

Hydroxyl radicals' production and ECG parameters during ischemia and reperfusion in rat, guinea pig and rabbit isolated heart

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Abstract. Ischemic and reperfusion injury is a serious condition related to numerous biochemical and electrical abnormalities of the myocardium. It has been repeatedly studied in various animal models. In this study, the production of hydroxyl radicals and electrophysiological parameters were compared in three species. Rat, guinea pig and rabbit isolated hearts were perfused according to Langendorff under strictly identical conditions. The heart rate and arrhythmias were monitored during ischemia and reperfusion periods at defined time intervals; the production of hydroxyl radical was determined by HPLC as 2,5-dihydroxybenzoic acid (2,5-DHBA) formed by salicylic acid hydroxylation. Relationship between arrhythmias and production of 2,5-DHBA was studied. The inter-species differences were observed in timing of arrhythmias onset and their severity, and in the production of 2,5-DHBA in both ischemia and reperfusion. The most considerable changes were observed in rats, where arrhythmias appeared early and with highest severity during ischemia on one side and the regular rhythm was restored early and completely during reperfusion. The corresponding changes in the production of 2,5-DHBA were observed. It can be concluded that rat isolated heart is the most suitable model for evaluation of ischemia/reperfusion injury under given experimental conditions.

Key words: Arrhythmia — Hydroxyl radical — Rat — Guinea pig — Rabbit

Introduction

Myocardial ischemia and reperfusion injury is known to be associated with biochemical, electrical and mechanical changes based on damage of cardiomyocytes (Bernier et al. 1986; Ojha et al. 2008). Enhanced generation of reactive oxygen species (ROS) and/or calcium overload have been repeatedly reported to participate in ischemia-reperfusion injury (Bolli 1990; Ferrari et al. 1991). During last two decades, the role of ROS was intensively studied. It has been demonstrated that superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl (OH^{\bullet}) radicals are formed during ischemia-reperfusion injury (Zweier 1988; Bolli 1990), all coming from various sources. Xanthine oxidase, mitochondrial respiratory chain and NADPH oxidase

belong to the most important systems generating radicals (Zweier et al. 2006). Generation of ROS is one of the factors which may play a role in the pathogenesis of cardiac arrhythmias often accompanying myocardial ischemia. It has been reported that ROS are involved in membrane injury leading to electrical abnormalities and arrhythmias (Bernier et al. 1986; Manning 1988; Jeong et al. 2012). ROS are produced during reperfusion period, mainly in its early phase by reintroduction of molecular oxygen into the ischemic myocardium (Bolli 1990). On the other hand, it has been observed recently that the ROS are also formed during ischemia when oxygen delivery is insufficient. Several studies have shown the formation of ROS in ischemic period in various models – in isolated guinea pig hearts (Kevin et al. 2003), in cats during brief ischemia (O'Neill et al. 1996) and in isolated cardiomyocytes during simulated ischemia (Becker 1999).

Among ROS, the hydroxyl radicals are extremely reactive and therefore are considered to be most potent ones in damaging the cells. Direct measurements of hydroxyl

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radicals have been limited primarily by the instability of these radicals. Electron paramagnetic spectroscopy (EPR) associated with spin trapping agents represents a technique usually used for the detection and determination of free radicals, including hydroxyl radicals (Zweier et al 1989). However, this method is rather expensive and requires costly instrumentation. Another approach is the use of trapping agents such as salicylic acid or phenylalanine and detection of the products of their aromatic hydroxylation (Onodera and Ashraf 1991; Sun et al. 1993).

