarct expansion in MP-treated animals may be secondary to slippage of myocytes [7]. In spite of a number of reports indicating the apparent positive outcome secondary to the use of glucocorticoids [8–12], our results coupled with those from the publications noted above clearly suggest the use of steroids is undesirable following MI. Interestingly, on average, early, and late DOX treatment resulted in trends suggestive of stiffer scars in the E_{11} direction.

Heart remodeling was also assessed via measurements of LV histomorphometry. MP-treated hearts tended to yield the largest LV volumes (Table 2). This contrasts with late DOX treatment, which yielded preservation of infarcted LV wall thickness and lessened the development of compensatory hypertrophy and chamber enlargement. It has been reported that tetracyclines have the capacity to concentrate at the site of tissue injury including, infarction [38–40]. This unusual feature may allow DOX to achieve local high concentrations at the site of injury that would tend to enhance its therapeutic efficacy. Of interest is the fact that scar-area collagen content was apparently not different between the vehicle, MP or DOX groups. As noted above, the increase in local scar mechanics as observed in the late MP group may be associated with changes in the biochemical composition of collagens and/or changes in noncollagenous constituents of the myocardial extracellular matrix.

MPO is abundantly expressed in neutrophils, and as a consequence, it has been used extensively as a marker of inflammation post-MI [41]. However, MPO is also expressed (albeit in lesser amounts) in macrophages and thus, tissue levels of MPO may also reflect chronic inflammation [42]. We utilized MPO activities at 3 and 7 days post-MI as indicators of late acute (3 days) and early chronic (7 days) phases of inflammation [30]. Results indicate that early MP treatment yields significant suppression of MPO activity versus vehicle at 3 days. The ability of an early single dose of MP to suppress inflammation 3 days after MI agrees with known pharmacologic properties of the drug in exerting prolonged effects [43]. Late MP treatment also yielded suppression of MPO activity at 3 and 7 days, in accordance with the repetitive dosing treatment used. Given that suppression of inflammation is observed at 3 days with early MP treatment, it is reasonable to infer that late treatment effects may be extended beyond the 7 day time point. As noted above, it has been proposed that suppression of inflammation may facilitate development of adverse ventricular remodeling and infarct expansion. Our early and late MP treatment results support this view. In fact, early MP treatment animals were the only group that revealed a trend ($p = 0.09$) towards greater post-operative mortality.

DOX has been reported to attenuate inflammation [17]. The mechanisms that relate to this effect have been linked to inhibition of human lymphocytic proliferation, phospholipase A2 activity, and decreases in nitric oxide synthetase levels. However, no direct evidence for anti-inflammatory actions of DOX has been reported and our MPO results agree with this. Given that DOX has been reported to possess anti-inflammatory properties, we investigated its capacity to modulate post-MI inflammation and compared its effects to those of MP. Overall, early and late DOX did not yield significant suppression of MPO activity. In fact, at some time points with early DOX treatment, MPO levels increased, albeit modestly. Our results are in agreement with those previously reported where use of DOX in animal models of abdominal aortic aneurysms limited further development of the aneurysm, but did not reduce local inflammation [44]. Thus, MPO activity results demonstrate a clear distinction between post-MI effects that can be derived from the use of known potent anti-inflammatory agents such as glucocorticoids and DOX treatment. In this case, it can be

argued that DOX should not be categorized with this general class of agents as it does not suppress inflammation in this model.

Global assessment of MMP activity was performed with the use of a broad-spectrum MMP substrate. Early MP treatment increased MMP activity in the septum and infarct region in 7 days tissue samples, but these effects did not translate into major changes in LV structure/function at 6 weeks (Fig. 3A). Hearts exposed to late MP treatment showed significant enhancement of septal MMP activity at 7 days, and as noted above, resulted in the development of adverse changes in global and scar LV structure/function (Fig. 3C). At 3 days, use of early or late DOX did not yield suppression of MMP activity in the septum relative to vehicle, but on average appeared to diminish it modestly in the infarct zone [28]. Early or late DOX at 7 days yielded values comparable to vehicle. Early DOX treatment failed to demonstrate significant suppression of MMP activity. This result may be due to the rather short-term treatment provided with the compound.

In conclusion, results derived from this study indicate that MP is an effective suppressor of post-MI inflammation, which yields adverse changes in LV structure/function. By contrast, DOX treatment does not suppress inflammation and is effective in ameliorating adverse post-MI ventricular remodeling. Given the proven and wide safety profile of tetracyclines, further investigation of DOX as a pharmacological therapy for pathological post-MI remodeling should be considered.

