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Myocardial protection by ischemic conditioning is already evident during ischemia: analysis of ST-segment elevation during ischemia/reperfusion in pigs

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1 Introduction

1.1 Myocardial ischemia – reperfusion

Myocardial infarction is defined as myocardial cell death due to sustained myocardial ischemia and subsequent reperfusion injury and in patients with acute myocardial infarction it is a major determinant of prognosis (Heusch & Gersh, 2017; Stone et al., 2016). The determinants of infarct size are the amount of ischemic myocardium, i.e. the area at risk, duration of ischemia, residual blood flow in the area at risk during ischemia, and to a smaller extent systemic hemodynamics. Temporal and spatial development of infarction largely depends on the interaction of these determinants and is species-dependent (Heusch, 2013). In humans, myocardial infarction begins to develop after 30 min, covers about 50% of the area at risk after 5 hours ischemia, and reaches its final extent presumably after more than 16 hours (Hedström et al., 2009). Thus, the primary therapeutic strategy for patients with acute myocardial infarction is a timely reperfusion which is the only way to stop progression of ischemic injury. In patients this was initially accomplished by pharmacological thrombolysis and more recently by percutaneous coronary intervention.

However, reperfusion can also induce irreversible damage to the myocardium, termed as reperfusion injury (Braunwald & Kloner, 1985; Heusch, 2013; Piper et al., 1998). Mechanisms contributing to reperfusion injury are the increase in intracellular calcium during ischemia which results in uncoordinated excess contractile activity upon reperfusion and leads to sarcolemmal disruption (Piper et al., 2003). With reperfusion, intracellular pH is rapidly restored by washout of catabolites of anaerobic metabolism and this rapid normalization of the intracellular pH induces further cellular damage. Experimental studies have shown that maintaining intracellular acidosis during early reperfusion is beneficial for the salvage of myocardial cells (Bond et al., 1991; Inserte et al., 2011). Reperfusion also leads to excess formation of reactive oxygen species which promotes additional cellular

injury (Schlüter et al., 1996; Zweier, 1988). All these events facilitate the opening of the mitochondrial permeability transition pore, a non-specific pore in the inner mitochondrial membrane (Bernardi & Di Lisa, 2015; Griffiths & Halestrap, 1995; Kim et al., 2006) resulting in the release of pro-apoptotic factors into the cytosol, the dissipation of mitochondrial membrane potential and loss of ATP production, ultimately leading to cell death during early reperfusion (Amanakis & Murphy, 2020; Hausenloy & Yellon, 2003). The existence of reperfusion injury was a long debate in the past but finally confirmed by success of protective interventions first applied during reperfusion, such as slow (Okamoto et al., 1986; Sato et al., 1997) or intermittent (Zhao et al., 2003) reperfusion.

1.2 Cardioprotection

Cardioprotection is the attenuation of irreversible myocardial ischemia/reperfusion injury and implicitly requires timely reperfusion to stop the ongoing ischemic damage (Garcia-Dorado & Piper, 2006). Cardioprotection on top of that by timely reperfusion can be achieved either through mechanical interventions that recruit endogenous protective mechanisms or pharmacologically (Heusch, 2020). The initial paradigm of cardioprotection was the discovery that brief nonlethal repeated cycles of myocardial ischemia/reperfusion, prior to a sustained injurious ischemic event, attenuates irreversible myocardial injury (Murry et al., 1986) and was termed as ischemic preconditioning (IPC). However, IPC has no clinical relevance. Clinical relevance is apparent with cardioprotective maneuvers which can be applied during reperfusion, such as ischemic postconditioning (PoCo), i.e. repetitive brief interruptions of blood flow to the reperfused myocardium (Zhao et al., 2003) or by gentle reperfusion (Okamoto et al., 1986; Sato et al., 1997). Transient periods of nonlethal ischemia in tissues remote from the myocardium, such as skeletal muscle (Thielmann et al., 2013) and the brain (Kleinbongard et al., 2017), can also induce a cardioprotective stimulus. This type of conditioning can be applied either prior to ischemia/reperfusion as remote ischemic preconditioning [RIPC, (Przyklenk et al., 1993)] or even during ongoing

ischemia as remote ischemic perconditioning [RPER, (Schmidt et al., 2007)] and is predestined to be used easily and without risk in patients with acute myocardial infarction (Bøtker et al., 2010) or patients undergoing cardiac surgery (Thielmann et al., 2010; Thielmann et al., 2013). Cardioprotection by all types of ischemic conditioning have been reported in all species studied so far, including humans, using a variety of endpoints and conditioning algorithms (Heusch, 2020).

1.3 Electrocardiogram

A basic characteristic of cardiomyocytes is their ability to generate an action potential with either spontaneous depolarizations (pacemaker cells) or in response to an electrical stimulus initiated from adjacent cells. The depolarization of the myocardium is a propagating phenomenon which occurs under normal conditions in a highly organized fashion involving a complex electrical cell-to-cell communication (Kléber & Rudy, 2004). As myocardial depolarization propagates over a larger amount of tissue, the difference in membrane potential between already depolarized and not-yet depolarized cells results in a vector of electrical potential, and the electrocardiogram (ECG) is the projection of the integral vector amplitude on the body surface, plotted against time. A typical ECG in the clinical setting usually covers 12 projections, also termed as leads. The depolarization/repolarization of distinct areas in the heart are reflected as distinct changes in ECG amplitude over time. Within a single cardiac cycle, the P-wave reflects atrial depolarization and the subsequent QRS-complex reflects ventricular depolarization. Repolarization of the atria coincides with the QRS-complex and is usually not detectable. The T-wave reflects ventricular repolarization (Figure 1).

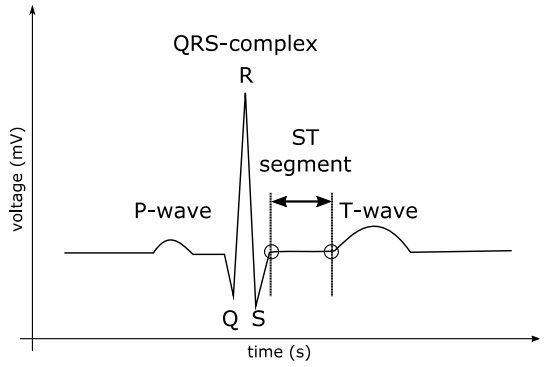

Figure 1: The normal ECG-waveform.

The ST-segment corresponds to the time interval during which the whole ventricular myocardium is depolarized, and repolarization has not yet begun. In an intact ventricle there is no potential difference between ventricular cells and therefore no electrical vector is generated, which is indicated by an isoelectric line in all ECG leads. During acute myocardial ischemia, ischemic cells have lower resting membrane potential and their action potential is characterized by smaller amplitude and shorter duration, resulting in earlier repolarization. Thus, while non-ischemic myocardium is still depolarized, the ischemic area already repolarizes. This difference in the membrane potential between non-ischemic and ischemic areas results in an electrical vector which becomes apparent during the ST-segment, and in the ECG recording the ST-segment deviates from the isoelectric line. With ongoing ischemia and loss of ATP production, the cytosolic Na^+/K^+ pump fails. The ischemic myocardium is no longer able to maintain a normal membrane/action potential and will finally undergo progressive cell death. These persistent differences between infarcted and viable myocardium again result in an electrical vector which, depending on the ECG-lead and the phase within the cardiac cycle, depresses or elevates the ECG amplitude during the TQ and/or ST-segments. These complex phenomena are summarized under the term ST-segment deviation (Samson & Scher, 1960). Due to the technical setup of an ECG recorder, amplitude changes in the TQ segment are not discernible but impact directly on ST-segment deviation. The ST-segment deviation is used widely in the clinic for diagnostic purposes in acute myocardial infarction (Ibanez et al., 2018). Notably, in transmural

ischemia an ST-segment elevation is apparent in ECG-leads that face the ischemic myocardium (Figure 2) while a reciprocal ST-segment depression is apparent in electrically opposite ECG-leads. In subendocardial ischemia an ST-segment depression is apparent in all standard ECG-leads.

