WORSENING OF LONG-TERM MYOCARDIAL FUNCTION AFTER SUCCESSFUL PHARMACOLOGICAL PRETREATMENT WITH CYCLOSPORINE

Pretreatment with cyclosporine (CsA) decreases infarct size 24h after myocardial ischemia/reperfusion (I/R). The goal of this study was to determine effects of CsA pretreatment on long-term cardiac function after I/R-injury. Rats were randomly assigned to group1: vehicle-only, group2: CsA-5mg/kg/day, and group3: CsA-12.5mg/kg/day given orally for three days prior to I/R-injury (30 min of left anterior descending coronary artery occlusion). Post-I/R survival and cardiac function were evaluated 14 days after I/R-injury by echocardiography and invasive hemodynamic measurements. Rats with I/R-injury showed increased left ventricular pressure (LVEDP) compared to rats without I/R-injury (p<0.005). Although CsA initially decreased infarct size, no differences of LVEDP were seen 14 days after I/R-injury (vehicle: 21.2±8.9 mmHg, CsA-5mg/kg/day: 21.5±0.7 mmHg, CsA-12.5mg/kg/day: 20.5±9.4 mmHg). Ejection fraction and fractional shortening were decreased compared to baseline, but showed no differences between groups. On day 14, a dose-dependent increase in left ventricular end diastolic diameter was seen (p<0.001). CsA pretreatment was associated with a dose-dependent decrease in post-I/R-survival (vehicle: 56%, CsA-5mg/kg/day: 32%, CsA-12.5mg/kg/day: 16%; p=0.017). CsA pretreatment did not improve long-term cardiac function despite decreased infarct size 24h after I/R-injury, but increased post-I/R mortality significantly. Poor cardiac function after CsA pretreatment might be caused by left ventricular dilation.

Keywords: myocardial infarction, ischemia/reperfusion, cyclosporine, preconditioning, outcome
INTRODUCTION

Ischemic cardiac preconditioning or pharmacological pretreatment have been widely studied as methods of cardioprotection against ischemia/reperfusion (I/R) injury. The effects of short episodes of ischemia (i.e. ischemic preconditioning) were first described by Murry et al. in 1986: brief ischemic events render the heart more resistant against a subsequent and prolonged ischemic period followed by reperfusion using infarct size as primary end point (1). These effects have been (partially) explained by indirect downstream inhibition of the irreversible opening of the mitochondrial permeability transition pore (MPT) (2). Accordingly direct inhibition of the opening of the MPT shows protective effects against I/R-injury (3,4).

The forming or opening of the MPT is caused by binding of cyclophilin D to a membrane component of the adenine nucleotide translocase in the inner mitochondrial membrane (5). The immunosuppressant cyclosporine (CsA) binds to cyclophilin D and thereby prevents the forming of the MPT (5,6). CsA has protective effects against I/R-injury in a wide variety of organs: spinal cord (7), kidney (8,9), liver (10-13), gut (14,15), lung (16), skeletal muscle (17), and heart muscle cells (18,19). However, with respect to the myocardium, studies have mainly focused on isolated myocytes (20) and on isolated perfused heart models (3,21). In vivo cardioprotective effects have been demonstrated only during the first few hours after reperfusion (22,23), and were measured using infarct size and biochemical injury markers: in in vivo models it has been reported that pretreatment with CsA diminished myocardial injury (measured by biochemical injury markers) and improves left ventricular function 5 hours after reperfusion; (23) in addition, it has been shown by our collaborators (22) and others (23) that pretreatment with CsA reduced infarct size 24 h after reperfusion.

In 2004, the National Heart, Lung, and Blood Institute (NHLBI) Working Group on the Translation of Therapies for Protecting the Heart from Ischemia declared that “over the past 30 years, several hundred experimental interventions have been claimed to limit myocardial infarct size in experimental animals. Unfortunately, few of these results have been reproducible and, with the exception of timely reperfusion, non has been translated into clinical practice.” (24). The NHLBI saw one major barrier for this failure in the limited survival time after reperfusion (24). Furthermore, the use of ‘secondary end-points such as limitation of infarct size are not sufficient’ to evaluate relevant outcome after I/R-injury (24).