The isolated heart perfused according to Langendorff belongs to golden standard in experimental cardiology. It enables to study various physiological as well as pathological situations including myocardial damage caused by various insults (Verdouw 1988). In the studies focused on ischemia and reperfusion, rat, guinea pig and rabbit are the most frequently employed species. However, their myocardium differs markedly, especially as the role of sarcoplasmic reticulum and equipment of cardiomyocyte membrane with specific ionic channels is concerned (Varro 1993). Moreover, the study comparing these three species at strictly identical experimental conditions during exposure to ischemia and reperfusion is missing in the literature. Therefore, in the present study we aimed to determine the most suitable animal model for evaluation of ischemia-reperfusion injury based on the electrophysiological changes and the production of high reactive hydroxyl radicals. An attention was also pointed on assessment of the relationship between arrhythmias' incidence and hydroxyl radicals' production.

Materials and Methods

Chemicals

All chemicals of analytical grade were obtained from Sigma Company (USA).

Animal models

All experiments were carried out with respect to recommendations of the European Community Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by Local Committee for Animal Treatment at Masaryk University, Faculty of Medicine.

Three species of laboratory animals, namely rat, guinea pig and rabbit, were used in this study. Fifteen Wistar rats (both sexes) weighting 277.5 ± 47.1 g, 10 guinea pigs (both sexes) weighting 325.7 ± 40.2 g, and 6 New Zealand rabbits (both sexes) weighting 3108.3 ± 406.7 g were included. Rats and guinea pigs were deeply anaesthetised by inhalation of ether. After subsequent cervical dislocation, the chest was quickly opened and the heart excised with a sufficiently long

segment of ascending aorta. The rabbits were pre-medicated with *i.m.* injection of diazepam (Apaurin, Krka) in the dose of 2 mg and then anaesthetized by *i.m.* injection of mixture of xylazin (Rometar, Spofa, 2 mg/kg) and ketamin (Narcamon, Spofa, 60 mg/kg). Then, the animals were artificially ventilated through tracheal cannula (ventilator for small laboratory animals, World Precision Instruments, USA), the chest was opened and the heart quickly excised with sufficiently long stump of ascending aorta.

After the isolation, the heart was firmly attached to perfusion set-up *via* the aorta and then placed in thermostat-controlled bath (37°C) filled with Krebs-Henseleit (K-H) solution of following composition (in mM): NaCl 118, NaHCO₃ 24, KCl 4.2, KH₂PO₄ 1.2, MgCl₂ 1.2, glucose 5.5, Taurine 10, and CaCl₂ 1.2. The solution was continuously oxygenated with 95% O₂ and 5% CO₂. The heart was then perfused in the mode of the constant perfusion pressure (80 mmHg) on Langendorff apparatus modified previously in our laboratory (Novakova et al. 2000). All hearts exhibiting any dysrhythmias during control period were discarded from the study.

The experiment consisted of three periods, 20 minutes duration of each: first, the heart was perfused with K-H solution containing salicylic acid (1 mmol/l) – control period. The duration of this stabilization period was sufficient to remove anaesthetic drugs from the heart tissue. Then, the perfusion was stopped – period of ischemia (flow ischemia). Next, the flow of solution was re-introduced – reperfusion period.

Electrogram recording

During the whole experiment, touch-less electrogram was recorded from three orthogonal bipolar leads – X, Y, and Z (Uematsu et al. 1987). Six silver-silver chloride disc electrodes (4 mm in diameter) were placed on the inner surface of the bath in which the heart was placed during the experiment. The signals were amplified by a set of three biological amplifiers DAM50 (World Precision Instruments, USA) and further simultaneously digitized by 16-bit AD converters at a sampling rate of 2000 samples/s using a data acquisition multifunction card PCI-6250 (National Instruments, USA). Data acquisition was controlled by subroutines of a software package LabView. The digital signals were stored on a hard disc for further off-line processing.

The recorded electrograms were analysed and the heart rate (HR) changes were evaluated from manually measured and averaged ten RR intervals at the end of each 5th minute during both ischemia and reperfusion periods. The results were then normalized to the end of control period (100%).