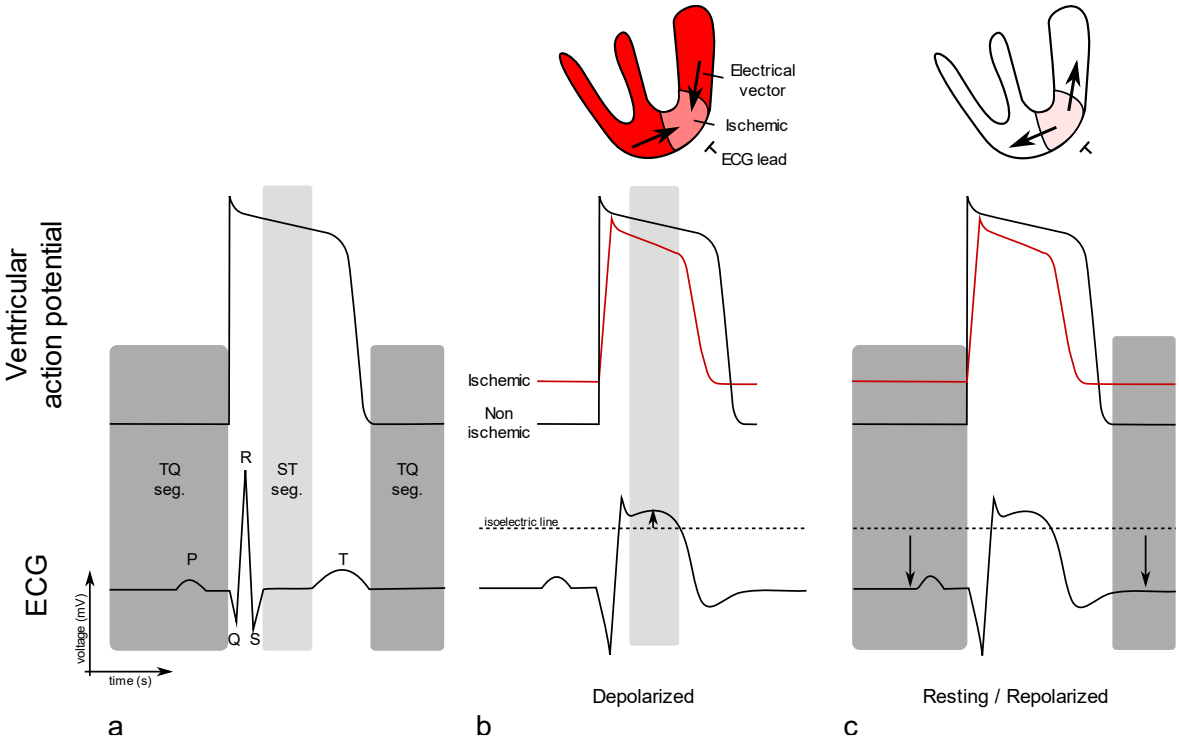

Figure 2: ST-segment elevation during ischemia is a complex phenomenon. When the non-ischemic myocardium is depolarized (b), the ischemic area due to its smaller and shorter action potentials begins to repolarize resulting in an electrical vector which displaces the ST segment from the isoelectric line; when the non-ischemic myocardium is in resting or repolarized state (c), the ischemic area exhibits lower resting membrane potentials resulting in an electrical vector which displaces the TQ segment from the isoelectric line; ECG: electrocardiogram, seg.: segment.

Several attempts have been made to link the magnitude of ST-segment elevation to the development of myocardial infarction and the salvage of ischemic/reperfused myocardium. However, the obtained results so far are limited to ST-segment elevation analysis only during the short ischemia/reperfusion cycles of an IPC maneuver. The attenuation of ST-segment elevation observed during the ischemic periods of IPC was associated with the cardioprotective effects (Cohen et al., 1997; Floyd et al., 2009; Shattock et al., 1996). This

attenuation still persists during the first 5 minutes of prolonged ischemia after IPC (Floyd et al., 2009). Whether such attenuated ST-segment elevation is a transient phenomenon limited to the initial minutes of ischemia or whether it persists for a prolonged time, is unknown.

1.4 Research objective and hypothesis

The general problem with the salvage of ischemic/reperfused myocardium by cardioprotection is that the success can be first estimated by determining final infarct size, which is usually performed late after the ischemia/reperfusion event. In the experimental setting infarct size is assessed by histology at least several hours after reperfusion (Birnbaum et al., 1997; Wolff et al., 2000) and in patients imaging techniques are applied days after the injurious event (Bulluck et al., 2017; Nensa et al., 2015). It would thus be desirable to have a robust marker which can be easily used to estimate the effectiveness of a cardioprotective maneuver already at an earlier stage.

In the present study I have therefore investigated the impact of different cardioprotective maneuvers, namely IPC, POCO, RIPC, and RPER, on ST-segment elevation prior to ischemia, during ischemia, and during subsequent reperfusion. The analysis was performed retrospectively in a well-established pig model of myocardial infarction (Heusch et al., 2011), where the major determinants of final infarct size were strictly controlled. The individual success of each cardioprotective maneuver was determined by histological measurement of myocardial infarct size. To ensure that the area at risk was indeed severely ischemic during the index ischemia, transmural myocardial blood flow was measured.

2 Material and methods

All experimental protocols were approved by the Bioethical Committee of the district of Duesseldorf (LANUV NRW, G1240/11, G1388/13, and G1407/14) and conformed with the "Position of the American Heart Association on Research Animal Use" adopted by the American Heart Association on November 11, 1984. All chemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany), unless otherwise specified.

2.1 Experimental preparation

The experiments were performed between November 2012 and November 2017 in male Goettingen minipigs (body weight 30-35 kg, age 14±2 months, Ellegaard, Dalmose, Denmark). The animals were provided standard chow (twice 300 g/day, #V4133, ssniff, Soest, Germany), had free access to water, and were kept in tiled rooms (~2 m^2/pig) with straw-bedding, at 12 h/12 h light/dark cycles. Pigs were sedated with flunitrazepam (0.4 mg/kg i.m., Sigma-Aldrich, Deisenhofen, Germany) and anesthesia was induced with etomidate (0.3 mg/kg i.v., Hypnomidat, Janssen-Cilag, Neuss, Germany) and sufentanil (1 µg/kg i.v., Sufenta, Janssen-Cilag, Germany) administered through a peripheral catheter in an ear-vein. After surgical tracheotomy, the pigs were mechanically ventilated with oxygen enriched air supplemented with isoflurane (2%, Forene, AbbVie, Ludwigshafen, Germany) to maintain anesthesia. Respiratory parameters were frequently adjusted to keep arterial blood gases and pH in the physiological range. The jugular veins were cannulated for saline volume replacement or drug administration. The common carotid arteries were cannulated to measure arterial pressure and to withdraw blood from the descending aorta as reference for the transmural myocardial blood flow measurement (TMBF). Before a left lateral thoracotomy, additional analgesia was provided with sufentanil (10 µg/kg i.v.), and neuromuscular blockade was induced with rocuronium (0.6 mg/kg i.v., Esmeron, MSD, Haar, Germany). This anesthesia protocol is identical to that used in our institution for

patients undergoing coronary artery bypass surgery (Thielmann et al., 2013). The heart was exposed, and a catheter connected to a micromanometer (CODAN pvb Medical, Lensahn, Germany) was inserted through the apex into the left ventricle to measure left ventricular pressure. A Teflon catheter was placed in the left atrium for injection of colored microspheres (Kowallik et al., 1991). A silk suture was placed around the left anterior descending coronary artery (LAD) distal of the second diagonal branch for later coronary occlusion. Occasionally, visible epicardial collaterals were ligated. During surgery, pigs were placed on a heated table and covered with drapes to keep body temperature (esophageal probe) at 37±1°C. Ventricular fibrillation was immediately terminated by internal defibrillation (Skyschally et al., 2017). Left ventricular pressure, aortic pressure, and a single channel ECG-lead similar to a V2 Wilson lead in humans were continuously recorded. Figure 3 depicts a sample of the hemodynamic and ECG monitoring during ischemia.

For cardioprotection by RIPC or RPER a rope-snare tourniquet was placed around one hindlimb proximal to the knee joint.

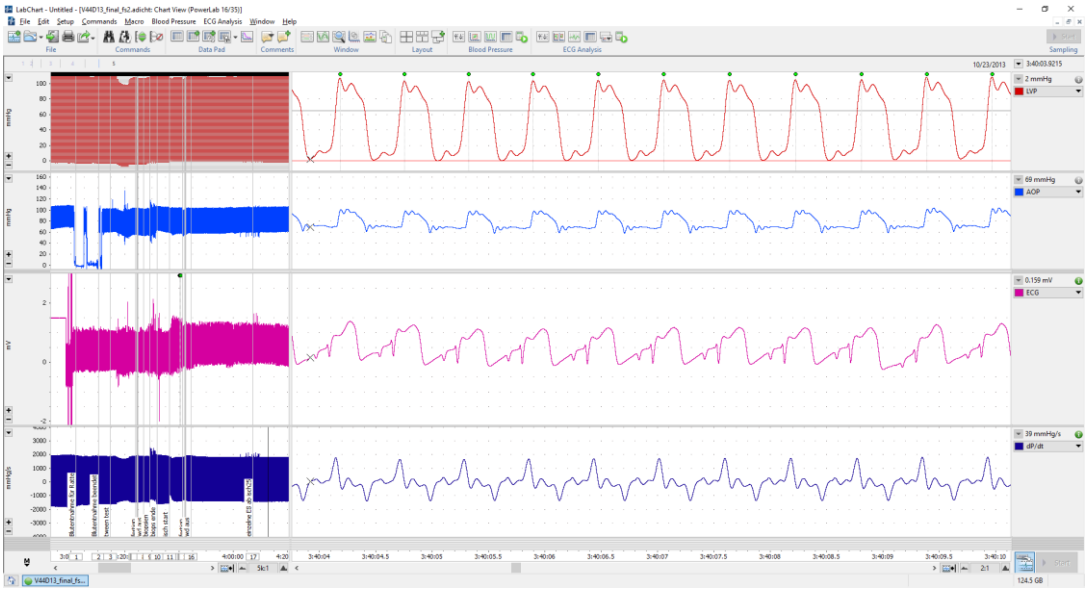

Figure 3: Hemodynamic and ECG monitoring 5 min after onset of ischemia in a placebo experimental protocol (Labchart, AD Instruments, Oxford, UK); from top to bottom: left ventricular pressure, aortic pressure, ECG-lead V2, and the first derivative of left ventricular pressure (dP/dt).

