Heart preservation

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2018

Document Version:
Publisher's PDF, also known as Version of record

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DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Belfragesalen, BMC D15, Lund

November 16th, 2018, 09:15.

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Organization LUND UNIVERSITY	Document name DOCTORAL DISSERTATION
	Date of issue 2018.11.16
Author: Guangqi Qin	Sponsoring organization

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In study I, pre-stretched coronary arterial segments were studied in organ baths. Contractions were induced with 16 combinations of four different concentrations of potassium (16, 23, 30, and 127 mmol/L) at four different temperatures (37, 22, 15, 8 °C). Magnesium with concentrations from 0-20 mmol/L was used to attenuate the contraction induced with potassium (16, 23, 30, and 40 mmol/L) at 37 °C. The contractions caused by potassium can be minimized when combining hypothermia and/or elevated magnesium.

In study II, a specially designed sealed perfusion system was used to investigate the oxygen consumption of aerobically perfused cardioplegic hearts. At 37 °C, each 100 g of heart tissue consumes 1.1 mL/min oxygen at rest; and the oxygen consumption is reduced to 53%, 30%, 19%, and 15%, when the temperatures were 30, 22, 15, and 8 °C, respectively.

In study III and IV, porcine hearts were perfused either 8 hours (continuously or intermittently) or 24 hours (with alternating pressures of 20 and 10 mmHg), using an oxygenated hyperkalemic perfusate containing erythrocytes, albumin, and hormones. The coronary arteries of the perfused hearts were investigated in organ baths and compared to fresh controls. The ventricular function of the 24 hours perfused hearts was similarly investigated. The intact coronary arterial endothelial function was preserved after both 8 hours and 24 hours of perfusion. The myocardial function was also fully preserved after 24 hours of perfusion.

In conclusion, with well-established perfusion preservation, the heart can be safely preserved for 24 hours.

Key words: Coronary artery contraction, Cardiac oxygen consumption, Heart preservation, Perfusion preservation, Coronary endothelial function, Myocardial function

Classification system and/or index terms (if any)

Supplementary bibliographical information	Language English
ISSN and key title: 1652-8220	ISBN: 978-91-7619-696-0

Recipient's notes	Number of pages: 92	Price
	Security classification	

Signature  Date 2018-10-05

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LUND
UNIVERSITY

Faculty of Medicine Doctoral Dissertation Series 2018:128
Department of Cardiothoracic Surgery

ISBN 978-91-7619-696-0
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2018

MADE IN SWEDEN

海纳百川，有容乃大

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林则徐

The reason for the sea being so wide is that it can accept all rivers

The reason for the cliff being so tall is that it can stand firm without any desire

Zexu Lin

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Qin G, Sjöberg T, Liao Q, Sun X, Steen S
Scandinavian Cardiovascular Journal, 2016; 50(5-6):362-366.

IV. **Intact coronary artery endothelial function and myocardial contractility after 24 hours of non-ischemic heart preservation**

Qin G, Wohlfart B, Zuo L, Hu J, Sjöberg T, Steen S
In submission

Abstract

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In conclusion, with well-established perfusion preservation, the heart can be safely preserved for 24 hours.

Popular Summary

Heart transplantation is a procedure to treat end-stage heart disease, and very often the only way. There are more than 5000 heart transplants performed each year in the world according to the registration of the society of heart and lung transplantation. According to the registration of the Scandiatransplant association (including Denmark, Finland, Iceland, Norway, Sweden, and Estonia), 86 heart transplants were performed the first half of 2018, and 113 patients were on the waiting list as of July 1st, 2018. There is a severe donor heart shortage.

At present, a donor heart to be used in transplantation is flushed with 4°C cardioplegic solution to induce cardiac arrest and then kept in the same solution, normally using an icebox, during transportation to the recipient. This is called cold ischemic storage, which means there is no oxygen or nutrient supply to the heart. In this way, the heart can be safely preserved for 4 hours. For donors less than 35 years old and recipients younger than 55 years, the preservation time may be extended to 5-6 hours. However, the longer preservation time is associated with higher mortality within one year after transplantation. If the safe preservation time can be extended, donor heart can be transported from far away, and this will increase the number of available hearts. A better preservation method may also allow hearts from older donors to be used, at least for recipients of a similar age.

A logical way to improve heart preservation time is to provide oxygen and nutrients during preservation. This can be achieved with perfusion preservation, in which the heart is perfused with solutions containing nutrients and oxygen during the transportation and implantation. Using this method, Steen and his team were able to safely transplant porcine hearts harvested 24 hours after brain death and then preserved for 24 hours before being transplanted. All the recipient pigs had normal heart function and urine production during the 24 hours follow-up period after transplantation.

The aim of the studies included in this thesis is to investigate this perfusion preservation method.

In study I, the effect of different concentrations of potassium and magnesium on the contractions of coronary arteries was investigated at different temperatures. Elevated potassium in the cardioplegic solution is to induce cardiac arrest. However, high concentrations of potassium also induce coronary ve ssel

contraction and hinder the perfusion of the heart. Reducing the temperature and/or increasing the magnesium content can counteract the contraction induced by potassium. Combinations of potassium, magnesium, and temperature were recommended for optimal coronary perfusion according to the results of this study.

In study II, the amount of oxygen needed by the arrested heart at different temperatures was investigated. A specially designed sealed perfusion system was used to accurately measure the oxygen consumption. The oxygen consumption of each 100 g heart tissue was shown to decrease significantly with temperature, from 1.1 mL/min at 37°C to 0.16 at 8°C.

In studies III and IV, pig hearts were stored and perfused for 8 and 24 hours with different perfusion strategies. Thereafter, the function of the heart tissue was studied in organ baths. The results show that the function of both the coronary arteries and the heart muscles were fully preserved after 24 hours.

Introduction

Heart transplantation is a procedure to treat intractable end-stage heart disease. There are more than 5000 heart transplants performed in the world each year according to the ISHLT registration [1]. According to the registration of the Scandiatransplant association (including Denmark, Finland, Iceland, Norway, Sweden, and Estonia), 86 heart transplants were performed the first half of 2018, 113 patients were on the waiting list as of July 1st, 2018 [2]. There is a severe donor heart shortage.

At present, the donor heart is preserved with a combination of cardioplegia and hypothermia. In practice, the heart is flushed with a 4°C cardioplegic solution followed with cold ischemic storage in the same solution. The safe preservation time is 4 hours. For donors less than 35 years old and recipient younger than 55 years, the preservation time may be extended to 5-6 hours. A prolonged ischemic time is associated with an increased risk of recipient 1-year mortality [1]. The limited preservation time is one of the causes of the donor heart shortage.

The logic way to extend the safe preservation time is non-ischemic cardioplegic perfusion. During the perfusion, the heart receives oxygen and the essential metabolic substrates. Therefore, it is important to know the oxygen consumption of the whole heart to establish safe perfusion preservation.

A prerequisite for a successful preservation of a donor heart is to protect the cardiomyocytes and the coronary vasculature during explantation, transportation, and implantation. The excitation-contraction coupling, contraction, and relaxation of cardiomyocytes strictly depend on the cellular calcium circulation [3]. Thus, the properties of the Ca^{2+} channels, exchangers, transporters, and their locations on the membranes of the cardiomyocytes, transverse tubules, and sarcoplasmic reticulum must be well preserved.

Coronary endothelium, a monolayer of cells on the inner surface of the coronary vessels, is probably the most vulnerable structure of the heart. It produces a variety of substances that regulate vascular permeability, vessel tone, coagulation, fibrinolysis, and inflammation response. The endothelium-dependent relaxation function is impaired after being preserved in 4°C cardioplegic solution for 6 hours [4]. The impaired endothelium-dependent relaxation function is associated with high coronary vascular resistance [5].

In a recent study, orthotopic transplantation was performed with porcine donor hearts harvested 24 hours after brain death and perfused at 8°C for 24 hours. The hearts were perfused with a hyperkalemic cardioplegic nutrition solution containing, albumin, oxygenated erythrocytes, and hormones. All recipient pigs had normal heart function during the 24 hours follow-up period after transplantation [6].

This perfusion preservation method seems to be a solution to preserve cardiac function for an extended time, and this thesis focuses on certain details in connection with extended heart preservation.

Aim

The overall aim of this thesis was to study the effects of non-ischemic perfusion preservation of the porcine heart for up to 24 hours. Detailed aims for the included works are:

I. To investigate how high concentrations of potassium can be safely used in cardioplegic solutions, without causing severe coronary artery vaso-contraction, and to evaluate the role of magnesium in avoiding potassium-induced vasoconstriction.

II. To measure the oxygen consumption of perfusion-preserved cardioplegic porcine hearts at 37, 30, 22, 15, and 8°C.

III. To study the endothelium-dependent relaxation and the contractile function of the coronary arteries after 8 hours of heart preservation with non-ischemic perfusion.

IV. To investigate the endothelium-dependent relaxation in the coronary arteries and the myocardial contractility after 24 hours of non-ischemic heart preservation.