It is not known if pharmacological pretreatment by CsA induces cardioprotective effects that last longer than 24 h and may influence intermediate to long-term consequences of myocardial I/R-injury. The goal of our study was to determine the effects of CsA pretreatment on long-term outcome after myocardial I/R-injury. To determine these long-term effects of CsA we used global parameters of cardiac function to evaluate if the previously demonstrated reduction of infarct size (22) translates into long-term functional improvement.
Since the NHLBI focuses on reproducibility of experimental results (24), we tested our setting to assess if it was comparable to former studies. We started with a small pilot-study to confirm previously reported short-term data. To our knowledge, there is only one study which analyzed functional long-term outcome after ischemic preconditioning in rabbits (25). Cardiac function was evaluated 21 days after I/R-injury using measurements of segment shortening in the ischemic area; no global parameters of cardiac function such as ejection fraction (EF) or left ventricular end-diastolic pressure (LVEDP) were used. Another study using ischemic preconditioning analyzed infarct size 7 days after I/R-injury without testing any global parameters of cardiac function (blood pressure and heart rate were obtained not beyond 15 min after reperfusion) (26).

The goal of our study was to follow the NHLBI postulations on experimental cardioprotective interventions and subsequently, to propose translation into clinical practice if these effects appear to be beneficial.

**MATERIAL AND METHODS**

Animals and treatment: All animal protocols were approved by Committee on Animal Research, and animal care was in agreement with the NIH guidelines for ethical animal research. Male Sprague-Dawley rats (age: 8-10 weeks, body weight: 280-320 g) were randomly assigned to 3 treatment groups: 1) control (vehicle only), 2) CsA, 5.0 mg/kg/day, and 3) CsA, 12.5 mg/kg/day. CsA was given by oral gavage (Neoral®, Novartis) diluted in skim milk. CsA or vehicle were administered for three days prior to I/R-injury.

*Pilot experiments:* To assure that we could reproduce the short-term reduction of infarct size, which was reported in the literature (21-23,27), we conducted a small pilot study measuring infarct size and CsA concentrations 24 hours after I/R-injury. We did not expect new data. Therefore group sizes were chosen as small as possible (n=4).

*Experimental protocols:* In subsequent experiments rats were randomly assigned to three groups. On day four, 4 hours after the last CsA dose, I/R-injury was induced. After I/R-injury, rats were observed for 24 hours (pilot experiments) or 14 days (main study). On day 14, myocardial function was measured by transthoracic echocardiography and invasive hemodynamic measurements.

*Induction of I/R-injury:* Rats were anesthetized with ketamine (80mg/kg i.p.) and xylazine (16mg/kg i.p.), endotracheally intubated, and mechanically ventilated (tidal volume 10-15ml/kg, FiO₂ 1.0) on a heated operating table. A blood sample (1 ml) was frozen in liquid nitrogen and stored at -80°C for measurement of CsA concentrations. The heart was exposed through the fourth left intercostal space, and the left anterior descending coronary artery (LAD) was occluded 1 mm below the left atrial appendage with a 4-0 silk suture. After 30 minutes, the ligation was released, and the chest closed. After reinstallation of spontaneous respiration, animals were extubated and allowed to recover.

*Areas of Risk and Infarction:* In the pilot-study, 24 hours after I/R-injury, area of risk (AoR) was stained using Evans blue (26,28,29). 50-100 mg tissue of the right ventricular free wall was removed, frozen in liquid nitrogen, and stored at -80°C for measurement of myocardial CsA concentrations. Area of infarction (AoI) was stained using TTC (30-32). AoR and AoI were measured using Adobe Photoshop 6.0® software.
Measurements of CsA concentrations: Heart tissue samples were weighed and homogenized in 0.5 ml of KH$_2$PO$_4$ (pH 7.4). For protein precipitation, 1 ml of methanol/1M ZnSO$_4$ (80/20 v/v) containing 100 µg/ml internal standard cyclosporine D was added to 0.25 ml of blood or heart tissue homogenate. After vortexing for 30 s and centrifugation (10,000 g for 10 min) 100 µl of the supernatant was used for HPLC analysis (22). For quantification of CsA, an established and fully validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay was used (33).

Echocardiography: Transthoracic echocardiography was performed using a 10 MHz ultrasound probe with a Vivid Five System (EchoPac 6.3.6 software, General Electrics). Ejection fraction, shortening fraction, left ventricular end-diastolic diameter, and cardiac output were obtained in standardized fashion (34-37). All echocardiographic measurements were performed in triplicate by one investigator and averaged.