2.2 Experimental protocols

A schematic diagram of all experimental protocols is shown in Figure 4.

Protocol 1 – Placebo (PLA, n=29)

Myocardial ischemia was induced for 60 min by tightening of the silk suture around the LAD against a silicone plate. Reperfusion was performed by quick release and removal of the suture and was continued for 3 h. Heart rate, maximal left ventricular pressure (LVP_{max}), maximal rate of rise of left ventricular pressure (dP/dt_{max}), and ST-segment elevation were measured at baseline, at 5 and 55 min after onset of ischemia, and at 10, 30, 60, and 120 min after onset of reperfusion. Regional myocardial blood flow was measured at baseline, 5 min after onset of ischemia, and at 10 min reperfusion. Area at risk and infarct size were determined post-mortem as described below.

Protocol 2 – local ischemic preconditioning (IPC, n=15)

Ischemic preconditioning was induced by two 3 min occlusions of the LAD, separated by 2 min reperfusion. The index ischemia was started 15 min later. Hereafter, the protocol was identical to that of PLA.

Protocol 3 – remote ischemic preconditioning (RIPC, n=21)

For remote ischemic preconditioning, the tourniquet around the left hindlimb was tightly closed and skin cyanosis was taken to indicate leg ischemia. Hindlimb reperfusion was induced by release of the tourniquet with skin blushing indicating reperfusion. Four cycles of 5 min ischemia / 5 min reperfusion, starting 40 min before thoracotomy were performed. The remaining protocol was identical to that of PLA.

Protocol 4 – remote ischemic perconditioning (RPER, n=18)

Remote ischemic perconditioning on the hindlimb was induced similar to RIPC but was started 20 min after coronary occlusion. The remaining protocol was identical to that of PLA.

Protocol 5 – ischemic postconditioning (POCO, n=9)

Ischemic postconditioning was induced by 4 cycles of 1 min LAD re-occlusion followed by 1 min LAD reperfusion each, starting at 1 min reperfusion. The remaining protocol was identical to PLA.

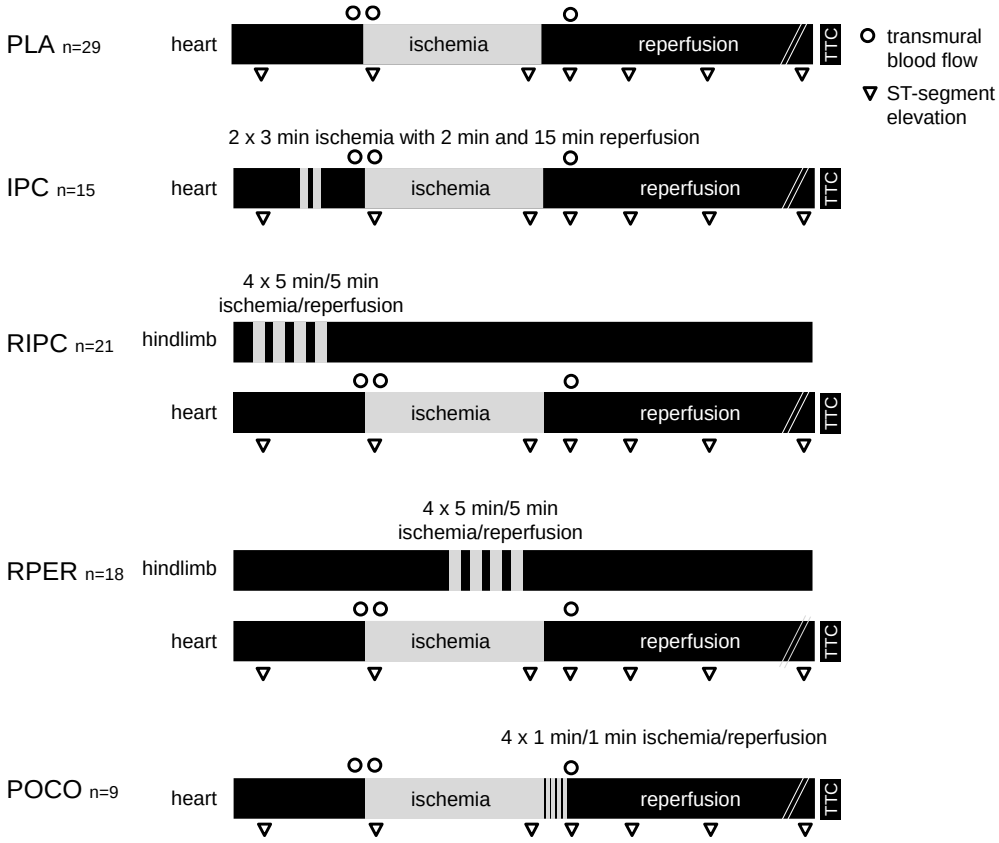

Figure 4: Conditioning protocols, time points of ST-segment elevation analysis (triangles) and transmural blood flow measurements (circles); 5 and 55 min after the onset of coronary occlusion, 10, 30, 60, and 120 min after the onset of reperfusion; PLA: placebo, IPC: ischemic preconditioning, RIPC: remote ischemic preconditioning, RPER: remote ischemic perconditioning, POCO: ischemic postconditioning.

2.3 Systemic hemodynamics

Heart rate, LVP_{max}, and dP/dt_{max} were averaged offline over a period of 10 subsequent cardiac cycles (CORDAT II) (Skyschally et al., 1993) at baseline, 5 and 55 min after the onset of coronary occlusion, and at 10, 30, 60, and 120 min reperfusion. Premature beats and periods of ventricular tachycardia/fibrillation were excluded from analysis.

2.4 Transmural myocardial blood flow

Transmural myocardial blood flow was measured with colored fluorescent microspheres (Fluospheres polystyrene 15 µm, Life technologies/Molecular probes, Darmstadt, Germany) which were injected into the left atrium (Kowallik et al., 1991). During microsphere injection, arterial blood was withdrawn with a constant rate (5 ml/min) through the catheter in the descending aorta as reference. Myocardial tissue samples weighing 0.7-1.0 g were cut post-mortem from the central area at risk and the posterior wall of the left ventricle. Residues of formazan dye which originated from TTC infarct size measurement (see section 2.5) were removed by washing the tissue samples with acetone and ethanol 96% solution (1:10). Myocardial tissue and reference blood samples were digested overnight in 4 mol/l and 16 mol/l potassium hydroxide respectively and the colored microspheres were recovered by filtration through an 8 µm polyester membrane filter (Pieper filter, Bad Zwischenahn, Germany). The fluorescence dye was dissolved from the recovered spheres by addition of 2-ethoxyethyl-acetate and its fluorescence was measured using a fluorescence photometer (Varian Cary Eclipse, Agilent Technologies, Santa Clara, USA). Transmural myocardial blood flow (TMBF) was calculated in ml/min according to the following equation (Kowallik et al., 1991):

$$\frac{Fluorescence\ in\ tissue\ sample\ (RFU)}{TMBF\ \left(\frac{ml}{min}\right)} = \frac{Fluorescence\ in\ blood\ sample\ (RFU)}{5\ \left(\frac{ml}{min}\right)}$$

Normalized for weight, TMBF is expressed in ml/min/g of tissue. TMBF was measured at baseline, 5 min after onset the onset of coronary occlusion, and 10 min reperfusion. TMBF >0.06 ml/min/g at 5 min of coronary occlusion was considered as non-severe ischemia, and these experiments were excluded from further analysis.

2.5 Area at risk and infarct size

At the end of each experimental protocol, the LAD was re-occluded and 5 ml blue dye (Patentblau V, Guerbet, Sulzbach, Germany) was injected into the left atrium to delineate the area at risk (AAR). The pig was then euthanized by rapid injection of 20 ml potassium chloride solution (2 mol/l), and the heart was excised and sectioned into 6 slices parallel to the atrioventricular groove. Slice shape and the demarcated area at risk were traced on transparent film. The slices were immersed in 0.09 mol/l sodium phosphate buffer containing 1% triphenyl-tetrazolium-choride (TTC, Sigma-Aldrich Chemie, Munich, Germany) and 8% dextran for 20 min at 37°C to demarcate infarcted areas. TTC reacts with NADH and NADPH in viable myocardial cells and forms formazan, a water insoluble deep red salt, demarcating vital from avital, infarcted tissue (Figure 5). The infarcted areas were traced again on transparent film (Figure 6). Area at risk, expressed as fraction of the left ventricle, and infarct size, expressed as fraction of the area at risk, were determined by computer assisted planimetry. The slices were weighted, and the mass of myocardium was calculated according to planimetry data.

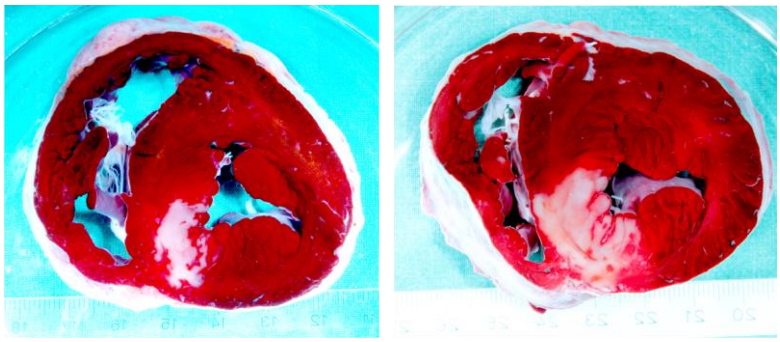

Figure 5: TTC staining of myocardial slices. Infarcted myocardium is not stained and appears white.