Materials and Methods

Designs of the studies (in brief)

I. The contractility and its attenuation of the distal part of the left anterior descending coronary arteries from fresh porcine hearts were studied in organ baths. The study consists of two parts. In the first part of the study, the contractions were induced with 127, 16, 23 and 30 mmol/L of potassium at 37 °C, and at either 22, 15 or 8 ℃ . In the second part, different concentrations of magnesium (0-20 mmol/L) were used to attenuate 16, 23, 30 and 40 mmol/L potassium-induced vaso-contractions at 37 °C.

II. The oxygen consumption of cardioplegic porcine hearts receiving continuous antegrade perfusion was studied. The perfusion system was temperature-controlled and sealed to prevent gas exchange with the environment. The oxygen consumption was measured at 37, 30, 22, 15, and 8 °C. With fully saturated erythrocytes in the perfusate, the oxygenator was excluded from the circuit and blood gases were drawn periodically until the saturation of erythrocytes was less than 20%. The oxygen consumptions were calculated when the oxygen saturation was between 80% and 60% during which time the desaturation was linear.

III. After either continuous or intermittent (15 minute perfusion and 60 minute non-perfusion) perfusion of the heart for 8 hours at 8°C, the contractility and endothelium-dependent relaxation of coronary artery segments from the preserved hearts were studied in organ baths and compared with those of fresh controls.

IV. Porcine hearts received perfusion for 24 hours at 8°C in cycles of 15 minute perfusion with a pressure of 20 mmHg, 40 minute perfusion with a pressure of 10 mmHg and 5 minute non-perfusion. The endothelium-dependent relaxation of coronary artery segments and myocardial contractility of ventricular trabeculae from the preserved hearts were studied in organ baths and compared with those of fresh controls.

Animals and Ethics

Swedish domestic pigs were used in all four studies. The mean body weight and numbers of the animals were 50 kg and 24 in study I; 50 kg and 30 in study II; 56 kg and 24 in study III; and 51 kg and 17 in study IV.

All animals received care in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Directive 2010/63/EU). The Ethics Committee for Animal Research of the University of Lund approved the studies (No. M389-12 and M174-15).

Anesthesia and Ventilation

Anesthesia was introduced with an intramuscular injection of atropine 0.5 mg, xylazine 100 mg, and ketamine 20 mg/kg body weight; and an intravenous injection of fentanyl 4 μg/kg body weight and midazolam 0.4 mg/kg body weight, andit was maintained with a continuous intravenous infusion of ketamine (10 mg/kg/h) and rocuronium bromide (1.5 mg/kg/h)

Tracheal cannulation was performed. A volume-controlled and pressure-regulated ventilation was managed with a FiO_2 of 0.5, PEEP 5 cmH_2O, and a tidal volume of 7-9 ml/kg body weight. The end-tidal CO_2 was between 4.8 to 5.3 kPa (36.0-39.7 mmHg).

Surgical preparation

After median sternotomy and intravenous injection of heparin 500 IU/kg body weight, 500 ml of either St. Thomas cardioplegic solution no. 2 (I, II, IV) or an albumin-containing cardioplegic solution (III, IV) were used to induce cardiac arrest. In the fresh control groups of study III, ventricular fibrillation was induced with a current pulse from a 9V battery applied to the surface of the heart. The left anterior descending coronary arteries were excised immediately and gently flushed with 4°C Krebs solution. The suitable thin trabeculae were excised after cardiac arrest induced with cold St. Thomas cardioplegic solution.

Organ Bath (I, III, IV)

The main components of the organ baths were: a heater-cooler unit, water mantled Perspex baths, one metal holder attached to a Grass FT03 transducer, another holder attached to a movable unit, a computer with a data recording software, a solution replacing system, and a gas bubbling system (Figure 1 and 3).

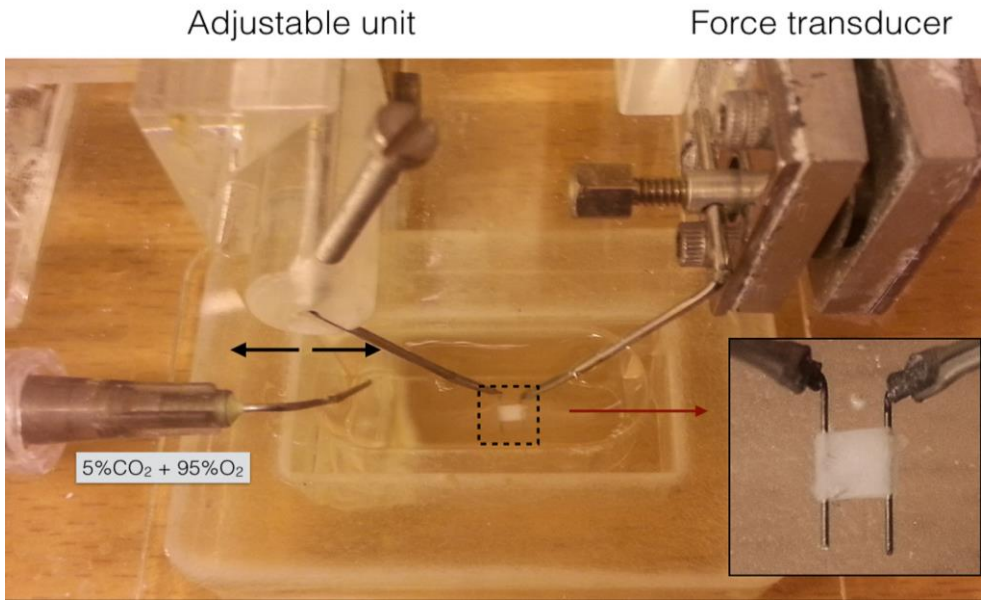

Figure 1. Organ Bath with a coronary artery segment mounted
The coronary artery segment was suspended between two needle holders and submerged in 5 ml Krebs solution bubbled with 5% CO_2 and 95% O_2. The adjustable unit is used to repeatedly stretch the vessel segment until the basal tension was reached. The force transducer measures the force (vessel tension) and sends the signal to a computer.

The solutions used in organ baths are shown in Table 1. In the modified Krebs solutions, the increase in potassium concentration was compensated by the same molar reduction of Na^+.

Table 1. The composition of the solutions used in the organ bath
To control pH and supply oxygen, a mixed gas of 5% CO_2 and 95% O_2 was used to bubble the solution.

Ingredients (mmol/L)	Normal Krebs	127 mM K^+-Krebs	16 Mm K^+-Krebs	23 mM K^+-Krebs	30 mM K^+-Krebs	40 mM K^+-Krebs
NaCl	119	0	107.6	100.6	93.6	83.6
NaHCO3	18	18	18	18	18	18
KCl	4.6	127	16	23	30	40
NaH2PO4	1.2	1.2	0-16	0-16	0-16	0-16
MgCl2	1.2	1.2	1.2	1.2	1.2	1.2
CaCl2	1.5	1.5	1.5	1.5	1.5	1.5
Glucose	11	11	11	11	11	11
Studies	I, III, IV	I, III, IV	I	I	I	I

For the coronary artery segments used in studies I, III and IV, a basal tension of 8-10 mN was used. Pilot studies have shown that the maximal response of the coronary segments could be achieved at this tension (Figure 2).

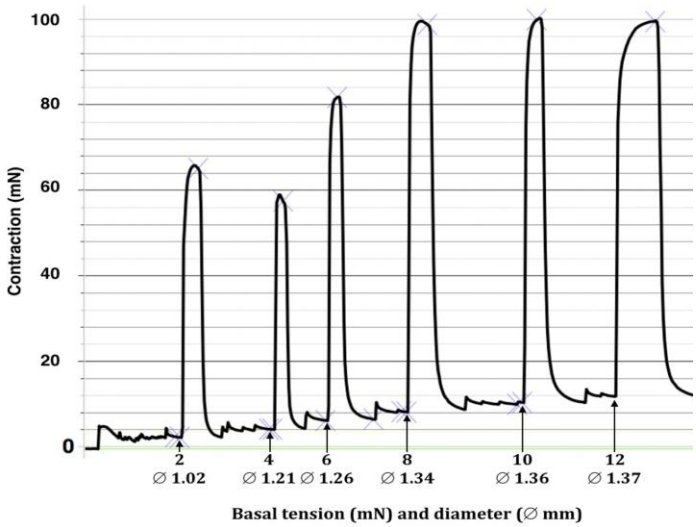

Figure 2. Basal tension and 127 mmol/L K^+ induced contractions
Maximal contraction could be obtained when the basal tension was 8-12 mN. Therefore, a basal tension of 8-10 mN was used.

In study I, the contractions were elicited by potassium with concentrations of 127, 16, 23 and 30 mmol/L at 37°C and at either 22°C, 15°C or 8°C. The relaxing effect of different Mg^{2+} concentrations on potassium-induced contractions were elicited at 37°C after using potassium concentrations of 16, 23, 30 and 40 mmol/L.

In study III and IV, coronary artery ring segments were contracted with a thromboxane A2 analog (U46619). After a plateau of the contraction was reached, substance P (10^{-9} - 10^{-4} M) was cumulatively added to the bath to elicit endothelium-dependent relaxations. Endothelium-independent relaxation was elicited with 10^{-4} Papaverine.