Invasive left ventricular function assessment: For left ventricular hemodynamic measurements, a microtip transducer (1.4 Fr., Millar Instruments) was inserted into the right carotid artery and advanced into the left ventricle. Systolic and diastolic pressures were recorded at closed chest (Hemodyn v1.1 software, Hugo Sachs). Untreated rats were used as negative controls.

Statistics: Values are expressed as mean ± standard deviation unless otherwise indicated. For survival analysis a Kaplan-Meier algorithm, for differences within groups paired t-tests and for differences between groups One-Way ANOVA were performed using SPSS 13.0 (SPSS Inc., Chicago, IL).

RESULTS

Only animals with a peripheral oxygen saturation above 90% while spontaneously breathing room air after recovery from anesthesia and after extubation were included in the final analysis. Intraoperative mortality was 26.2% with no differences between groups (comparable to data reported in the literature (38,39)). Animals that did not qualify for follow-up were replaced. Due to the high mortality in the CsA groups, group sizes differed between groups to allow a sufficient group size to perform a functional analysis at day 14 after I/R-injury (Fig. 6).

Pilot-study results

A dose dependent increase of blood CsA concentrations (CsA 5mg/kg/day: 247.2±208.0 ng/ml; CsA 12.5 mg/kg/day: 428.9±416.4 ng/ml) and myocardial tissue CsA concentrations (CsA 5mg/kg/day: 0.49 ± 0.16 ng/mg; CsA 12.5 mg/kg/day: 4.48 ± 2.13 ng/mg) was seen (n=4 samples per group). In the group of rats receiving 12.5 mg/kg/day CsA a reduction of infarct size was observed. In the group of rats receiving 5.0 mg/kg/day CsA almost no reduction of infarct size was present (Fig. 1).

Main study results

Left ventricular end diastolic pressure (LVEDP): Compared to rats without I/R-injury (LVEDP 5.2 ± 1.5 mmHg), vehicle treated rats after I/R-injury had a significantly increased LVEDP (21.2 ± 8.9 mmHg, p=0.001). CsA treated rats
also had a significantly increased LVEDP compared to rats without I/R-injury (CsA 5.0 mg/kg/day: LVEDP = 21.5±0.7 mmHg, p<0.001; CsA 12.5 mg/kg/day: LVEDP = 20.5±9.4 mmHg, p=0.004). Between CsA pretreated rats and vehicle treated rats, no significant differences in LVEDP were observed 14 days after I/R-injury (Fig. 2). Therefore, cyclosporine pretreatment did not ameliorate the increase of LVEDP 14 days after I/R-injury.

**Ejection fraction (EF):** EF after CsA pretreatment, but before induction of I/R-injury was not significantly different between groups and ranged between 68% and 77%. Fourteen days after I/R-injury, EF was significantly decreased in each group (55.0±7.3% [p=0.006], 45.5±8.1% [p=0.009], and 54.0±11.3% [p=0.048] compared to baseline in rats receiving vehicle, CsA 5 mg/kg/day, and CsA 12.5 mg/kg/day, respectively). EF after 14 days was not significantly different between groups (Fig. 3). In summary, CsA pretreatment did not improve EF 14 days after I/R-injury.

**Fractional Shortening (FS):** FS after pretreatment with CsA, but before induction of I/R-injury ranged between 44% and 52%. There were no significant differences between the treatment groups. Fourteen days after induction of I/R-injury was decreased in all groups (range of 28% to 33%), but there were no significant differences between groups. Compared to baseline before I/R-injury, the decrease of FS was only significantly different in the vehicle treated group.
**Fig. 2:** Left Ventricular End-Diastolic Pressure (LVEDP) in rats 14 days after I/R-injury. Negative control animals had no I/R-injury; vehicle treated animals had I/R-injury without CsA treatment. CsA treated rats received drug orally prior to I/R-injury. Data is presented as mean ± SD (n= 6; CsA 12.5 mg/kg/day: n=4); *: p<0.005.

**Fig. 3:** Ejection Fraction in rats before and 14 days after I/R-injury. Vehicle treated animals received no CsA prior to I/R-injury. CsA treated rats received drug orally prior to I/R-injury. Data is presented as mean ± SD (n= 6; CsA 12.5 mg/kg/day: n=4, BL: baseline, 14d: day 14); *: p<0.05.
(p=0.014, Fig. 4), but CsA pretreatment did not improve fractional shortening in any group 14 days after I/R-injury.