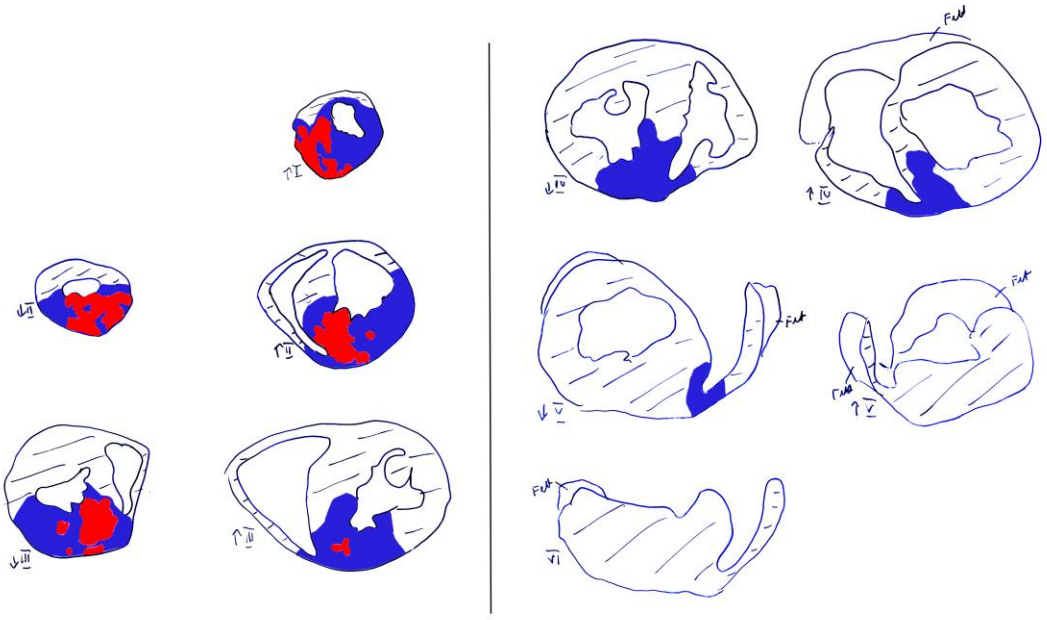

Figure 6: Transparent film with traces of slice shape, area at risk (solid blue) and infarcted myocardium (solid red).

2.6 ECG analysis

Surface ECG was continuously recorded using a single channel, calibrated (1 mV reference) amplifier (Kleinbongard et al., 2018). Due to the surgical preparation and use of a metal rib retractor the recorded ECG-lead appeared similar to a V2 Wilson lead in humans facing the anteroseptal wall. Thus, when the LAD was occluded causing transmural ischemia an ST-segment elevation was apparent in the recorded ECG-lead. The ST-segment elevation was defined as the amplitude difference between a point 30 ms before the P wave and a second point 20 ms after the J-point (Figure 7). In addition, QRS duration, QT interval and R amplitude (Sun et al., 2013; Wagner et al., 1988) were measured. Analysis was performed offline using digital calipers (Labchart 8, AD Instruments, New South Wales, Australia) at baseline, 5 and 55 min after the onset of ischemia, and at 10, 30, 60, and 120 min reperfusion. For each measurement data from 30 consecutive cardiac cycles were averaged; premature beats or periods of ventricular tachycardia/fibrillation were

excluded. Of note, the position of the animal remained the same throughout the course of ECG registration to avoid changes in ECG amplitude.

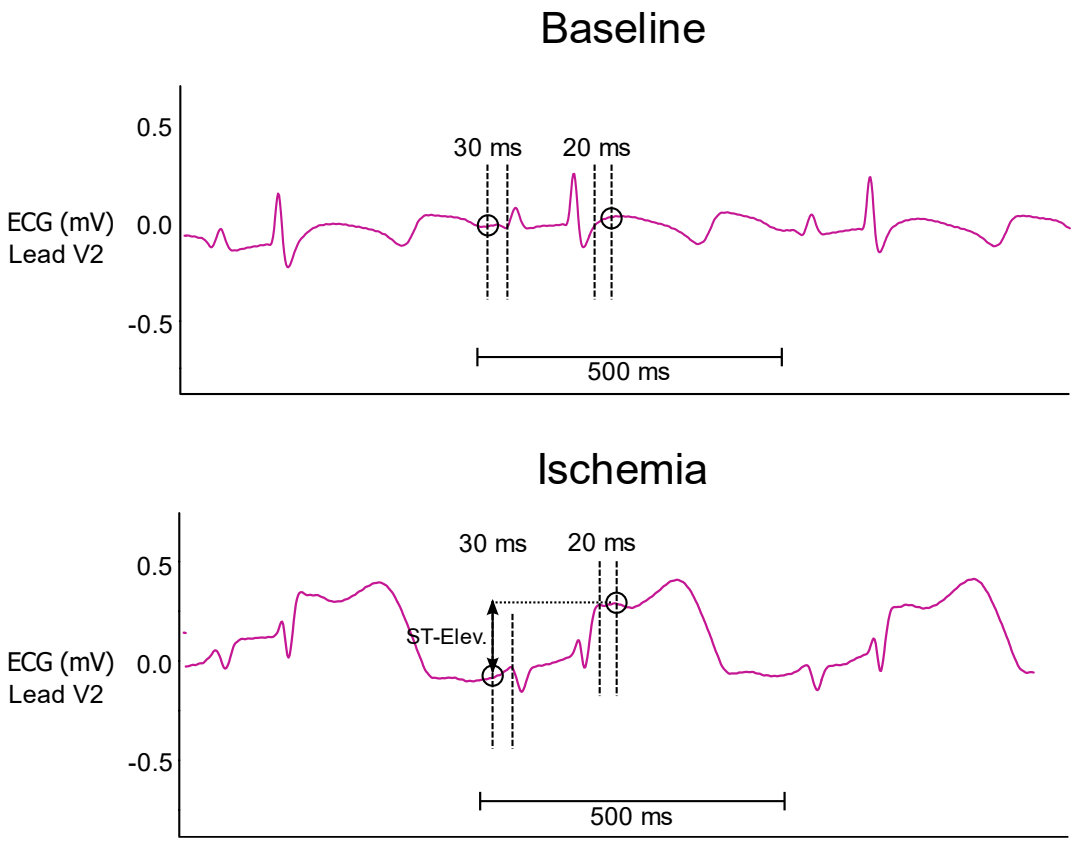

Figure 7: Measurement of ST-segment elevation; the ST-segment elevation was defined as the amplitude difference between two points, 30 ms before the P-wave and 20 ms after the J-point; ST-Elev.: ST-segment elevation.

2.7 Statistical analysis

All data are presented as mean ± SD. Area at risk, and infarct size were analyzed by 1-way ANOVA with Fisher's least significant difference post-hoc test. Transmural myocardial blood flow, heart rate, LVP_{max}, dP/dt_{max}, were analyzed by 2-way ANOVA for repeated measures with Fisher's least significant difference post-hoc test (SigmaStat 3.5; Systat Software, Erkrath, Germany). Normal distribution of ST-segment elevation data was tested with the Shapiro-Wilk method. Outliers were identified using the Boxplot method and were excluded from statistical analysis when off by more than 1.5 times the interquartile range. One pig of

the PLA group and two of the IPC group were excluded retrospectively from the analysis as their ST-segment elevation met the criteria for multiple outliers (≥5 time points). ST-segment elevation, QRS duration, QT interval, and R amplitude were analyzed by a mixed model analysis (SAS 9.4, SAS Institute Inc., Cary, NC, USA) for the fixed effects “group”, “time”, and the interaction “group*time”, considering random effects induced by the individual animal. Least square means were computed as post-hoc tests to identify differences between single mean values. Differences were considered significant at the level of $p<0.05$.

I tested whether the magnitude of attenuation of ST-segment elevation was related to the magnitude of infarct size reduction on an individual level. For that, a linear regression between myocardial blood flow in the area at risk at 5 min ischemia and infarct size in pigs subjected to ischemia/reperfusion only was calculated. Then the individual infarct size reduction for each pig in the IPC, RIPC, and RPER groups was assessed as the difference between the observed infarct size and the expected infarct size without a protective intervention, given the individual blood flow at 5 min ischemia.