The incidence of arrhythmias was noted, especially their severity, timing and frequency of appearance. Each examined heart was given a score from 0 to 5 according to scale by Haworth et al. (2010). This dysrhythmia score (DS)

is based on Lambeth score (Walker et al. 1988) modified for a determination of the arrhythmia score *per* minute, by using a maximum upper ventricular tachycardia (VT) duration of 60 s. While some VT episodes were thus split between adjacent minutes, this allowed the time course of arrhythmias to be assessed. The DS was as follows: 0, no arrhythmias; 1, 10 s VT or other arrhythmias, no ventricular fibrillation (VF); 2, 11–30 s VT or other arrhythmias, no VF; 3, 31–90 s VT or other arrhythmias, no VF; 4, 91–180 s VT or other arrhythmias and/or 10 s reversible VF; 5, 80 s VT or other arrhythmias and/or 10 s reversible VF; and 6, irreversible VF.

Measurement of hydroxyl radical production

Determination of hydroxyl radicals in perfused hearts was performed using salicylic acid trapping method (Onodera and Ashraf 1991). This method is based on the detection of the hydroxylated derivatives of the salicylic acid added to the perfused medium. It has been reported that 1 mmol/l salicylic acid can trap approximately 10% of the theoretically generated hydroxyl radicals, producing 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) and catechol in proportions of 49, 40, and 11%, respectively (Floyd et al. 1984; Grootwelt and Halliwell 1986). In this study, 2,5-DHBA was used rather than 2,3-DHBA because of its greater sensitivity for quantification of hydroxyl radicals, as previously reported (Wang et al. 2004; Novakova et al. 2008). 2,5-DHBA was analysed in the coronary effluents collected at the end of the control period (after the 20th min of stabilization) and during the 1st, 5th, 10th, 15th, and 20th minutes of the ischemic period and during the 1st, 5th, 10th, 15th, and 20th minutes of the reperfusion period. The samples were collected always during the last 10 s of particular interval. As a control group, rat, guinea pig and rabbit hearts – which were not subject to ischemia-reperfusion insult – were perfused for equal time (60 min) and electrogram recordings were done and coronary effluents were collected at the corresponding intervals. All coronary effluent samples were frozen at –80°C until further analysis.

Determination of 2,5-DHBA was performed by the HPLC coupled with the electrochemical detection (McCabe et al. 1997) with our modifications (Novakova et al. 2008). The HPLC system consisted of high pressure LC-20AD pump (Shimadzu), electrochemical detector Coulochem II with analytical cell model 5010 (ESA, Chelmsford, MA, USA) and Rheodyne 7125 syringe injector (Cotati, CA, USA) with sample loop of 20 μ l. The reversed-phase column was used (LiChrospher 100 RP 18.5 μ m, 100 \times 3 mm I.D., Merck) with precolumn (Separon SGX, 5 μ m) for the separation. Detection of 2,5-DHBA was performed by isocratic elution with the mobile phase containing 20% (v/v) MeOH in the buffer

pH 3.6 (0.03 mol/l citric acid and 0.06 mol/l NaH₂PO₄), flow rate 0.5 ml/min. The mobile phase was filtered through nylon membrane filter (0.2 μ m pore size, SolVac, USA). The analytes were detected on a dual electrode analytical cell with the first electrode set to oxidize the 2,5-DHBA at +250 mV and the second electrode set to oxidize salicylic acid at +750 mV. A guard cell (model 5020) was placed between the pump and sampler at a potential of +775 mV to oxidize contaminants in mobile phase. The ClarityTM (Data Apex, Prague, Czech Republic) was used for the quantification of analytes.

Concentration of 2,5-DHBA in effluents during ischemic period was expressed in nmol/l. The production of 2,5-DHBA during the control and the reperfusion periods was expressed as an amount of 2,5-DHBA accumulated *per* minute (nmol/min).

Statistical analysis

All data are expressed as mean \pm SEM. Paired Student's *t*-test was applied to test significance of data in experimental group. Statistical differences of $p < 0.05$ were considered to be significant. For description of continuous parameters, the average completed by 95% confidence interval was used. The relationship between the radicals' production and DS was described by Pearson correlation coefficient and its statistical significance. Statistical significance of change during ischemic and reperfusion period compared to baseline values was tested by paired *t*-test.