References

1. Pfeffer MA, Braunwald E (1990) Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. Circulation 81:1161–72

2. Hobbs RE (2004) Management of decompensated heart failure. Am J Ther 11:473–9

3. Nian M, Lee P, Khaper N, Liu P (2004) Inflammatory cytokines and post-myocardial infarction remodeling. Circ Res 94:1543–53

4. Silverman HS, Pfeifer MP (1987) Relation between use of anti-inflammatory agents and left ventricular free wall rupture during acute myocardial infarction. Am J Cardiol 59:363–4

5. Hammerman H, Schoen FJ, Braunwald E, Kloner RA (1984) Drug-induced expansion of infarct: morphologic and functional correlations. Circulation 69:611–7

6. Van Kerckhoven R, Kalkman EA, Saxena PR, Schoemaker RG (2000) Altered cardiac collagen and associated changes in diastolic function of infarcted rat hearts. Cardiovasc Res 46:316–23

7. Mannisi JA, Weisman HF, Bush DE, Dudeck P, Healy B (1987) Steroid administration after myocardial infarction promotes early infarct expansion. A study in the rat. J Clin Invest 79:1431–9

8. LeGal YM, Morrissey LL (1990) Methylprednisolone interventions in myocardial infarction: a controversial subject. Can J Cardiol 6:405–10

9. Libby P, Maroko PR, Bloor CM, Sobel BE, Braunwald E (1973) Reduction of experimental myocardial infarct size by corticosteroid administration. J Clin Invest 52:599–607

10. Spath JA Jr, Lane DL, Lefer AM (1974) Protective action of methylprednisolone on the myocardium during experimental myocardial ischemia in the cat. Circ Res 35:44–51

11. Hafezi-Moghadam A, Simoncini T, Yang Z, Limbourg FP, Plumier JC, Rebsamen MC, Hsieh CM, Chui DS, Thomas KL, Prorock AJ, Laubach VE, Moskowitz MA, French BA, Ley K, Liao JK (2002) Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. Nat Med 8:473–9

12. Valen G, Kawakami T, Tahepold P, Dumitrescu A, Lowbeer C, Vaage J (2000) Glucocorticoid pretreatment protects cardiac function and induces cardiac heat shock protein 72. Am J Physiol Heart Circ Physiol 279:H836–43

13. Frangogiannis NG, Smith CW, Entman ML (2002) The inflammatory response in myocardial infarction. Cardiovasc Res 53:31–47

14. Entman ML, Youker KA, Frangogiannis N, Lakshminarayanan V, Nossuli T, Evans A, Kurrelmeyer K, Mann DL, Smith CW (2000) Is inflammation good for the ischemic heart–perspectives beyond the ordinary. Z Kardiol 89 Suppl 9:IX/82–7

15. Jugdutt BI (2003) Ventricular remodeling after infarction and the extracellular collagen matrix: when is enough enough? Circulation 108:1395–403

16. Dovi JV, He LK, DiPietro LA (2003) Accelerated wound closure in neutrophil-depleted mice. J Leukoc Biol 73:448–55

17. Sapadin AN, Fleischmajer R (2006) Tetracyclines: nonantibiotic properties and their clinical implications. J Am Acad Dermatol 54:258–65

18. Golub LM, Sorsa T, Lee HM, Ciancio S, Sorbi D, Ramamurthy NS, Gruber B, Salo T, Konttinen YT (1995) Doxycycline inhibits neutrophil (PMN)-type matrix metalloproteinases in human adult periodontitis gingiva. J Clin Periodontol 22:100–9

19. Amin AR, Attur MG, Thakker GD, Patel PD, Vyas PR, Patel RN, Patel IR, Abramson SB (1996) A novel mechanism of action of tetracyclines: effects on nitric oxide synthases. Proc Natl Acad Sci U S A 93:14014–9

20. Hoyt JC, Ballering J, Numanami H, Hayden JM, Robbins RA (2006) Doxycycline modulates nitric oxide production in murine lung epithelial cells. J Immunol 176:567–72

21. Schmidt HH, Walter U (1994) NO at work. Cell 78:919–25

22. Robbins RA, Hadeli K, Nelson D, Sato E, Hoyt JC (2000) Nitric oxide, peroxynitrite, and lower respiratory tract inflammation. Immunopharmacology 48:217–21

23. Cross RK, Wilson KT (2003) Nitric oxide in inflammatory bowel disease. Inflamm Bowel Dis 9:179–89

24. Scarabelli TM, Knight R, Stephanou A, Townsend P, Chen-Scarabelli C, Lawrence K, Gottlieb R, Latchman D, Narula J (2006) Clinical implications of apoptosis in ischemic myocardium. Curr Probl Cardiol 31:181–264