3 Results

3.1 Systemic hemodynamics

Heart rate did not differ significantly between the groups (Table 1). Within all groups, there was a drop in LVP_{max} 5 min after onset of ischemia as compared to baseline which persisted up to 2 h after reperfusion onset. The LVP_{max} of RIPC was significantly lower in comparison to PLA starting 10 min after onset of reperfusion and persisted until 30 min. POCO exhibited a lower LVP_{max} throughout the protocol compared to PLA.

	time	HR [1/min]	LVP_{max} [mmHg]	dP/dt_{max} [mmHg/s]
PLA	baseline	117±16	89±9	1793±370
(n=29)	5 min ischemia	113±14	80±8*	1508±291*
	55 min ischemia	117±15	82±9*	1564±347*
	10 min reperfusion	118±16	81±9*	1622±432*
	30 min reperfusion	119±14	81±8*	1675±446
	60 min reperfusion	118±13	81±7*	1669±317*
	120 min reperfusion	118±12	78±9*	1520±332*
IPC	baseline	112±18	87±8	1629±319
(n=15)	5 min ischemia	105±17	79±8*	1416±244*
	55 min ischemia	107±13	79±10*	1422±256*
	10 min reperfusion	110±19	80±7*	1525±368
	30 min reperfusion	108±14	78±5*	1429±288*
	60 min reperfusion	107±14	78±5*	1411±268*
	120 min reperfusion	106±15	74±6*	1303±237*
RIPC	baseline	112±11	88±7	1669±389
(n=21)	5 min ischemia	109±12	79±7	1374±307
	55 min ischemia	114±18	79±8	1393±269
	10 min reperfusion	118±20	77±10	1418±286
	30 min reperfusion	117±17	78±9	1485±309
	60 min reperfusion	116±16	76±9	1477±394
	120 min reperfusion	113±15	76±8	1437±394
RPER	baseline	112±11	88±10	1733±417
(n=18)	5 min ischemia	107±12	81±9*	1327±272*
	55 min ischemia	107±13	75±9*	1370±276*
	10 min reperfusion	111±13	78±8*	1456±379*
	30 min reperfusion	109±13	78±9*	1499±414*
	60 min reperfusion	112±13	79±10*	1529±382*
	120 min reperfusion	112±13	78±9*	1499±435*

	time	HR [1/min]	LVP_{max} [mmHg]	dP/dt_{max} [mmHg/s]
POCO (n=9)	baseline	117±12	85±10	1833±502
	5 min ischemia	108±15	75±6*	1276±195*
	55 min ischemia	113±11	73±5*	1449±500*
	10 min reperfusion	114±10	74±7*	1545±535*
	30 min reperfusion	117±12	73±6*	1725±585
	60 min reperfusion	118±16	71±6*	1521±351*
	120 min reperfusion	115±10	70±6*	1420±343*

Table 1: Heart rate (HR), maximal left ventricular pressure (LVP_{max}), and maximal rate of rise of left ventricular pressure (dP/dt_{max}); *p<0.05 vs. baseline, 2-way repeated measures ANOVA; PLA: placebo, IPC: ischemic preconditioning, RIPC: remote ischemic preconditioning, RPER: ischemic perconditioning, POCO: ischemic postconditioning.

3.2 Transmural myocardial blood flow

Transmural myocardial blood flow in the area at risk was not different between groups at baseline, 5 min ischemia and 10 min reperfusion (Figure 8).

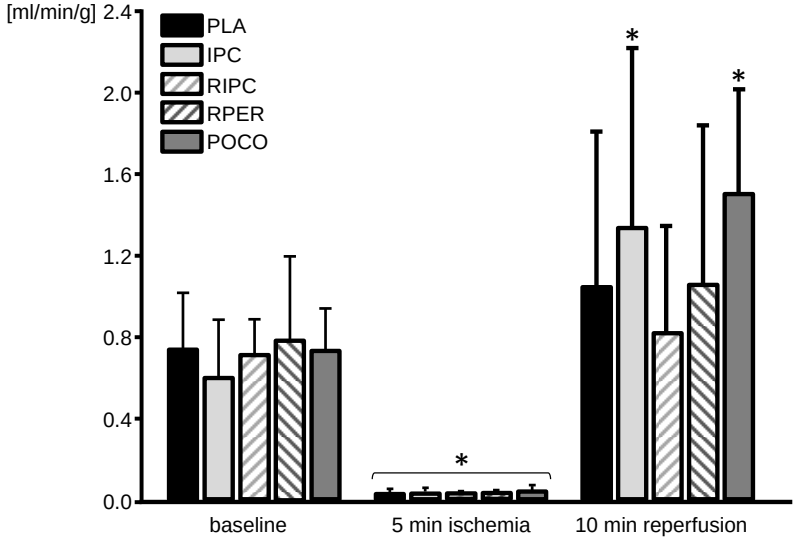

Figure 8: Transmural myocardial blood flow in the area at risk at baseline, 5 min ischemia and 10 min reperfusion. No significant differences between groups; *p<0.05 vs. baseline, 2-way ANOVA and Fisher's least significant difference post-hoc test; PLA: placebo, IPC: ischemic preconditioning, RIPC: remote ischemic preconditioning, RPER: remote ischemic perconditioning, POCO: ischemic postconditioning.

3.3 Area at risk and infarct size

The area at risk (AAR), expressed as fraction of the left ventricle, was not different between groups (Figure 9). Compared to PLA all four cardioprotective maneuvers reduced infarct size (Figure 10).

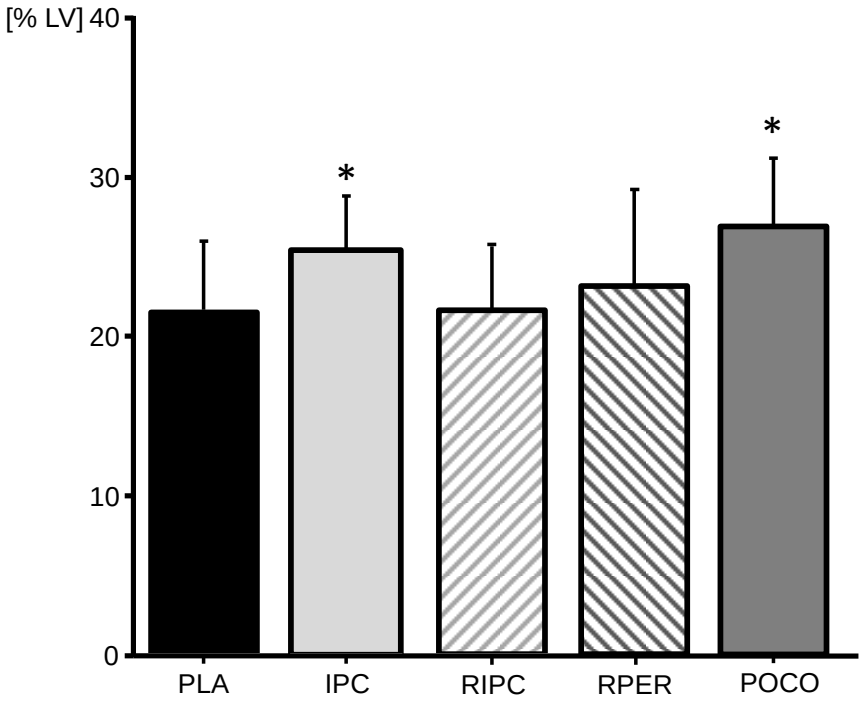

Figure 9: Area at risk (AAR) expressed as percentage of the left ventricle; *p<0.05 vs. PLA; 1-way ANOVA and Fisher's least significant difference post-hoc test; LV: left ventricle, PLA: placebo, IPC: ischemic preconditioning, RIPC: remote ischemic preconditioning, RPER: ischemic perconditioning, POCO: ischemic postconditioning.

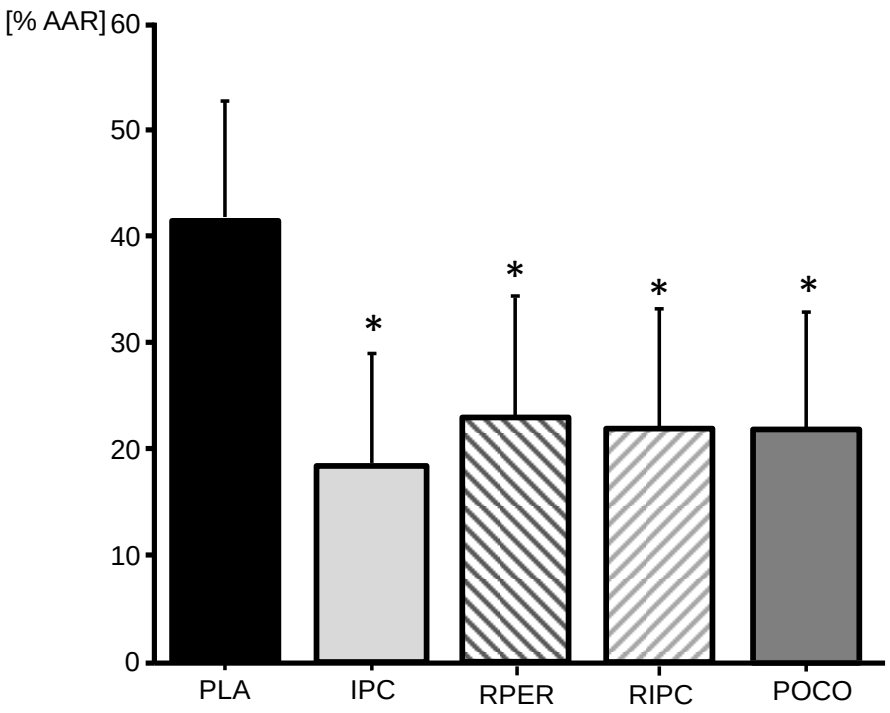

Figure 10: Infarct size expressed as percentage of the area at risk; all conditioning maneuvers reduced infarct size; *p<0.05 vs. PLA; 1-way ANOVA and Fisher's least significant difference post-hoc test; AAR: area at risk, PLA: placebo, IPC: ischemic preconditioning, RIPC: remote ischemic preconditioning, RPER: ischemic perconditioning, POCO: ischemic postconditioning.