Results

All hearts from control group did exhibit neither electrophysiological changes (such as heart rate changes and/or arrhythmias occurrence) nor production of hydroxyl radicals.

Absolute values of HR at the end of control period in the hearts included in this study were 285 \pm 35, 210 \pm 19 and 157 \pm 23 in rat, guinea pig and rabbit hearts, respectively. All hearts exposed to ischemic insult responded immediately with profound prolongation of RR intervals (Figure 1). In guinea pigs and rabbits, the normalized HR decreased to 28% and 18% of resting values at the end of ischemic period, respectively. In rat, all hearts but one even stopped beating after 5 min of flow ischemia. In rat and guinea pig, this decrease of HR was highly significant ($p < 0.001$), in rabbits it was less intense, however still significant ($p < 0.01$).

During the first five minutes of reperfusion, all rat hearts reinstated beating. At the end of reperfusion period, the HR reached 90%, 80%, and 95% of resting values in rat, guinea pig, and rabbit, respectively. This HR restoration was incomplete in all species after 20 min from perfusion onset;

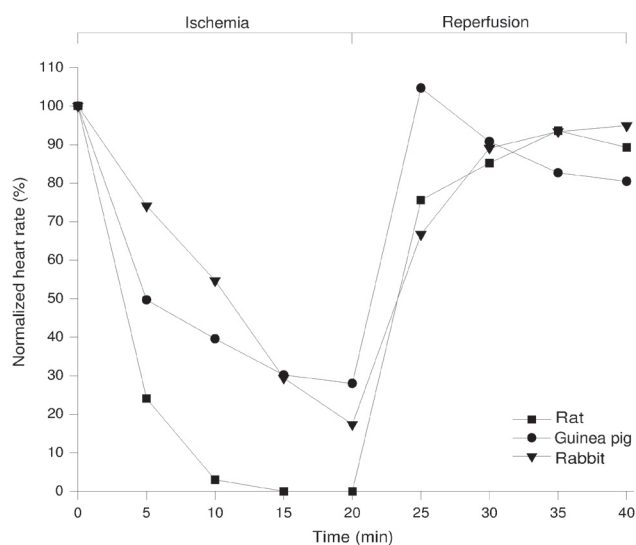


Figure 1. Heart rate changes in rat, guinea pig, and rabbit isolated hearts ($n = 12, 5$ and 5 , respectively) during ischemia and reperfusion. Normalized to the end of control period.

however, the differences from the values at the end of control period were insignificant.

All examined hearts were then classified according to the most severe arrhythmia observed during each experimental period. The values of modified DS are overviewed in Figure 2. As the inter-species differences are concerned, it is obvious that rat hearts respond to ischemia early and with the highest intensity (see Figure 2). On the other hand, the arrhythmias disappear during the first few minutes of reperfusion. The guinea pig hearts respond to ischemia a couple of minutes later and with lower intensity; however, they do not recover from ischemia during the whole reperfusion period (score around 1 at the end of the reper-

fusion). The rabbit hearts react even later, after ten minutes of ischemia, and their full recovery takes approximately 10 min of reperfusion.

As noted above, in rat hearts the incidence of arrhythmias during ischemia was high and the arrhythmias were severe. The most frequent was ventricular fibrillation; it was present in all studied hearts and the total duration of this type of arrhythmia was 47% of ischemic period. Also premature ventricular beats and episodes of AV block of the second and the third degree were frequently observed. All hearts restored spontaneous beating during reperfusion. Most arrhythmias in reperfusion phase were detected within first 10 minutes – ventricular fibrillation (6% of ischemia duration), frequent premature ventricular beats (24 *per* one heart on average) and short episodes of ventricular tachycardia.