**Left ventricular end-diastolic diameter (LVEDD):** LVEDD was 7.28 ± 0.39 mm in animals treated with vehicle, 6.90 ± 0.39 mm in animals treated with 5 mg/kg/day CsA, and 6.98±0.44 mm in animals treated with 12.5 mg/kg/day CsA immediately before I/R-injury (not significant). Fourteen days after I/R-injury, LVEDD was 7.28 ± 0.10 mm, 8.11 ± 0.49 mm, and 9.55 ± 0.73 mm in animals treated with vehicle, CsA 5 mg/kg/day, and CsA 12.5 mg/kg/day, respectively (Fig. 5). In vehicle-treated animals LVEDD was constant before and 14 days after induction of I/R-injury. In contrast, CsA treated rats had a highly significant dose-dependent increase in LVEDD compared to vehicle-treated animals (p<0.001).

**Cardiac output:** Cardiac output and heart rate were not different between and within groups before and 14 days after induction of I/R-injury.

**Survival:** CsA pretreatment was associated with a statistically significant, dose dependent decrease in the post- I/R survival; in the vehicle group survival was 56%, 32% in rats which received 5 mg/kg/day CsA and 16% in rats which received 12.5 mg/kg/day 14 days after I/R-injury (p=0.017, Fig. 6). Ninety percent of all deaths occurred within the first 24 hours after I/R-injury. 24 hour

![Fractional Shortening](image.png)

**Fig. 4:** Fractional Shortening in rats before and 14 days after I/R-injury. Vehicle treated animals received no CsA prior to I/R-injury. CsA treated rats received drug orally prior to I/R-injury. Data is presented as mean ± SD (n= 6; CsA 12.5 mg/kg/day: n=4, BL: baseline, 14d: day 14); *: p<0.05.
Fig. 5: Left ventricular end-diastolic pressure in rats before and 14 days after I/R-injury. Vehicle treated animals received no CsA prior to I/R-injury. CsA treated rats received drug orally for 3 days prior to I/R-injury. Data is presented as mean ± SD (n= 6; CsA 12.5 mg/kg/day: n=4, VT: vehicle treated; BL: baseline, 14d: day 14); *: p=0.014; **: p=0.004; #: p<0.001.

Fig. 6: Survival analysis over the course of 14 days after I/R-injury. Vehicle treated animals received no CsA prior to I/R-injury. CsA treated rats received drug orally prior to I/R-injury; n: animals per group; s: surviving animals per group; *: p=0.017.
survival rate in the vehicle group was 72%, 32% in rats pretreated with 5 mg/kg/day CsA, and 20% in rats pretreated with 12.5 mg/kg/day CsA (p=0.002).

**DISCUSSION**

The main result of our study is that CsA pretreatment before myocardial I/R-injury did not improve cardiac function 14 days after I/R-injury despite a decreased early infarct size. That cardiac function 14 days after I/R-injury was not improved appears to be related to a left ventricular (post-infarct) dilatation. Additionally, CsA pretreatment increased post-I/R mortality. Therefore, it is questionable if functional outcome after myocardial I/R-injury depends on infarct size (40,41). Similar to our pilot study, CsA, when administered before the onset of ischemia (4,22,42,43) or during ischemia (21), decreased the size of infarcted myocardium afterwards.

Based on the literature reporting that an oral CsA dose between 10 and 15 mg/kg/day administered for three days prior to I/R-injury in rats causes an optimal reduction of infarct size (22), we used 12.5 mg/kg/day CsA. Lower as well as higher doses of CsA were reported to be less effective or protective (18). Since bioavailability of orally administered CsA is highly variable, we measured blood and myocardial tissue levels to document the degree of systemic availability. Measured CsA concentrations in blood and myocardial tissue were similar to previous reports (44-46). The short-term effects of our pilot experiments correlated well with the known data from the literature and the previous results of our collaborators, i.e. reduction of infarct size 24 hours after I/R-injury through CsA pretreatment (4,22,42,43). Our study did not yield repeating experiments addressing the mechanisms causing a reduction of infarct size (22).

The main question of our study was to assess whether pretreatment with CsA and its effects on infarct size translate into significant improvement of later myocardial function and outcome or not (24,25). Functional outcome after infarct seems to depend to a great extent on ventricular remodeling, i.e. alterations in the ventricular architecture (47). Generalized cardiac dilatation, measured by LVEDD, is one form of ventricular remodeling after infarct or I/R injury, leading to increased wall stress. The increased wall stress serves as an stimulus for reactive hypertrophy of the viable myocardium (47). However, this reactive hypertrophy may be inadequate to fully compensate for the degree of myocardial lost (47,48).