In study IV, trabeculae were suspended by means of preformed suture loops on the metal holders in the baths and stimulated with electric pulses (Figure 3). The optimum muscle length for force production was established for each sample by stretching the muscle in small steps and monitoring the force (Frank-Starling law). Peak force (PF), time from stimulus to peak force (TPF) and time from peak force to half relaxation (THR) were measured. Test contractions were induced with stimuli at 10 different intervals (from 0.2 s to 1.0 s), to measure post-extrasystolic potentiation and its calcium recirculation (Figure 4).

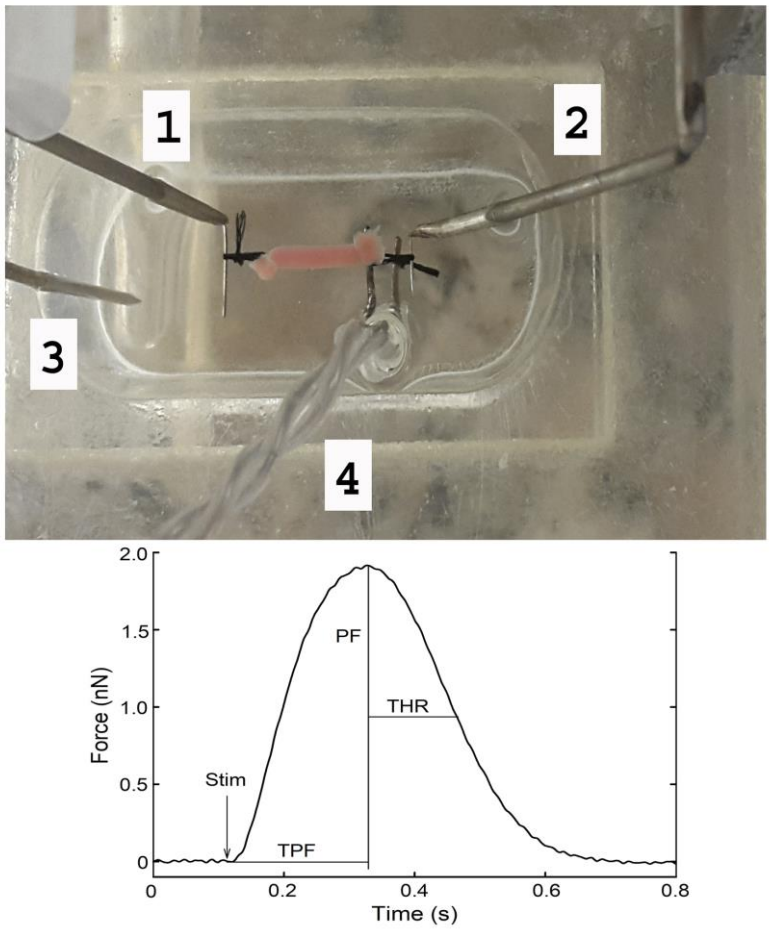

Figure 3. Trabecula in an organ bath and a typical curve of contraction.
In the upper panel, 1 and 2 are needle holders to attach the preformed loops. 3 is the tube for gas supply consisting of 5% CO_2 and 95% of O_2. The trabecular was submerged in 5ml Krebs solution. The stimulus was delivered using a pair of electrodes (4) placed under the muscle. The stimulus frequency was 30/min and had an amplitude of 150% of the threshold value. Measurements were made at a frequency of 60/min. A typical contraction curve is shown in the lower panel, where Stim is the stimulus, TPF is the time from stimulus to peak force, PF is the peak force, and THR is the time from peak force to half relaxation.

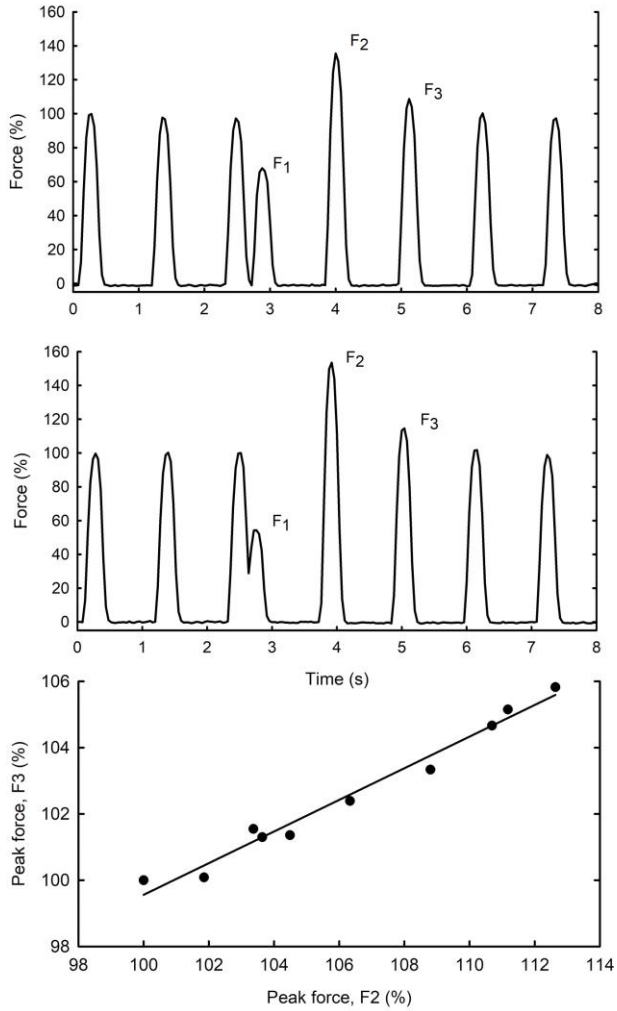

Figure 4. Electrically induced test contractions
The test contraction (10 different intervals in each experiment) was denoted F1 and followed by F2 and F3 back at 60/min. The lower panel shows an example of the linear relation between F3 and F2 based on ten pairs of values. The slope of the regression line was used to quantify the recirculation.

Airtight perfusion system (II)

The main components of the perfusion system were two roller pumps, one oxygenator, two heater-cooler units, a 3-liter resealable plastic bag, and a water bath (Figure 5). One pump perfused the heart (140 ml/min) while the other pump continuously mixed the perfusate (500 ml/min).

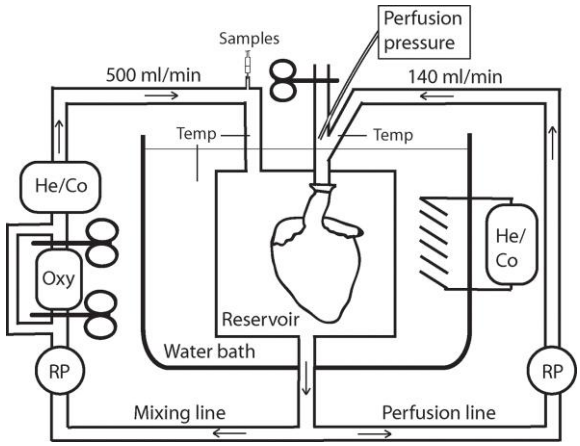

Figure 5.Schematic drawing of the sealed perfusion system.
The reservoir was submerged into a temperature controlled water bath to maintain the temperature and heat distribution. Oxygenator was bypassed with clamps during sample time. Temp is temperature probe; He/Co is heater-cooler unit; Oxy is oxygenator; RP is roller pump.

Non-ischemic perfusion system (III, IV)

The main components of the system were an automatic pressure and flow-controlled perfusion system, an automatic gas exchange system, a leucocyte filter, an arterial filter, a heater-cooler unit, an autonomous power unit, and a programmable sequencer (Figure 6). During the experiment, the perfusion pressures were set at either 20 mmHg (III) or alternating between 20 mmHg and 10 mmHg (IV). The system was filled with 3 L of perfusate.

Figure 6. The non-ischemic perfusion system
The single-use unit on the right was built before each experiment.

Perfusate (II, III, IV)

The perfusate was an albumin-containing hyper-oncotic potassium-cardioplegic nutrition-hormone solution with washed and leucocyte-filtered erythrocytes from a compatible pig blood donor (Table 2). Samples of the perfusate were collected periodically for oxygen consumption calculation (II), blood gas analysis (II, III, and IV), and hemolysis test (III, IV).

Table 2. The composition of the perfusate
The perfusate contains a physiological amount of Noradrenaline, Adrenalin, Triiodothyronine, and Cortisol. High oncotic pressure is achieved with albumin to avoid edema during the perfusion.