In guinea pig hearts, the most frequent arrhythmia was AV blockade of the second and the third degree, present exclusively during ischemia (25% of ischemia period). Short-lasting episodes of ventricular fibrillation were present in two animals out of five; they terminated spontaneously. During reperfusion, very frequently single or multiple premature ventricular beats were observed (47 *per* one heart on average). In one case, short ventricular tachycardia appeared.

The most frequent arrhythmia in rabbit hearts was AV blockade of the second and third degree, present almost entirely in the second part of ischemia and lasting 20% of ischemia duration. In reperfusion period, premature ventricular beats (three animals) and short ventricular tachycardia (one case) were observed.

The basal levels of 2,5-DHBA collected during the 20th minute of control period were negligible in all isolated hearts. The values of 2,5-DHBA concentration produced during 20 minutes of ischemia are shown in Figure 3. It is obvious that remarkable increase of 2,5-DHBA concentration was detected already after the first minute of ischemia in

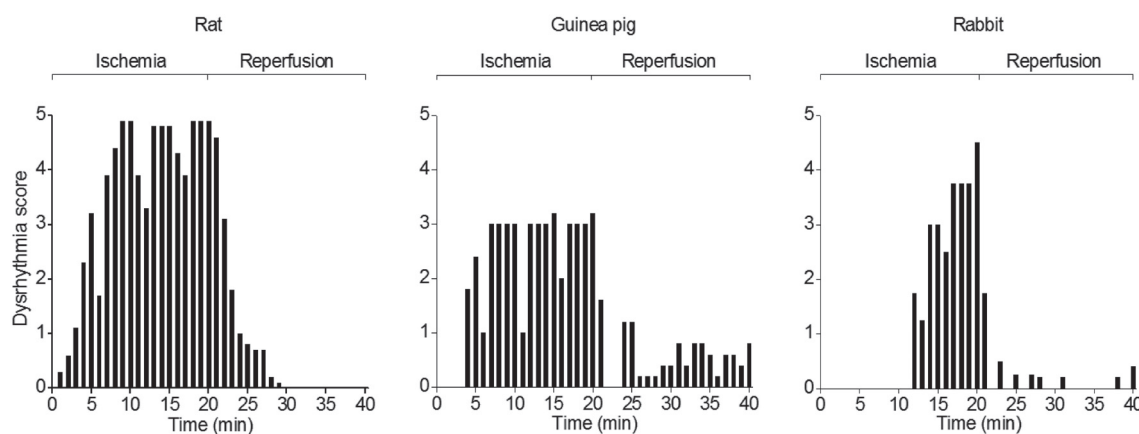


Figure 2. Dysrhythmia score of rat, guinea pig, and rabbit isolated hearts ($n = 12, 5$, and 5 , respectively) during particular minutes of ischemia and reperfusion.