We observed a significant dose-depending increase of LVEDD after CsA pretreatment which may be a sign of detrimental remodeling. This cardiac dilatation may very well correlate to poor cardiac function (47).

One factor which can worsen post-ischemic cardiac dilatation may be an increased ventricular afterload. It is known that CsA can cause systemic hypertensive effects, however, Sprague-Dawley rats usually do not develop arterial hypertension in response to CsA (49,50). Short-term acute CsA-mediated
hypertension can not be completely excluded since CsA activates sympathetic vasoconstriction lasting hours after a single CsA dose (49). Increased cardiac afterload induced by CsA may be responsible for an increased LVEDD with deleterious effects, but whether these are sufficient to result in myocardial remodeling is questionable (47).

An additional factor of poor functional outcome after infarct is insufficient reactive hypertrophy of the non-infarcted myocardium (48). Myocardial hypertrophy can be mediated by calcineurin and inhibited by CsA (51). Decreased global myocardial function may be mediated through calcineurin inhibition by CsA and subsequently inadequate reactive myocardial hypertrophy (39,52,53). However, inhibition of reactive cardiac hypertrophy and its consequences on functional outcome have been reported to be time-dependent. To initiate CsA treatment before and to continue it after infarct appears to be beneficial with respect to long-term outcome, whereas the start of CsA treatment after infarct seems to be deleterious, but both administration sequences and their functional effects as well as the outcome observed appear to be mediated by calcineurin inhibition (39,52,54). In our model, pretreatment with CsA (without continuation after reperfusion) caused deleterious effects with respect to survival, myocardial remodeling and ventricular dilatation. Given the pharmacokinetic data on CsA in rats (44-46) and the measured CsA levels 24 hours after I/R-injury, calcineurin-inhibiting levels of CsA are expected to be present for a maximum of 36 hours after ischemia. Since we observed a dose-dependent increase in LVEDD and a poor cardiac function in all treatment groups, calcineurin inhibition at the time of I/R-injury may inhibit the early stimulus for reactive hypertrophy, which therefore may be considered to be one critical step for functional outcome (48). However, we would like to point out that most data of previous reports were derived from models of ischemia without reperfusion. Comparisons to data obtained from I/R-injury models like ours should be done with caution since calcineurin activity seems to be highly sensitive to oxidation and may interfere with the oxidative burst during reperfusion (55,56).

In addition to the ventricular dilatation, we observed an increase of mortality from 44% to 84% (Fig. 6) after CsA pretreatment and I/R-injury, with 90% of all deaths occurring within the first 24 hours after I/R-injury. Death after I/R-injury usually is caused by malignant arrhythmias or ruptured ventricular aneurysms (60). However, since ventricular aneurysms develop over a longer time period, and 90% of the deaths in our study occurred during the first 24 hours after reperfusion, it seems very likely that most of the animals died from arrhythmias. This assumption is supported by Michael et al (60) who reported increased rates of arrhythmias after I/R-injury in comparison to permanent occlusions of the LAD in mice. Since we observed a CsA dose-dependent increase in early post-I/R death, it is possible that CsA has pro-arrhythmogenic properties post-I/R even though it may decrease arrhythmias at the time of reperfusion (61). Additionally, CsA pretreatment might decrease cardiac energy metabolism (22) and therefore may
render the heart more susceptible to arrhythmias. The ratio of stunned to infarcted myocardium is larger in CsA pretreated hearts and one may hypothesize that inhomogenities in electrophysiological properties of these ischemically injured, but still viable cells may act pro-arrhythmogenic (62-64).

On major limitation of our study is that the early high mortality associated with CsA pretreatment allowed us only long-term functional examination of surviving animals but no physiological assessment of early death.

In summary, pretreatment with CsA did not improve myocardial function 14 days after I/R-injury despite a decreased infarct size 24 hours after I/R-injury. CsA increased the risk of early death during the post-I/R phase. The 24 hour infarct size seems not to be related to long term cardioprotection or improved cardiac function. Outcome seems to depend on complex post-injury mechanisms and pretreatment can interfere with these mechanisms. Therefore, long-term studies of pretreatment strategies are necessary to evaluate clinical relevant pretreatment strategies.

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