3.4 ST-segment elevation

Representative ECG recordings and the numerical analysis of ST-segment elevation from the five protocols are presented in Figures 11-12. At baseline there were no differences between the groups. With PLA, ST-segment elevation was increased at 5 min after the onset of ischemia and remained unchanged at 55 min ischemia. At 10 min reperfusion ST-segment elevation increased further and then gradually recovered to values near baseline level after 120 min (Figure 12).

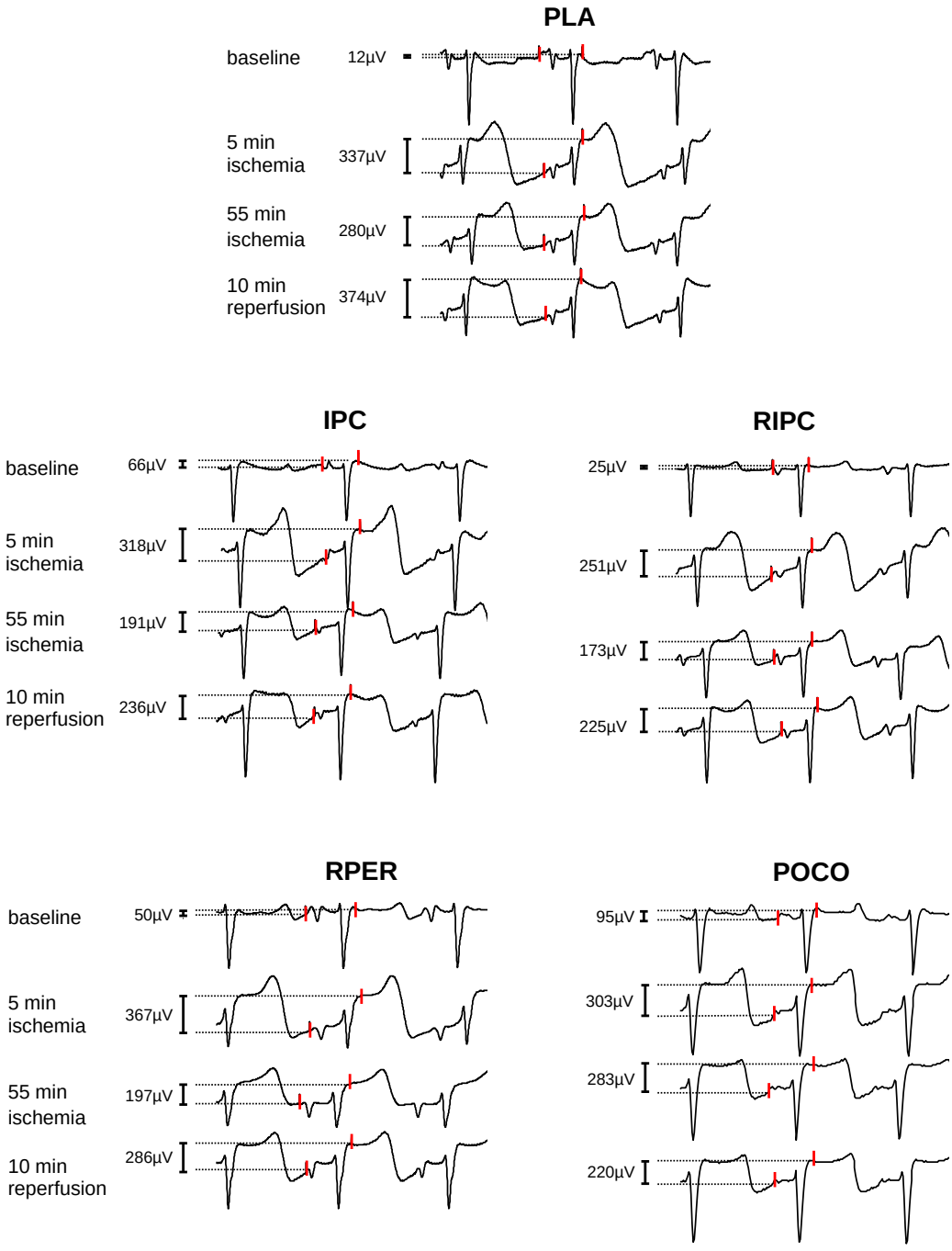

Figure 11: Representative original ECG recordings from one pig of each experimental group; ST-segment elevation is displayed as amplitude difference between 2 points (vertical lines) 30 ms before the P-wave and 20 ms after the J-point; PLA: placebo; IPC: ischemic preconditioning; RIPC: remote ischemic preconditioning; RPER: remote ischemic perconditioning; POCO ischemic postconditioning.

IPC and RIPC did not attenuate ST-segment elevation compared to PLA at 5 min of ischemia, but did so at 55 min. This attenuation remained apparent up to 30 min after reperfusion (Figure 12A-B).

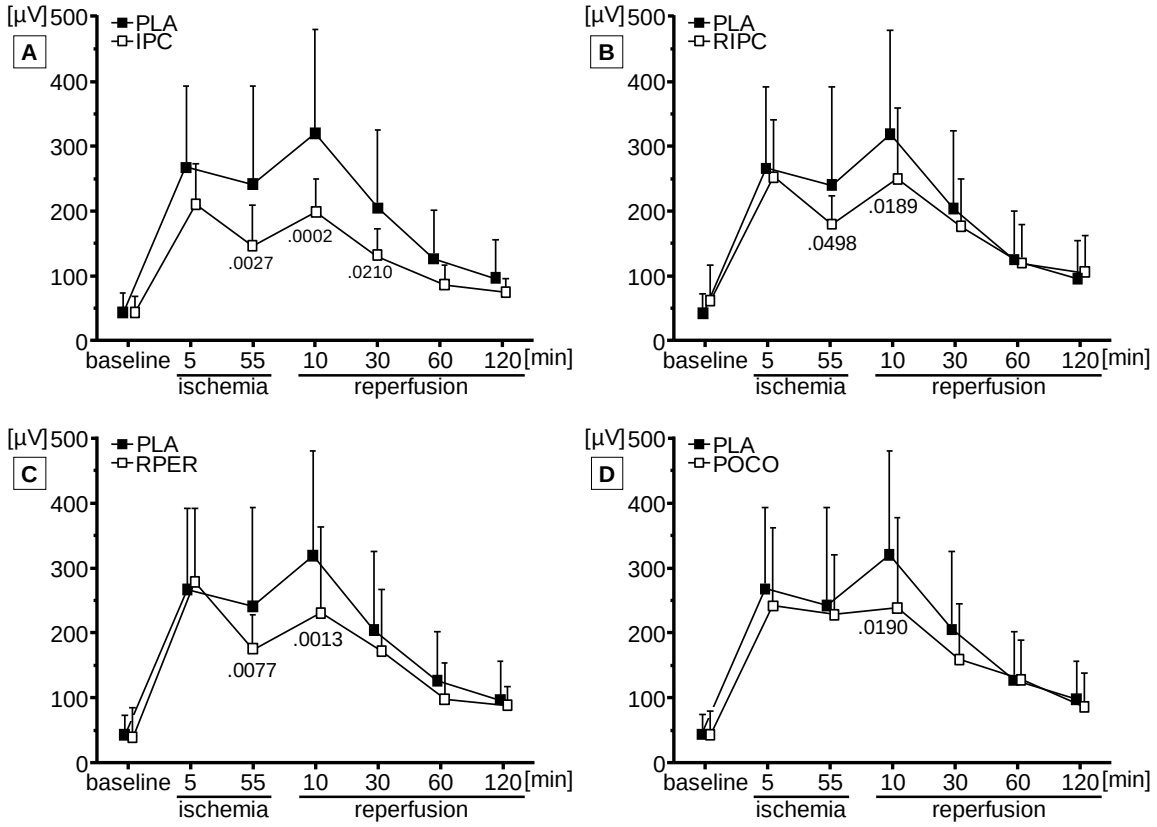

Figure 12: Time course of ST-segment elevation with ischemic conditioning; A: ischemic preconditioning (IPC), B: remote ischemic preconditioning (RIPC), C: remote ischemic perconditioning (RPER), and D: ischemic postconditioning (POCO); mixed model analysis and least square means post-hoc test; p values vs. placebo (PLA) are given below the respective data point; for comparison, the time course of PLA is displayed in each panel.

With RPER, ST-segment elevation was attenuated immediately after the conditioning maneuver at 55 min ischemia and up to 10 min reperfusion (Figure 12C).

With POCO, ST-segment elevation similar to PLA at baseline and during ischemia, but the further increase in ST-segment elevation at 10 min reperfusion was abrogated (Figure 12D).

There was no notable correlation between the individual change in ST-segment elevation between 5 and 55 min of ischemia and the calculated individual reduction in infarct size (r=0.004). QRS duration and QR interval did not change during the experimental protocols and were not different between groups (Figures 13-14). R amplitude was decreased at 5 min ischemia and then gradually recovered without difference between groups (Figure 15).