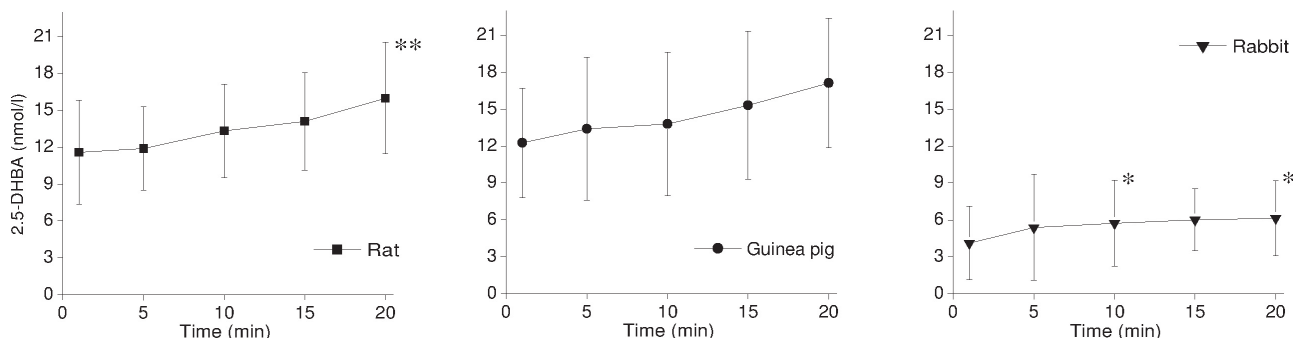


Figure 3. Production of 2.5-DHBA in rat, guinea pig and rabbit ($n = 15, 10,$ and $6,$ respectively) during ischemic period. The results are presented as a mean, 95% CI. Differences of $p < 0.05$ were considered statistically significant. * $p < 0.05$ vs. the first minute of ischemia, ** $p < 0.01$ vs. the first minute of ischemia.

all examined hearts (11.587 ± 8.376 nmol/l, 12.252 ± 7.200 nmol/l, and 4.117 ± 2.850 nmol/l in rats, guinea pigs and rabbits, respectively). The final concentrations with values of 15.991 ± 8.977 nmol/l, 17.119 ± 8.510 nmol/l, and 6.135 ± 2.919 nmol/l were obtained during the 20th minute of ischemia in rat, guinea pig, and rabbit group, respectively. Thus statistically significant, although low increase of 2.5-DHBA between the 1st and the 20th minute was found only in rats and rabbits.

During the reperfusion period the values are expressed as an amount of 2.5-DHBA accumulated *per minute* (Figure 4) because the experiments were performed at the constant perfusion pressure and the flow of the perfusate through coronary system was not constant. The highest amounts of 2.5-DHBA were detected during the 1st min of reperfusion in all examined hearts (0.038 ± 0.020 nmol/min, 0.224 ± 0.141 nmol/min, and 0.059 ± 0.034 nmol/min in rats, guinea pigs and rabbits, respectively). Then, the concentration of 2.5-DHBA was decreasing during reperfusion in all three

groups and the lowest values of 2.5-DHBA were obtained at the end of the reperfusion period. The amounts of 2.5-DHBA during the 20th minute of reperfusion were 0.016 ± 0.011 nmol/min, 0.206 ± 0.157 nmol/min, and 0.053 ± 0.029 nmol/min in rats, guinea pigs and rabbits, respectively. Only in rat, considerable decrease ($p < 0.001$) of the production of hydroxyl radicals was observed. To compare the production of hydroxyl radicals during ischemia and at the beginning of reperfusion, 2.5-DHBA in the 1st minute of reperfusion was expressed also in nmol/l (5.70 ± 3.42 nmol/l, 28.26 ± 14.38 nmol/l, 3.88 ± 2.22 nmol/l in rat, guinea pig and rabbit groups, respectively).

In order to evaluate relationship between the 2.5-DHBA concentration and arrhythmias incidence, the averaged DS (calculated for particular time interval) and subsequent production of 2.5-DHBA has been expressed as Pearson coefficient for both ischemia and reperfusion periods. In ischemia, relationship between averaged DS and corresponding 2.5-DHBA concentration was found in all species.