Na^+	136 mmol/L
K^+	23 mmol/L
Ca^{2+}	1.3 mmol/L
Mg^{2+}	8.0 mmol/L
Cl^-	142 mmol/L
HCO_3^-	25 mmol/L
PO_4^{2-}	1.3 mmol/L
D-Glucose	6.3 mmol/L
Albumin	75 g/L
Cocaine	6 nmol/L
Noradrenaline	6 nmol/L
Adrenalin	6 nmol/L
Triiodothyronine	3 nmol/L
Cortisol	420 nmol/L
Insulin	8 IU/L
Heparin	5000 IU/3L
Imipenem	20 mg/L
Hematocrit	10-15%
$95\%O_2+5\%CO_2$	0.2 L/min

Oxygen Consumption Calculation (II)

The formula used to calculate oxygen consumption (MVO_2), measured in mL/min/100g heart weight, was:

$$MVO_2 = (\Delta sO_2 \times Hb \times 1.34 + \Delta pO_2 \times 7.5 \times 0.03) \times 100/W \times volume / time.$$

Where Δ is the difference between two consecutive samplings, sO_2 is oxygen saturation (%), pO2 is the partial pressure of oxygen (kPa), W is the heart weight (g), and volume is the perfusate volume (L).

Wet weight / Dry weight (III, IV)

After 8 or 24 hours of perfusion, the myocardium was sliced into small pieces and weighed before and after being parched in the heating cabinet. The water content was (weight before – weight after)/weight before.

Data analysis

Results were expressed as the mean ± standard error of the mean, if not otherwise is stated as SD (standard deviation). Two-tailed Student's *t*-test for unpaired data was used for single comparison between two groups. Comparisons of basal tension at different temperatures between four groups were made using one-way ANOVA with Tukey's Multiple Comparison Test. ($p = 0.05$).

Results

The sizes of the coronary artery segments (I, III, IV) and trabeculae preparation (IV).

The size of the coronary artery segments (Table 3) and trabeculae used in the organ baths did not vary significantly between experimental groups and their control groups in all three studies. The trabeculae preparations had a length of 3.8 ± 1.2 (SD) mm and a diameter of 0.59 ± 0.17 (SD) mm.

Table 3. The sizes of the coronary artery segments.
Results are given as Mean ± SEM. (n) is the number of the animals.

Studies	Diameter (mm)			Length (mm) (n)
	Group 1 (n)	Group 2 (n)	Group 3 (n)	
Study I	1.43 ± 0.02 (6)	1.46 ± 0.03 (6)	1.44 ± 0.04 (6)	2.57 ± 0.02 (18)
Study III	1.51 ± 0.04 (8)	1.51 ± 0.04 (8)	1.56 ± 0.08 (8)	2.57 ± 0.02 (24)
Study IV	1.27 ± 0.04 (6)	1.26 ± 0.05 (6)	------	2.09 ± 0.02 (12)

Potassium-induced contraction of the coronary artery (I)

The basal tension of the coronary segments at different temperatures are 9.92 ± 0.09, 9.12 ± 0.22, 8.64 ± 0.42, and 7.85 ± 0.17 mN at 37, 22, 15, and 8 °C, respectively (Figure 7). When cooling the vessel segments from 37 °C to lower temperatures, the basal tension decreased with decreasing temperatures. The differences were significant between all groups, except 22 and 15 °C. This indicates that reducing temperature decreases the tone of the coronary arteries.

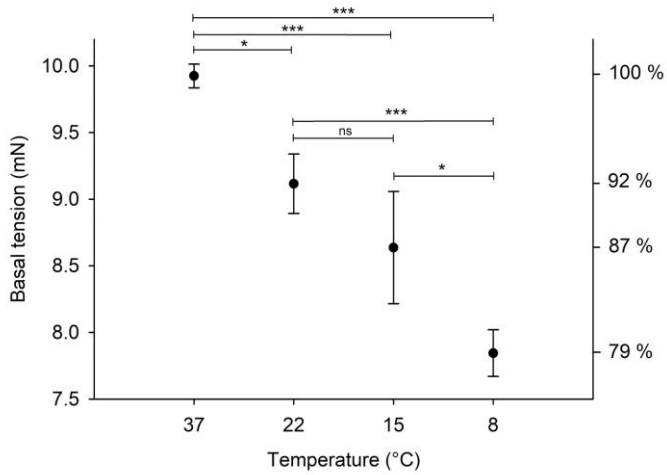

Figure 7. Basal tension at different temperatures
The right axis shows the percentage of the different contractions when the basal tension at 37 °C was defined as 100%. Mean ± SEM, n = 6. * p<0.05, ** p<0.01, *** p<0.001.

Potassium-induced contractions at different temperatures

The contraction (in mN) and its percentage (Figure 8) of contraction induced with 127 mmol/L potassium at 37 °C are shown in Table 4. At the same temperature, higher concentrations of potassium induced stronger contractions. With the same concentration of potassium, the higher temperatures did the same.

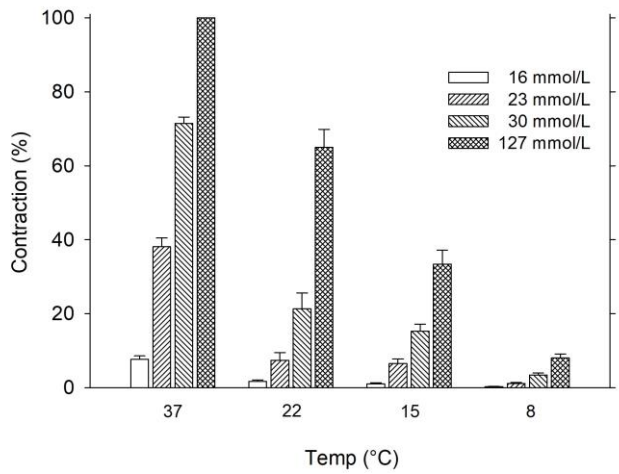

Figure 8. Contractions induced with different concentrations of potassium at different temperatures
The left axis shows the percentage of the contraction when the contraction induced with 127 mmol/L K^+ at 37 °C was defined as 100%. Mean ± SEM, n = 6.

Table 4. The contractions induced with different concentrations of potassium at different temperatures
The values are given in mN and the percentage of the 127 mmol/L K^+ induced contraction at 37 ℃ . Mean ± SEM.

Temp.	n	127 mmol/L		16 mmol/L		23 mmol/L		30 mmol/L	
37°C	18	60.1 ± 1.6	100%	4.7 ± 0.6	7.7%	22.8 ± 1.6	38%	42.8 ± 1.4	72%
22℃	6	42.8 ± 3.2	65%	1.1 ± 0.3	1.7%	4.7 ± 1.2	7.4%	13.8 ± 2.5	21%
15℃	6	19.0 ± 2.4	33%	0.6 ± 0.2	1%	3.7 ± 0.9	6.6%	8.8 ± 1.5	15%
8℃	6	8.0 ± 1.1	14%	0.3 ± 0.	0.6%	1.2 ± 0.3	2.1%	3.4 ± 0.5	6%

Magnesium-induced vaso-relaxation

Increasing concentrations of magnesium caused concentration-depended reduction of potassium-induced vasocontraction of pre-contracted vessel segments at 37 °C (Figure 9).

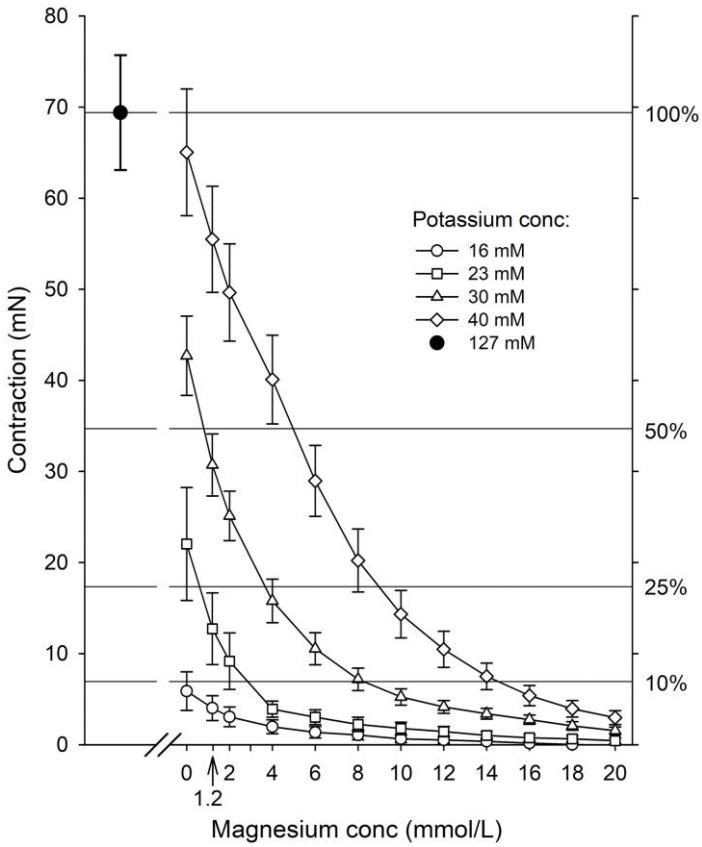

Figure 9. Magnesium-induced vaso-relaxation
The symbol with the filled circle is the 127 mmol/L K^+ induced contraction, defined as 100%. 1.2 mmol/L magnesium, which is physiological level was also used. Mean ± SEM, n = 6.

Oxygen consumption (II)

Blood gas analysis

Blood gas analysis results when the oxygen saturation (sO_2) of the hemoglobin in the perfusate were close to 80% and 60% are shown in Table 5. The values of pH, the partial pressure of oxygen (pO_2) and sO_2 were used for oxygen consumption calculation.