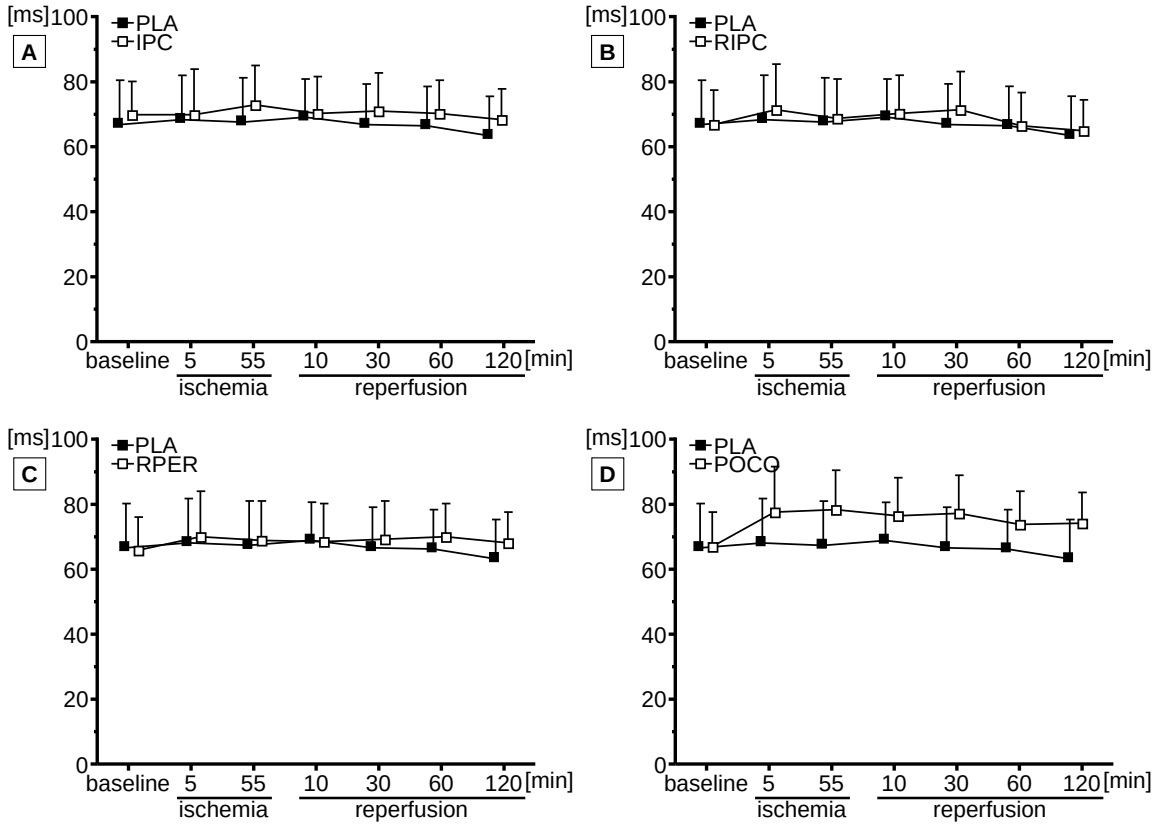

Figure 13: Time course of QRS duration with ischemic conditioning; A: ischemic preconditioning (IPC), B: remote ischemic preconditioning (RIPC), C: remote ischemic perconditioning (RPER), and D: ischemic postconditioning (POCO); mixed model analysis; no significant differences between groups; for comparison, the time course of placebo (PLA) is displayed in each panel.

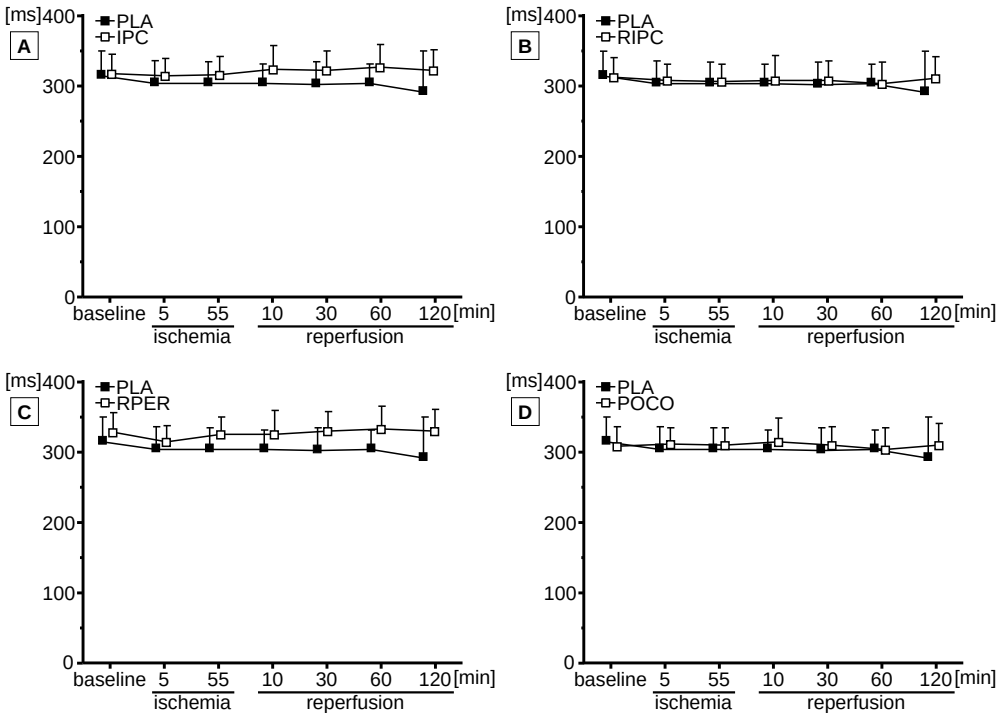

Figure 14: Time course of QT interval with ischemic conditioning. A: ischemic preconditioning (IPC), B: remote ischemic preconditioning (RIPC), C: remote ischemic perconditioning (RPER), and D: ischemic postconditioning (POCO); mixed model analysis; no significant differences between groups; for comparison, the time course of placebo (PLA) is displayed in each panel.

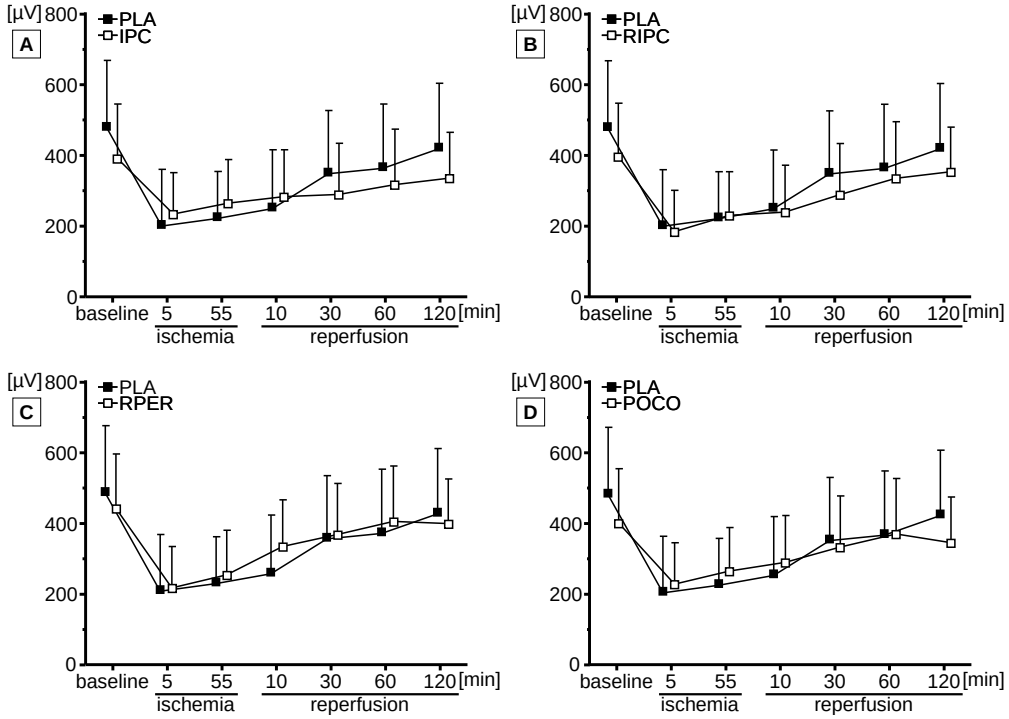

Figure 15: Time course of R amplitude with ischemic conditioning. A: ischemic preconditioning (IPC), B: remote ischemic preconditioning (RIPC), C: remote ischemic perconditioning (RPER), and D: ischemic postconditioning (POCO); mixed model analysis; no significant differences between groups; for comparison, the time course of placebo (PLA) is displayed in each panel.

3.5 Critique of methods

All data were analysed retrospectively from experiments performed in a single institution over a period of 5 years. However, this approach is in line with the 3R (replace, reduce, refine) principle to reduce the number of animal experiments. All experiments have been reported in another context (Gent et al., 2017; Kleinbongard et al., 2018; Skyschally et al., 2017; Skyschally et al., 2015; Skyschally et al., 2018; Skyschally et al., 2013). The algorithms for ischemic conditioning were adapted from published maneuvers in the clinical setting [IPC (Buyukates et al., 2005), RIPC (Munk et al., 2010), RPER (Bøtker et al., 2010), POCO (Staat et al., 2005)], and have been shown to robustly reduce infarct size in prior studies in our institution (Gent et al., 2017; Kleinbongard et al., 2018; Skyschally et al., 2017; Skyschally et al., 2015; Skyschally et al., 2018; Skyschally et al., 2013). The cardioprotective algorithms were not optimized for further infarct size reduction (Johnsen et al., 2016). Anesthesia was maintained with isoflurane which might facilitate cardioprotection (Haroun-Bizri et al., 2001). However, the anesthetic regimen was identical in all groups. Biomarkers of myocardial infarction such as LDH, troponin, or CK were unavailable. The use of a single lead in ECG recording prevented a reliable analysis of QRS distortion as proposed as a predictor of a large area at risk and infarct size (Valle-Caballero et al., 2016).