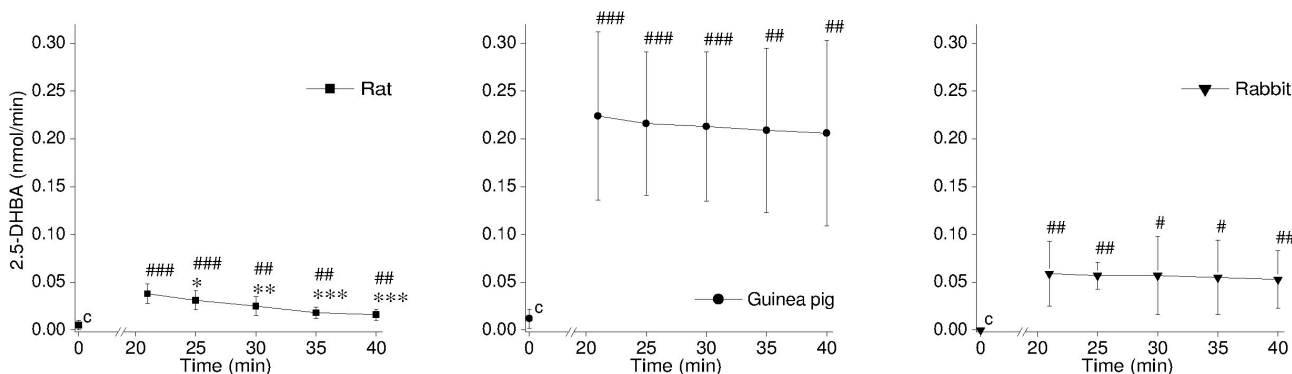


Figure 4. Accumulated amount of 2.5-DHBA in rat, guinea pig and rabbit isolated hearts ($n = 15, 10,$ and $6,$ respectively) during 20 min of reperfusion period. The results are presented as a mean, 95% CI. Differences of $p < 0.05$ were considered statistically significant. c, control value; * $p < 0.05$ vs. the first minute of reperfusion; ** $p < 0.01$ vs. the first minute of reperfusion; *** $p < 0.001$ vs. the first minute of reperfusion; # $p < 0.05$ vs. control period; ## $p < 0.01$ vs. control period; ### $p < 0.001$ vs. control period.

In rat hearts, DS (6–10 min) is related to 2.5-DHBA (in the 10th min), $r = 0.422$. In guinea pig hearts, DS (11–15 min) is related to 2.5-DHBA (in the 15th min), $r = 0.879$. In rabbit hearts, DS (11–15 min) is significantly related to 2.5-DHBA (in the 15th min), $r = 0.436$ ($p < 0.001$) and DS (16–20 min) is related to 2.5-DHBA (in the 20th min), $r = 0.799$. In reperfusion period, the relationship between DS and 2.5-DHBA concentration was found in guinea pigs. In these hearts, DS (11–15 min) is related to 2.5-DHBA (in the 15th min), $r = 0.354$.

Discussion

In this study, the production of hydroxyl radical and electrogram parameters were evaluated during ischemia-reperfusion injury in the model of isolated heart perfused according to Langendorff. Special attention has been paid to inter-species differences. It is worth mentioning that all experiments at isolated heart – electrogram recording and perfusate collection – were performed at strictly identical conditions.

The changes of HR and the incidence, severity and timing of arrhythmias were analysed from electrogram recorded by touch-less method. Significant slowing of spontaneous cardiac activity has been observed in all hearts. This result was expected and served for verification of our model, especially as a proof of sufficient duration of ischemic period. No inter-species differences have been found in this part of the study.

As the arrhythmias are concerned, marked differences were present namely in timing of arrhythmias onset and their severity. These inter-species differences can be most probably attributed to different equipment of cardiac cells with specific ionic channels. Their impaired gating during ischemia-reperfusion may result in increased arrhythmias incidence. Rat, guinea pig and rabbit hearts differ mainly in repolarization part of action potential. Guinea pig ventricular cardiomyocytes do not develop transient outward current; on the other hand, it is present in rat and in about half of the rabbit cardiac ventricular cells. Delayed rectifier current of quite high amplitude is reported in guinea pig cardiomyocytes, but it is negligible or even absent in rat and rabbit. Inward rectifier potassium current is similar in rabbit and guinea pig (Varro et al. 1993). Thus, different response to ischemia and reperfusion in our experiments may be caused by the effect on characteristics of delayed rectifier and/or transient outward currents.

Increased production of ROS in ischemic animal heart has been reported in various models (O'Neill et al. 1996; Becker 1999; Kevin et al. 2003) and it has been explained as a consequence of derangement of the mitochondrial electron transport system and decrease of energy status

due to lack of oxygen (Dhalla and Duhamel 2007). Among ROS, hydroxyl radicals are the most reactive ones. Salicylic trapping method was chosen for their detection in our experiment (Onodera and Ashraf 1991) and production of 2.5-DHBA was determined as a measure of hydroxyl radical formation.