Table 5. Blood gas analysis of the perfusate
The values of pH, the partial pressure of oxygen (pO_2), and the partial pressure of CO_2 (pCO_2) are temperature corrected. ΔpO_2 is the difference of the partial pressure of oxygen when oxygen saturation (sO_2) was close to 80% and 60%. Mean ± SEM, n = 6.

Temp (°C)	sO_2 (%)	pH	pO_2 (kPa)	ΔpO_2	pCO_2 (kPa)	Glucose (mmol/L)	Lactate (mmol/L)
37	79.0 ± 1.6	7.36 ± 0.01	6.91 ± 0.22	1.7	6.02 ± 0.15	6.6 ± 0.0	0.3 ± 0.1
	60.7 ± 2.2	7.35 ± 0.01	5.21 ± 0.16		6.20 ± 0.17	6.5 ± 0.1	0.3 ± 0.1
30	78.9 ± 0.8	7.37 ± 0.01	4.70 ± 0.06	1.28	5.52 ± 0.07	6.3 ± 0.1	0.3 ± 0.1
	60.8 ± 1.0	7.36 ± 0.01	3.42 ± 0.05		5.67 ± 0.08	6.3 ± 0.1	0.3 ± 0.0
22	78.2 ± 1.6	7.36 ± 0.01	3.08 ± 0.12	0.87	5.05 ± 0.02	6.4 ± 0.1	0.3 ± 0.0
	60.3 ± 0.7	7.35 ± 0.01	2.21 ± 0.05		5.08 ± 0.03	6.4 ± 0.1	0.3 ± 0.0
15	78.7 ± 0.8	7.33 ± 0.01	2.13 ± 0.04	0.63	4.65 ± 0.09	6.5 ± 0.1	0.4 ± 0.1
	60.8 ± 0.7	7.33 ± 0.01	1.51 ± 0.03		4.70 ± 0.10	6.5 ± 0.1	0.4 ± 0.1
8	79.4 ± 1.2	7.23 ± 0.01	2.40 ± 0.08	0.76	5.88 ± 0.08	6.4 ± 0.1	0.2 ± 0.0
	59.6 ± 1.4	7.22 ± 0.01	1.64 ± 0.04		5.95 ± 0.07	6.4 ± 0.1	0.2 ± 0.0

The oxygen saturation curve and the disassociation curve based on all the results of the blood gas analysis are shown in Figures 10 and 11. To control the experimental time, 700 mL perfusate was used in the 8 °C group; and 1200 ml was used in the 37 °C group; 1000 mL was used in the other three groups. It took 180 minutes to desaturate the erythrocyte to 20% saturation, even though the perfusate volume was 700 mL, whereas the 37 °C group reached 20% saturation after only 40 minutes, even with a perfusate volume of 1200 mL.

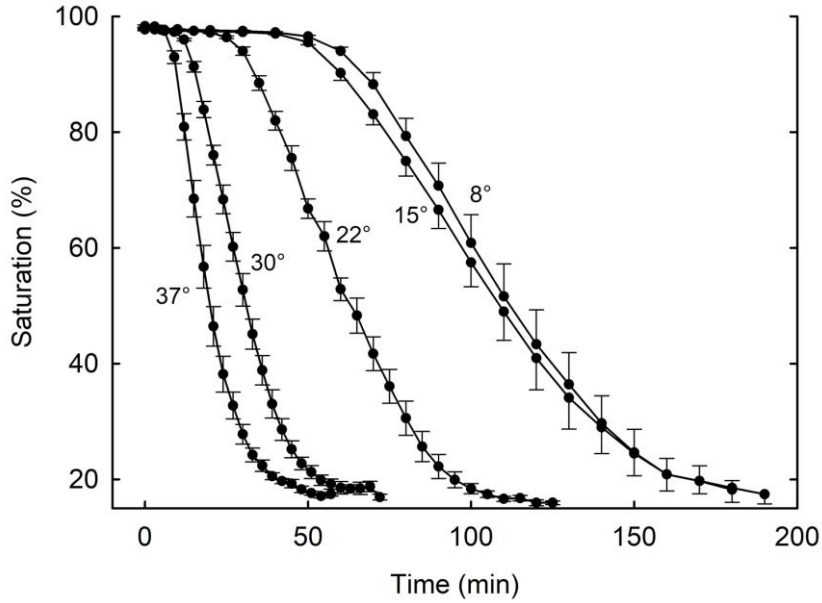

Figure 10. Oxygen saturation of the hemoglobin in the perfusate
The rate of the oxygen desaturation decreased with decreasing temperature. Mean ± SEM, n = 6.

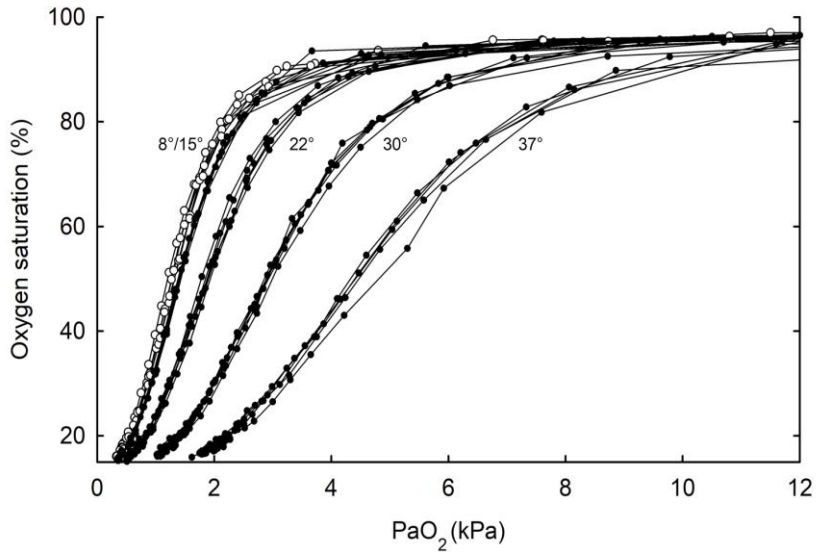

Figure 11 Dissociation curve of the hemoglobin in the perfusate
The curves are based on all the blood gas analysis. PaO_2 is the partial pressure of oxygen. The curves marked with empty circles are from the 8 °C group.

Oxygen consumption

The oxygen consumptions given as mL/min/100 g heart tissue were 1.10 ± 0.04 at 37 °C, 0.58 ± 0.02 at 30 °C, 0.33 ± 0.01 at 22 °C, 0.21 ± 0.01 at 15 °C and 0.16 ± 0.02 at 8 ℃ (Figure 12). Decreasing the temperature from 37 to 8 °C reduced the oxygen consumption of a cardioplegic-perfused heart by 85%.

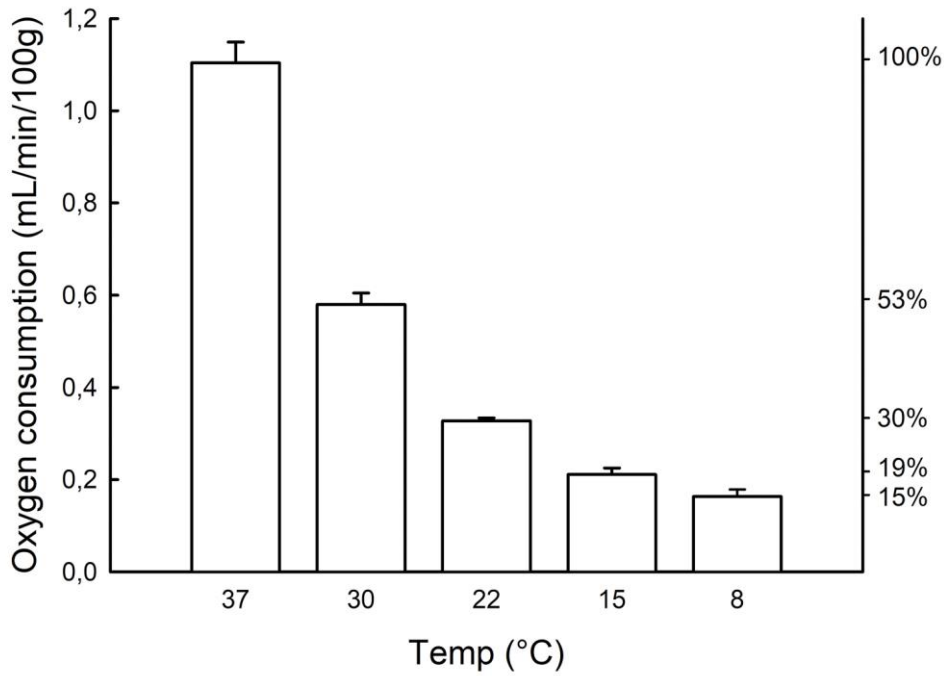

Figure 12. Oxygen consumption of cardioplegic perfused heart
The right axis shows the percentage of the oxygen consumption compared the consumption at 37 °C. Mean ± SEM, n= 6.