4 Discussion

The present study demonstrated that cardioprotection by IPC, RIPC, RPER, and POCO attenuates the ST-segment elevation in an experimental setting of acute ischemia/reperfusion, suggesting that the ECG might indeed serve as an online marker reflecting cardioprotection. Cardioprotection by ischemic conditioning maneuvers prior to ischemia, i.e. IPC and RIPC, did not attenuate ST-segment elevation at early ischemia but, similar to RPER, did so at 55 min ischemia and during early reperfusion. POCO abrogated a further increase in ST segment elevation seen in PLA between 55 min ischemia and 10 min reperfusion.

4.1 Attenuation of ST-segment elevation

Attenuation of ST-segment elevation with repeated brief coronary occlusions has been reported in patients undergoing elective percutaneous coronary interventions (Deutsch et al., 1990; Edwards et al., 2002; Tomai et al., 1994) and in experimental studies which reported reduced ST segment elevation during repeated brief ischemia/reperfusion cycles of an IPC maneuver (Cohen et al., 1997; Shattock et al., 1996) and during the first minutes of sustained ischemia (Floyd et al., 2009). This trend for an attenuated ST-segment elevation was also seen with IPC at 5 min ischemia in the present study. However, in patients undergoing elective percutaneous coronary intervention, myocardial infarction is not present, and the referred animal studies did not report infarct size data, such that no link between infarct size reduction and attenuated ST-segment elevation can be suggested. The ST-segment elevation is a complex phenomenon attributed to altered ionic currents in acute myocardial ischemia. The membrane potential differences between the non-ischemic myocardium with a normal depolarization/repolarization cycle and the ischemic myocardium, which is in an attenuated or even inactivated depolarized state, generates a so called injury current. During electrical diastole, there is a current flow from the ischemic

myocardium to the non-ischemic myocardium which is reversed during electrical systole. Such contribution of both non-ischemic and ischemic myocardium to the injury current is reflected in the apparent ST-segment elevation and makes it impossible to attribute the observed attenuation of the ST-segment amplitude to a specific ionic current.

The ST-segment elevation largely reflects the ongoing ischemia- and reperfusion-induced injury. It does not distinguish between reversible (at 5 min coronary occlusion) and irreversible (55 min coronary occlusion / 10 min reperfusion) injury. In addition, it also does not reflect the amount of ischemia and reperfusion injury since it recovers almost completely at 120 min reperfusion, despite the definite presence of infarcted tissue in the area at risk. Likewise, the attenuation of ST-segment elevation by an ischemic conditioning maneuver reflects the ongoing cardioprotection, but not its final result. Thus, unsurprisingly, the magnitude of attenuation of ST-segment elevation did not reflect the magnitude of infarct size reduction.

However, because the attenuation of ST-segment elevation by the ischemic conditioning maneuver reflects ongoing cardioprotection, it can indeed serve as an online marker of cardioprotection. In RPER cardioprotection is initiated during ongoing coronary occlusion as seen previously in pigs (Kleinbongard et al., 2018). Such a protection during ongoing coronary occlusion is in line with prior clinical studies using RPER (Pryds et al., 2016) or metoprolol (Garcia-Ruiz et al., 2016), where patients with acute ST-segment elevation myocardial infarction tolerated longer coronary occlusion but had less infarcted tissue when the cardioprotective intervention was applied during coronary occlusion. Attenuation of ST-segment elevation may reflect the potential of conditioning to achieve protection not only from reperfusion but also from ischemic injury, as recently highlighted (Rossello & Ibanez, 2018).

Regarding RIPC and RPER, the transfer of the cardioprotective signal from the peripheral organ to the heart has to be accounted for. Available data support both humoral and neuronal transfer signals (Kleinbongard et al., 2017). RIPC in pigs involves a humoral cardioprotective signal, as blood plasma taken from pigs after remote ischemic

preconditioning protects isolated rat hearts from ischemia/reperfusion injury (Skyschally et al., 2015). In the present study, the RPER maneuver was performed entirely during ischemia and humoral transmitters released from the remote skeletal muscle into the bloodstream could not have reached the central ischemic myocardium before the onset of reperfusion, since transmural myocardial blood flow was minimal and did not change during the coronary occlusion. The data rather suggest a mechanism involving neuronal pathways which are also assumed to be involved in cardioprotection afforded by RIPC (Donato et al., 2013; Gho et al., 1996; Lieder et al., 2018).

4.2 Clinical considerations

Of all ischemic conditioning maneuvers, only RPER and POCO are feasible in patients with acute myocardial infarction. However, POCO requires manipulation of the culprit coronary lesion that has initiated acute myocardial infarction, and as such always carries the risk of inducing coronary microembolization with additional myocardial injury (Heusch, 2013). The clinical significance of cardioprotection by remote ischemic conditioning as measured by infarct size reduction, has been shown in several smaller clinical trials (Bøtker et al., 2010; Cao et al., 2018; Crimi et al., 2013; Eitel et al., 2015; Liu et al., 2016; Munk et al., 2010; Prunier et al., 2014; White et al., 2015; Yamanaka et al., 2015; Yellon et al., 2015).

Unfortunately, the effectiveness of remote ischemic conditioning in the clinical scenario of acute myocardial infarction was recently questioned in a large multicenter trial. Remote ischemic conditioning did not improve the clinical outcomes cardiac death or hospitalization for heart failure at 12 months in patients with STEMI undergoing primary percutaneous coronary intervention (Hausenloy et al., 2019).

However, the disappointing data do not necessarily mean that remote ischemic conditioning may not still be beneficial in other settings. With focus on patients who really need protection on top of reperfusion, e.g. those with underlying heart failure, shock, or severe

hemodynamic symptoms or those with delayed reperfusion, e.g. in developing countries, cardioprotection by ischemic conditioning is still of clinical interest (Heusch & Gersh, 2020).

In this context, the availability of an indicator of successful cardioprotection, like the attenuation of ST-segment elevation, appears to be reasonable and indicated. As an improved outcome is not necessarily associated with infarct size reduction (Gaspar et al., 2018) the acute attenuation of the ST-segment elevation already during ischemia, regardless of its cause (smaller area at risk, higher residual blood flow, shorter duration of ischemia or cardioprotection by ischemic perconditioning), might provide a significant marker of myocardial protection.

5 Abstract

Ischemic conditioning maneuvers reduce myocardial infarct size. However, infarct size reduction can first be assessed hours after established reperfusion. The present study tested the hypothesis that ST-segment elevation and its attenuation reflect cardioprotection by ischemic conditioning online. Pigs were subjected to regional myocardial ischemia/reperfusion (1 h/3 h). Ischemic conditioning was induced prior to ischemia either locally (preconditioning; IPC; n=15) or remotely (remote preconditioning; RIPC; n=21), or remotely during ischemia (remote perconditioning; RPER; n=18), or locally at reperfusion (postconditioning; POCO; n=9). Pigs without conditioning served as controls (PLA; n=29). Area at risk and infarct size were measured postmortem, and ST-segment elevation was analyzed in a V2-like electrocardiogram lead. All ischemic conditioning maneuvers robustly reduced infarct size (PLA 42 ± 11% of area at risk; IPC 18 ± 10%; RIPC 22 ± 12%; RPER 23 ± 12%, POCO 22 ± 11%). With PLA, ST-segment elevation was increased at 5 min ischemia, sustained until 55 min ischemia and further increased at 10 min reperfusion. IPC and RIPC did not impact on ST-segment elevation at 5 min ischemia, but attenuated ST-segment elevation at 55 min ischemia. With RPER, ST-segment elevation was not different from that with PLA at 5 min but attenuated at 55 min ischemia. POCO abolished the further increase of ST-segment elevation with reperfusion. Cardioprotection by ischemic conditioning is robustly reflected online by attenuation of ST-segment elevation.

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9 Abbreviations

AAR	area at risk
ANOVA	analysis of variance
CK	creatinine kinase
dP/dt_{max}	maximal rate of rise of left ventricular pressure
ECG	electrocardiogram
HR	heart rate
PLA	placebo
IPC	ischemic preconditioning
IS	infarct size
i.v.	intravenously
LAD	left anterior descending coronary artery
LDH	lactate dehydrogenase
LV	left ventricle
LVP	left ventricular pressure
LVP_{max}	maximal left ventricular pressure
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
PLA	placebo
RPER	remote ischemic perconditioning
POCO	ischemic postconditioning
RIPC	remote ischemic preconditioning
TMBF	transmural myocardial blood flow
s.c.	subcutaneously
TTC	triphenyl-tetrazolium-choride

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12 Curriculum Vitae

The curriculum vitae is not included in the online version for data protection reasons.