During the ischemia onset remarkable increase of 2.5-DHBA concentration was detected in all examined hearts, indicating presence of oxidative stress. During the following 19 min, further increase was detected in all hearts; however, it was statistically significant only for rats and rabbits. These values are comparable with results of Bao et al. (2007) who reported that in rabbits the production of 2.5-DHBA measured by microdialysis technique reached during 30 min of ischemia was in tens of nmol/l.

During the reperfusion period, the production of 2.5-DHBA was measured in the first minute and then in each consecutive 5-min interval. The highest values of 2.5-DHBA concentration in effluent were obtained in the first minute; however, these values at least partially corresponded to 2.5-DHBA that was accumulated in the perfusate during the previous ischemic period. Nevertheless, the increased level of 2.5-DHBA in the effluent after the 5th minute of reperfusion and later can be ascribed to its new formation and implicates the production of hydroxyl radicals also as a consequence of reperfusion. This finding corresponds with previous reports where reintroduction of oxygen into an ischemic heart is related to accelerated production of ROS in the first few minutes of reperfusion (Zweier et al. 1988; Cai et al. 1995; Ravingerova et al. 1999; Merrill G. 2001). It is difficult to assess whether the production of hydroxyl radicals was higher during ischemia or at the beginning of reperfusion since the experiment was done at constant perfusion pressure and thus the coronary flow was not controlled. Therefore, the values of 2.5-DHBA were expressed in different units in ischemia and reperfusion periods. In order to compare at least partially 2.5-DHBA production, we expressed its values – measured in the first minute of reperfusion – also in nmol/l. Taken from this point of view, only in guinea pig higher production of hydroxyl radicals was detected in reperfusion than in ischemia. The production of 2.5-DHBA during on-going reperfusion had descending character in all three groups and the lowest values of 2.5-DHBA were obtained at the end of the reperfusion period. Only in rat, considerable decrease of the production of hydroxyl radicals during 20 min reperfusion was observed. This inter-species difference might indicate that rat hearts are more resistant to reperfusion injury and their recovery is therefore faster.

Also other inter-species differences have been observed in our model. In ischemia, the production of hydroxyl radicals was at least twice higher in rat and guinea pigs than in rabbits. During reperfusion, the hydroxyl radical production was lower in rabbit and rat comparing to guinea pig. In the

first minute of reperfusion, production of hydroxyl radicals was around 5 times higher in guinea pig than in the other two studied species and the increased values persisted over all reperfusion period. These differences might be explained by various sensitivity of animal hearts to the oxidation stress and ischemia-reperfusion injury.

It was found in this study that the incidence of ischemic and reperfusion arrhythmias is associated with the production of hydroxyl radicals. In ischemia, tight relationship between dysrhythmia score and 2.5-DHBA was observed in the hearts of all studied species. The same relationship was observed in guinea pig hearts during reperfusion. These results indicate that the hydroxyl radicals might participate in the pathogenesis of arrhythmias. It corresponds with study of Ravingerova et al. (1999) who described that reperfusion induced arrhythmias are coincided with generation of H₂O₂.

In this paper, the model for studying ischemia-reperfusion injury in isolated heart perfused according to Langendorff was proposed and used under strictly identical experimental conditions in three species of laboratory animals. Since the response to ischemia-reperfusion insult monitored by arrhythmias incidence, their severity and timing, hydroxyl radicals' production and recovery from ischemia was most considerable in rat isolated heart, it may be concluded that rat heart is the most suitable model for evaluation of ischemia-reperfusion injury in this experimental model. Moreover, tight relationship between the arrhythmias incidence and severity and hydroxyl radicals' production in this experimental model has been proven.

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