Non-ischemic preservation (III, IV)

Blood gas analysis and free hemoglobin during 8 hours of preservation (III)

The results from the blood gas analysis are shown in Table 6. A slight increase of lactate was observed during 8 hours of intermittent perfusion, while there is no increase in lactate during 8 hours of continuous perfusion. The acid-base balance

was well maintained during the preservation in both groups. The perfusion didn't cause any hemolysis.

Table 6. Blood gas after 2 and 8 hours of perfusion
The values of pH, the partial pressure of O_2 (pO_2), and the partial pressure of CO_2 (pCO_2) are temperature corrected. Mean ± SEM, n = 6.

	Intermittent perfusion		Continuous perfusion	
	2 Hours	**8 Hours**	**2 Hours**	**8 Hours**
Hematocrit (%)	16.9 ± 0.6	17.1 ± 0.4	10.9 ± 0.4	10.9 ± 0.5
Oxygen Saturation(%)	96.4 ± 0.5	96.8 ± 0.3	97.5 ±0.2	97.6 ± 0.2
pCO_2(kPa)	6.1 ± 0.1	6.4 ±0.1	5.3 ± 0.3	6.4 ± 0.1
pH	7.26 ± 0.01	7.23 ± 0.01	7.30 ± 0.02	7.22 ± 0.01
$HCO3^-$ (mmol/L)	23.9 ± 0.5	23.1 ± 0.4	24.0 ± 0.3	24.3 ± 0.5
Glucose (mmol/L)	7.9 ± 0.5	7.9 ± 0.5	7.1 ± 0.2	7.2 ± 0.2
Lactate (mmol/L)	1.0 ± 0.3	1.9 ± 0.3	0.3 ± 0.1	0.2 ± 0.1
Free Hemoglobin (g/L)	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.3 ± 0.1

Blood gas analysis during 24 hour preservation (IV)

No hemolysis occurred during 24 hours of preservation. Lactate was slightly increased. Acid-base was well maintained. (Table 7).

Table 7. Blood gas analysis during 24 hour perfusion
Base values were obtained before the heart was connected to the system. pH, the partial pressure of O_2 (pO_2), and the partial pressure of CO_2 (pCO_2) are temperature corrected values. Mean ± SEM, n = 6.

	Base	0.5 hours	4 hours	8 hours	12 hours	24 hours
Hematocrit (%)	10.2±0.3	10.2±0.3	11.2±0.3	11.3±0.3	11.5±0.0	11.9±0.0
Oxygen Saturation(%)	97.7±0.1	97.6±0.2	97.3±0.1	97.6±0.1	97.4±0.1	97.3±0.1
pCO_2(kPa)	5.3±0.2	5.4±0.2	5.9±0.1	6.0±0.0	6.0±0.0	6.0±0.1
pH	7.26±0.02	7.26±0.01	7.22±0.01	7.21±0.05	7.21±0.01	7.23±0.01
$HCO3^-$ (mmol/L)	22.2±0.9	22.7±0.4	22.1±0.4	22.0±0.5	22.0±0.4	23.0±0.4
Glucose (mmol/L)	6.5±0.2	6.5±0.2	6.5±0.2	6.5±0.2	6.4±0.1	6.4±0.2
Lactate (mmol/L)	0.00	0.16±0.06	0.16±0.06	0.18±0.06	0.20±0.04	0.20±0.04
Free Hemoglobin (g/L)	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0

Coronary artery contraction and endothelium-dependent relaxation after 8 hour preservation (III)

There were no significant differences in the contraction, endothelium-dependent relaxation, or endothelium-independent relaxation (induced with papaverine) among the three groups (Table 8). The endothelium-dependent relaxation curve is shown in Figure 13.

Table 8. Coronary artery contraction and endothelium-dependent relaxation (EDR) after 8 hours of preservation
pEC50 is the negative logarithm of the concentration of substance P giving half-maximal relaxation. Mean ± SEM, n= 8.

	Fresh controls	Intermittent	Continuous
Contraction with 127mM K [b]-Krebs (mN)	53.01 ± 3.18	52.09 ± 3.74	57.37 ± 3.21
Contraction with U-46619 (mN)	36.30 ± 3.11	41.04 ± 1.42	43.57 ± 2.55
Max EDR (%)	93.3 ± 1.3	95.7 ± 1.5	95.8 ± 0.5
pEC50	6.21 ± 0.11	6.31 ± 0.06	6.44 ± 0.07
Relaxation with Papaverine (%)	104.2 ± 0.3	103.3 ± 0.7	101.7 ± 0.2

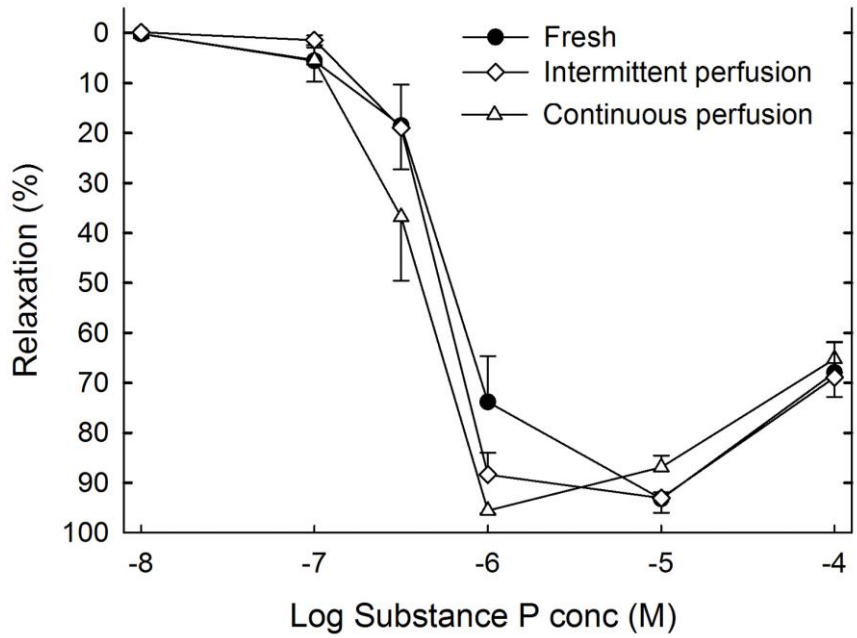

Figure 13. Coronary endothelium-dependent relaxation after 8 hour perfusion
Endothelium-dependent relaxation was elicited by Substance P on a thromboxane A_2-induced contraction. Log Substance P conc is the logarithm of the concentration of substance P. Mean ± SEM, n= 8.

Myocardial contraction and coronary artery endothelium-dependent relaxation after 24 hour preservation (IV)

There were no significant differences of the endothelium-dependent relaxation, endothelium-independent relaxation, the peak force of myocardium contraction, the time from stimulus to peak force, the time from peak to half relaxation, potentiation, or calcium recirculation between the 24 hour perfusion group and the fresh control group (Table 9). The endothelium-dependent relaxation curve is shown in Figure 14.

Table 9. Coronary endothelium-dependent relaxation (EDR) and characteristics of myocardial contractions after 24 hours of preservation

pEC50 is the negative logarithm of the concentration of substance P giving half-maximal relaxation. PF is the peak force. TPF is the time from stimulus to the peak force. THR is the time from the peak force to half relaxation. Each calculation of the recirculation was based on 10 different test intervals. Mean ± SEM, n is the number of hearts.

	Fresh (n)	**24 hours (n)**	***p*= 0.05**
Max-EDR (%)	93.1 ± 1.8 (6)	91.2 ± 1.2 (6)	ns
pEC50	6.36 ± 0.10 (6)	6.43 ± 0.08 (6)	ns
Relaxation with Papaverine	104 ± 0.5%	103 ± 0.5%	ns
PF (mN/mm2)	17.3 ± 1.7 (11)	15.6 ± 2.5 (6)	ns
TPF (ms)	226 ± 16 (11)	225 ± 15 (6)	ns
THR(ms)	128 ± 11 (11)	155 ± 15 (6)	ns
Potentiation (%)	137 ± 5 (11)	129 ± 5 (6)	ns
Recirculation (fraction)	0.44 ± 0.06 (11)	0.46 ± 0.01 (6)	ns

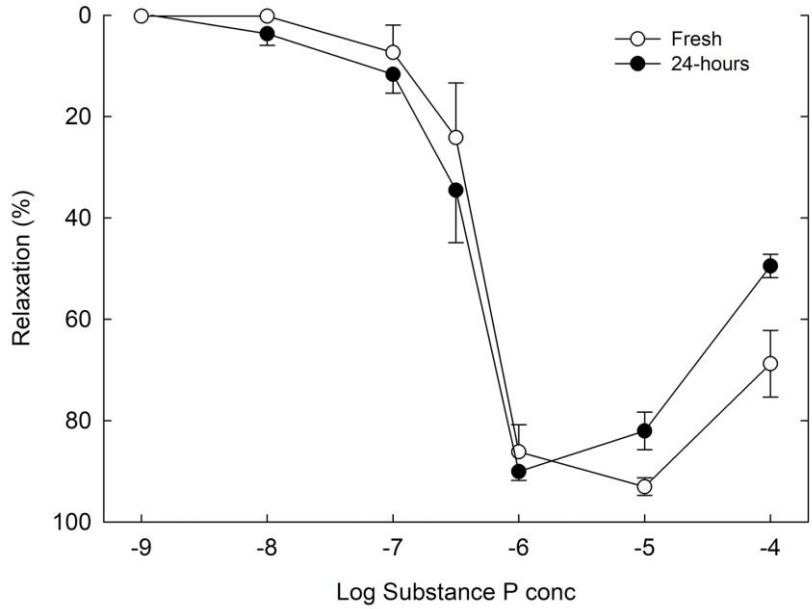

Figure 14. Coronary endothelium-dependent relaxation after 24 hours of preservation
Endothelium-dependent relaxation was elicited by Substance P on a thromboxane A2-induced contraction. Log Substance P conc is the logarithm of the concentration of substance P. Mean ± SEM, n= 6.