25. Scarabelli TM, Stephanou A, Pasini E, Gitti G, Townsend P, Lawrence K, Chen-Scarabelli C, Saravolatz L, Latchman D, Knight R, Gardin J (2004) Minocycline inhibits caspase

activation and reactivation, increases the ratio of XIAP to smac/DIABLO, and reduces the mitochondrial leakage of cytochrome C and smac/DIABLO. J Am Coll Cardiol 43:865–74

26. Cheung PY, Sawicki G, Wozniak M, Wang W, Radomski MW, Schulz R (2000) Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. Circulation 101:1833–9

27. Sawicki G, Leon H, Sawicka J, Sariahmetoglu M, Schulze CJ, Scott PG, Szczesna-Cordary D, Schulz R (2005) Degradation of myosin light chain in isolated rat hearts subjected to ischemia-reperfusion injury: a new intracellular target for matrix metalloproteinase-2. Circulation 112:544–52

28. Villarreal FJ, Griffin M, Omens J, Dillmann W, Nguyen J, Covell J (2003) Early short-term treatment with doxycycline modulates post-infarction left ventricular remodeling. Circulation 108:1487–92

29. Garcia RA, Pantazatos DP, Gessner CR, Go KV, Woods VL Jr, Villarreal FJ (2005) Molecular interactions between matrilysin and the matrix metalloproteinase inhibitor doxycycline investigated by deuterium exchange mass spectrometry. Mol Pharmacol 67:1128–36

30. Fishbein MC, Maclean D, Maroko PR (1978) Experimental myocardial infarction in the rat: qualitative and quantitative changes during pathologic evolution. Am J Pathol 90:57–70

31. Villarreal F, Omens J, Dillmann W, Risteli J, Nguyen J, Covell J (2004) Early degradation and serum appearance of type I collagen fragments after myocardial infarction. J Mol Cell Cardiol 36:597–601

32. Garcia RA, Brown KL, Pavelec RS, Go KV, Covell JW, Villarreal FJ (2005) Abnormal cardiac wall motion and early matrix metalloproteinase activity. Am J Physiol Heart Circ Physiol 288:H1080–7

33. Vivaldi MT, Eyre DR, Kloner RA, Schoen FJ (1987) Effects of methylprednisolone on collagen biosynthesis in healing acute myocardial infarction. Am J Cardiol 60:424–5

34. Hammerman H, Kloner RA, Hale S, Schoen FJ, Braunwald E (1983) Dose-dependent effects of short-term methylpred-nisolone on myocardial infarct extent, scar formation, and ventricular function. Circulation 68:446–52

35. Hammerman H, Kloner RA, Schoen FJ, Brown EJ Jr, Hale S, Braunwald E (1983) Indomethacin-induced scar thinning after experimental myocardial infarction. Circulation 67:1290–5

36. Brown EJ Jr, Kloner RA, Schoen FJ, Hammerman H, Hale S, Braunwald E (1983) Scar thinning due to ibuprofen administration after experimental myocardial infarction. Am J Cardiol 51:877–83

37. Hammerman H, Kloner RA, Schoen FJ, Brown EJ Jr, Hale SL, Braunwald E (1982) The effects of early indomethacin administration on late scar formation following myocardial infarction. Trans Assoc Am Physicians 95:104–9

38. Lichey J, Charissis G, Braunsdorf M, Franke J, Hahn W (1979) Accumulation of 99mTc-tetracycline in lung infarction area. An experimental study. Respiration 37:220–3

39. Ryan CF, Athari-Nejad A, Holcslaw TL (1979) Quantitation of isoproterenol-induced myocardial necrosis with 3H-tetracycline. Res Commun Chem Pathol Pharmacol 25:489–501

40. Modrak JB, Rovang KS (1981) Estimation of infarct size by determination of myocardial 3H-tetracycline accumulation in the coronary ligated rat. Res Commun Chem Pathol Pharmacol 34:149–52

41. Griffin MO, Jinno M, Miles LA, Villarreal FJ (2005) Reduction of myocardial infarct size by doxycycline: a role for plasmin inhibition. Mol Cell Biochem 270:1–11

42. Daugherty A, Dunn JL, Rateri DL, Heinecke JW (1994) Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. J Clin Invest 94:437–44

43. Jackson RT, Waitzman MB (1967) The long-term effects of a single dose of methyl prednisolone on 35S uptake in ocular and nasal tissue. Biochem Pharmacol 16:1115–7

44. Franklin IJ, Harley SL, Greenhalgh RM, Powell JT (1999) Uptake of tetracycline by aortic aneurysm wall and its effect on inflammation and proteolysis. Br J Surg 86:771–5