Water content of the myocardium (III, IV)

There was no significant difference of myocardial water content after neither 8 hours nor 24 hours of preservation compared to their controls (Table 10). This indicates that no myocardial edema occurred.

Table 10. The water content of myocardium after preservation
Mean ± SEM, n is the number of hearts.

Groups	Water content
Fresh Controls	79.5 ± 0.2 % (8)
8 hour Intermittent	79.0 ± 0.4 % (8)
8 hour continuous	79.0 ± 0.3 % (8)
24 hour variable	79.3 ± 0.2 % (6)

Discussion

Currently, the most commonly used cardioplegic and heart preservation solutions contain potassium of a concentration of over 15 mmol/L [7], such as St. Thomas cardioplegic solution no. 2, which contains 16 mmol/L potassium and 16 mmol/L magnesium. The elevated concentrations of potassium depolarize the membrane potential of cardiomyocytes from -85 mV to around -55 mV, where the fast Na^+ channels are inactivated, resulting in diastolic arrest [8]. High potassium concentrations may induce coronary vasocontraction. A study on isolated porcine pulmonary arterial segments shows that high potassium content in the organ preservation solution causes strong vasocontraction, especially at normal body temperature [9]. When measuring the oxygen consumption of dog hearts at different temperatures using in situ perfusion with a heart-lung machine, Chitwood found that transmural blood flow decreased significantly (from 1.27 to 0.74 ml/min/g heart weight) just by replacing normal blood flow with blood cardioplegia. However, after cooling below 32 °C, the flow returned to the control level [10]. Magnesium, nature's physiological calcium blocker, is able to attenuate the effect of calcium on myocardial contractility, impulse formation, and smooth muscular tone [11]. Within the muscle cells, magnesium appears to inhibit sarcoplasmic reticulum (SR) from releasing calcium, it even seems to accelerate the SR's uptake of calcium. Magnesium can also compete with calcium to bind troponin C and myosin [12, 13]. By increasing the magnesium concentration in a hyperkalemic cardioplegic solution, a strong contraction of the coronary arteries can be avoided. However, the relationship of the potassium concentration and the magnitude of the coronary arterial contraction at different temperatures remains unknown, as well as the relationship between the magnesium concentrations and the coronary contractions.

In study I, when decreasing the temperature of the baths, a reduction of basal tension was observed. This indicates that hypothermia relaxes coronary vascular tone, and by doing so, may prevent potential coronary vasoconstriction during the introduction of hyperkalemic cardioplegia.

During warm blood cardioplegia, using potassium over 23 mmol/L may induce strong vasocontraction if magnesium is not included. Magnesium at 8 mmol/L effectively attenuates vasocontraction for potassium concentrations up to 30 mmol/L.

Oxygen consumption of a heart is normally measured with either perfusion of an isolated whole heart or in situ during cardiopulmonary bypass [14]. By measuring the heart weight, the coronary flow, and the oxygen content in the aorta and coronary sinus, the oxygen consumption is calculated as mL/min per 100 g of heart tissue [5, 10, 15]. However, coronary flow cannot be accurately measured with this method. It is well known that not all the blood supplying the myocardium enters the heart through the left and right coronary arteries. Functional coronary collaterals are present in 23% of normal coronary vessels, and complete filling of main epicardial recipient vessels is seen in approximately 85% of patients with chronic total occlusion of coronary arteries [16]. In addition, there are small cardiac veins draining directly into any of the four chambers of the heart. This means the methods mentioned above give an indication, but cannot accurately measure the oxygen consumption of the whole heart. By the previously-used methods, it has been estimated that the arrested heart requires 0.6-1.85 ml O_2/min/100g, 80-90% less than does the normal beating heart. The additional hypothermia reduces the oxygen consumption further to 0.31 ml/min/100g at 22 °C, and 0.135 ml/min/100g at 10-12 °C [17].

For accurate measurement of the oxygen consumption, we designed a sealed perfusion system as described in the materials and methods [II].

To control if the system was airtight, the sealed perfusion system was setup without a heart, and the erythrocytes in the perfusate were desaturated to less than 20% by supplying a mixed gas of 93 % nitrogen and 7% carbon dioxide to the oxygenator. The oxygenator was then bypassed, and blood samples were taken every 10 minutes for blood gas analysis. A minor oxygen leakage could be observed as an oxygen re-saturation in the perfusate. When the oxygen saturation had reached to 60-80%, the oxygen partial pressure was between 0.63-1.7 kPa in the perfusate, the leakage was 0.082, 0.072, 0.066, 0.060, and 0.042 mL/min at temperatures of 37, 30, 22, 15, and 8 °C, respectively. These oxygen leakage values were used to correct the results obtained during the perfusion of the whole isolated heart.

As shown in the results, the effect of temperature on the oxygen consumption was not linear. A cardioplegic heart requires 1.1 mL/min/100g to maintain the basal metabolism at 37 ℃ . When defining the oxygen consumption of a cardioplegic heart at 37 °C as 100%, it was found that temperature steps from 37 to 30, 30 to 22, 22 to 15, and 15 to 8 °C corresponded to reductions in oxygen consumption of 47%, 23%, 11%, and 4%, respectively.

For successful non-ischemic perfusion preservation, several factors need to be taken into consideration: the composition and temperature of the perfusate; the

perfusion pressure and flow; and the perfusion strategy (continuous or intermittent). The perfusate shall meet the following criteria. First, it must have the capacity to carry adequate oxygen during the perfusion and reserve a sufficient amount if the perfusion is interrupted. In addition, the perfusate shall have the capacity to counterbalance the perfusion pressure with oncotic pressure to prevent edema. Furthermore, it must have a buffering capacity to maintain acid-base balance throughout the preservation time. The preservation method used in studies III and IV has been used in a recently published study demonstrating safe preservation for 24 hours at 8℃ [6].

Erythrocytes have a large capacity to carry oxygen. For a healthy person, only about 3% of the total oxygen is transported in a dissolved state, while 97% is transported bound to hemoglobin. Moreover, a large amount of HCO_3^- is produced inside the erythrocytes [18]. Therefore, in addition to carrying sufficient oxygen, erythrocytes, when exposing to carbogen (5% CO_2 + 95% O_2), help maintain the acid-base balance. Human albumin can increase the oncotic pressure to prevent potential edema caused by the perfusion. It can also bind water, cations, fatty acids, hormones, thyroxine, and pharmaceuticals. The physiological concentration of noradrenaline, adrenaline, cortisone, insulin, and thyroxine were used to mimic the condition the heart was normally exposed to. Cocaine was used to block the reuptake of the noradrenaline into the synaptic nerve terminals to maintain noradrenaline concentration at the receptor level [19].

In clinical practice, ischemic heart storage using 4°C cardioplegic solution is the gold standard [20]. As shown in study II, profound hypothermia during cardioplegia can minimize metabolic activity, which is crucially important for cold ischemic storage. However, the ideal preservation temperature is still under debate, especially for perfusion preservation, where the oxygen demand can be fully met. This may allow temperatures higher than 4 °C, even up to normothermia, to be used during perfusion preservation. Studies indicate that preservation of lungs at 10°C is superior to preservation at 15 and 4℃ [21]. It is also suggested that cardiac function is better preserved at temperatures higher than 8°C during blood cardioplegia [22-24] A recently published study using perfusion preservation successfully preserved heart function for 24 hours at 8°C [6]. The same method was adopted in studies III and IV.

In study III and IV, we used a perfusion pressure of 20 mmHg for several reasons. Firstly, with antegrade perfusion through the aorta, 20 mmHg perfusion pressure is able to maintain aortic valve closure. Secondly, no edema occurs with 20 mmHg perfusion pressure using a perfusate containing 75 g/L albumin, as demonstrated in a recent study [6]. In addition, the perfusion pressure at 20 mmHg gives a coronary flow of 75-100 ml/min/100g heart tissue; this can guarantee thorough

perfusion of the whole heart even in the presence of aortic valve insufficiency, if the left ventricle is effectively drained.

The vascular endothelium plays a critical role in multiple processes, including regulation of vascular tone, anticoagulation, and inflammation response. Endothelial cells produce endothelium-derived relaxing factors, including nitric oxide (NO), prostacyclin and endothelium-derived contracting factors, to regulate the vascular tone. When the endothelium is impaired, its ability to release NO and prostacyclin decreases and even worse, its ability to release endothelium-derived contracting factors may be enhanced [25, 26]. Consequently, vasospasm may occur, leading to inadequate organ perfusion after transplantation [27]. In the present studies, after perfusion preservation, the endothelium-dependent relaxation function of coronary arterial segments was studied in organ baths. U46619, substance P, and papaverine were used to induce contractions and relaxations.

U46619, a thromboxane A_2 analog, is able to induce contraction in different types of smooth muscles via the thromboxane A_2 receptor [28]. In the present studies, a stable contraction of the coronary artery segments was induced with 3×10^{-8} mol/L U46619, corresponding to 60-80% of the contraction induced by a total depolarization of the smooth muscle membranes by potassium. This contraction can be counteracted with endothelium-dependent relaxing factors. Substance P is an endothelium-dependent vasodilator, which acts on an endothelial NK-1 receptor and induces the release of the endothelium-dependent relaxing factor NO, leading to relaxation of vascular smooth muscles [29]. This relaxing effect is strictly dependent on the presence of endothelial cells [30, 31]. Papaverine is a non-selective smooth muscle relaxant, which is commonly used in the hospital to treat vessel spasm [32, 33]. The relaxing effect, which is endothelium-independent, may be due to papaverine stabilizing the L-type Ca^{2+} channels in the inactivated state [34]. Papaverine at a concentration of 10^{-4} mol/L was used in the present studies after endothelium-dependent relaxation was induced with substance P, to confirm the total relaxation capacity of coronary artery segments.

After both 8 hours of continuous (III) or intermittent (IV) and 24 hours variable perfusion (IV), no significant differences in endothelium-dependent relaxation function were found compared to fresh controls, indicating the endothelium was intact after the perfusion.

Myocardial function, including contractility and relaxing ability, is the first priority for heart preservation. Calcium plays a major role in excitation-contraction coupling, contraction, relaxation, energy consumption, and cell death of the cardiomyocytes [35]. It is crucially important to preserve all the membranes

(membranes of myocytes, transverse tubules (T-tubules), the sarcoplasmic reticulum, and mitochondria); including their ion-channels, ion-exchanger, and transporters, to maintain normal intracellular and transmembrane calcium circulation [36].

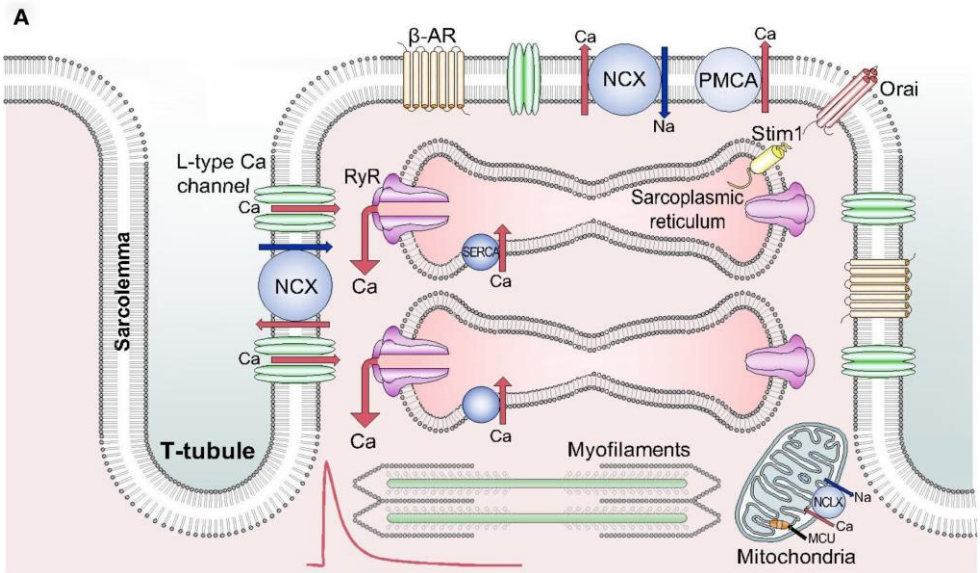

Figure 15. Structures involved in [Ca^{2+}] circulation [3]. [Eisner 2017]
Schematic diagram. This shows cell membrane (sarcolemma), transverse tubule (T-tubule), sarcoplasmic reticulum, and mitochondria, as well as the various channels exchangers and transporters.

The membrane depolarization induced by an action potential opens the L-type calcium channels (Figure 15) on the cell membrane and the membrane of T-tubules, which leads to an influx of Ca^{2+}. The increasing level of Ca^{2+} causes an opening of calcium-releasing channels on the sarcoplasmic reticulum, resulting in a large release of Ca^{2+} release from the sarcoplasmic reticulum. Ca^{2+} binds to troponin, which initiates the contraction process. Thereafter, while the L-type calcium channels and calcium-releasing channels on the SR close, the Ca^{2+} is pumped back into SR by the means of Ca^{2+}-ATPase, and out of the cell mostly by means of the Sodium-calcium exchange (NCX). [3] The strength and duration of a cardiac contraction depends on the amount of calcium that binds to troponin. During diastole, a complete relaxation relies on the quick decline of cytosol Ca^{2+}. The disruption of any step of the calcium circulation will lead to myocardial dysfunction. Injured T-tubules and/or SR is commonly observed in ventricular myocytes of failing human and animal hearts [37, 38], which are associated with compromised contractility [36].

Ventricular trabeculae are intact collections of longitudinal cardiomyocytes covered by endocardium, which, according to their size, makes them ideal for myocardial studies in organ baths [39, 40]. To investigate myocardial function, ventricular trabeculae with diameters less than 0.9 mm were used in study IV, which allows the whole preparation to get oxygen and nutrients by direct diffusion. In study IV, both the coronary vascular endothelial and myocardial functions were studied in preparations obtained from the same heart after 24 hours of perfusion preservation.

By means of electrical stimulation of myocardium, the peak force, time from stimulus to the peak force, and time from peak force to half relaxation of the trabeculae were recorded. To further investigate if the calcium circulation is fully preserved, the potentiation and the calcium recirculation fraction (decline of potentiation) were obtained with a series of test stimuli, which were given with 10 different intervals (from 0.2s to 1.0s). The intervals of the test stimuli were shorter than regular stimuli, like an extra heartbeat. The test contraction (F1) (Figure 4) caused by the test stimulus started during the decline of Ca^{2+}, and consequently, was reduced in amplitude and duration compared to regular contractions. The first contraction (F2) and second contraction (F3) following the test contraction were stronger than regular contraction due to the superimposing of Ca^{2+}. The linear relationship between F3 and F2, representing the decline of potentiation, was also calculated and compared to fresh controls. The results indicate that contractility, the relaxing ability, and the calcium circulations of myocardium were well preserved after 24 hours of perfusion.

Conclusions

- For pre-stretched coronary arteries, both temperature and potassium concentration affect the vascular tone. When the potassium concentration is higher than 16 mmol/L at normothermia, the risk of coronary vasoconstriction increases. The effect can be mitigated with hypothermia.

- Magnesium is able to eliminate the coronary vascular contraction induced with potassium at 37 °C. When using a potassium concentration lower than 23 mmol/L, 8 mmol/L magnesium is enough to attenuate 90% of the contraction.

- The oxygen consumption of cardioplegic hearts is 1.1 mL/min/100g at 37 ℃ ; it reduced by 47%, 70%, 79% and 85% at temperatures of 30, 22, 15, 8 °C, respectively.

- Endothelium-dependent relaxation function can be fully preserved with perfusion preservation for 24 hours.

- The contractility and the relaxing ability of cardiac muscle, as well as the calcium circulation of the cardiomyocytes, can be fully preserved with 24 hours of perfusion preservation.

Acknowledgments

This work will not be possible without the help and support of many people. I wish to express my sincere appreciation to them all.

First and foremost, I would like to thank my main supervisor Stig Steen and my co-supervisor Trygve Sjöberg, who gave me the opportunity to be part of their research team, for their guidance and support. They led me through the Ph.D. program with their profound knowledge and enormous patience.

I would also like to thank Audrius Paskevicius, Björn Wohlfart, Qiuming Liao and everybody of our research team, who have always been pleasant to work with, for all their invaluable contribution to the studies.

I am very grateful to Ådne, Matt, Gunilla, and everybody of the big family at Igelosa, who have always been helping and supporting me since the day I came to Sweden.

My sincere thanks goes to Harry Pigot for the proofreading of this thesis.

Finally, my great gratitude goes to my family, for their endless support and love.

The studies in this thesis were supported by grants from the Hans-Gabriel and Alice Trolle-Wachtmeister's Foundation for Medical Research, Skåne University Hospital, and the Swedish Heart Lung Foundation.

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Printed by Media-Tryck, Lund 2018 NORDIC SWAN ECOLABEL 3041 0903

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Department of Cardiothoracic Surgery

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2018:128
ISBN 978-91-7619-696-0
ISSN 1652-8220