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ORIGINAL ARTICLE

Oral β-glucan reduces infarction size and improves regional contractile function in a porcine ischaemia/reperfusion model

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Abstract

OBJECTIVES: We previously reported a cardioprotective effect of oral β-glucan in patients who underwent coronary artery bypass grafting. The present study was conducted to determine whether oral β-glucan could reduce myocardial infarction size and whether these changes would be reflected by better preservation of contractile indices measured by speckle tracking echocardiography (STE).

METHODS: Fourteen pigs were randomized to receive oral β-glucan 50 mg/kg ($n = 7$) or placebo (control, $n = 7$) 10 days before they were anaesthetized and subjected to 1 h clamping of the left anterior descending coronary artery followed by reperfusion for 3 h. Longitudinal strain, circumferential strain and radial strain were assessed by STE after 3 h of reperfusion. Infarction size and area at risk were determined by Evans blue and 2,3,5-triphenyltetrazolium chloride staining.

RESULTS: Pretreatment with β-glucan reduced the infarct area/area at risk ratio by 36% ($P < 0.05$) and the total necrotic area of the left ventricle by 37% ($P < 0.05$) compared with controls. Viable myocardium at risk was 30% higher in the β-glucan vs. control group ($P < 0.05$). Anterior apical strain values for β-glucan vs. control were −4.7 ± 9.4 vs. 5.9 ± 6.1% ($P < 0.05$) for longitudinal strain, −14.7 ± 6.6 vs. −7.7 ± 4.3 ($P < 0.05$) for circumferential strain, 15.1 ± 7.7 vs. 7.1 ± 11.8 (ns) for radial strain.

CONCLUSIONS: Oral β-glucan pretreatment reduces infarction size and improves regional contractile function in a porcine ischaemia/reperfusion model.

Keywords: β-Glucan • Infarction • Ischaemia • Pigs • Reperfusion • Speckle-tracking echocardiography

BASIC SCIENCE

INTRODUCTION

The innate immune response plays a central role in myocardial ischaemia/reperfusion injury and represents a coveted target for those involved in the treatment of ischaemic heart disease [1]. β-Glucan is a glucose polymer found in the fungal cellular wall, which because of its conserved microbial structure will trigger host immunity through germ-line-encoded receptors such as dectin-1, complement receptor 3, scavenger receptors, lactocylceramide and toll-like receptors [2, 3]. Although the detailed mechanisms behind the effect remain elusive, pretreatment with β-glucan has been introduced into different ischaemia/reperfusion models with promising results [4, 5]. We previously demonstrated the safety and tolerability of pretreatment with oral β-glucan in patients scheduled for coronary artery bypass grafting (CABG) [6]. In the previous study, patients pretreated with a relatively low dose of β-glucan exhibited a dose-dependent reduction in postoperative cardiac troponin T and creatine kinase myocardial bound levels, indicating a dose-dependent cardioprotective effect of oral β-glucan. However, measures of cardiomyocyte injury markers following cardiac surgery may be an inaccurate method to evaluate responses to cardioprotective therapy, since the release of these markers are influenced not only by ischaemia/reperfusion per se, but also by the CPB-induced systemic inflammation, mechanical cardiac trauma and re-transfusion of mediastinal shed blood [7, 8]. The aim of the present study was therefore to exclude these potential confounding mechanisms and to evaluate the effect of β-glucan pretreatment in a standardized porcine ischaemia/reperfusion model with infarction size and regional myocardial function measured by speckle tracking echocardiography (STE) as end points.

MATERIALS AND METHODS

Animal care

The experimental protocol was approved by the local steering committee of the Norwegian Animal Experiments Authority. All animals received humane care in compliance with the European

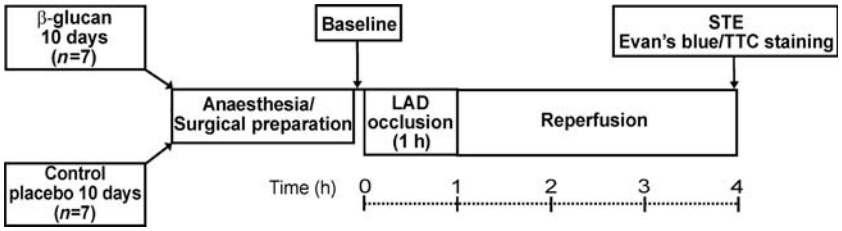

Figure 1: Study outline. Following a pretreatment period of 10 days, pigs in the β-glucan ($n = 7$) and control ($n = 7$) groups were anaesthetized and subjected to ligation of the LAD for 1 h followed by reperfusion for 3 h. After ischaemia and 3 h of reperfusion, regional myocardial function was measured by STE and infarct size and risk zones were estimated by Evans blue and 2,3,5-triphenyltetrazolium chloride staining. LAD: left anterior descending artery; STE: speckle tracking echocardiography; TTC: 2,3,5-triphenyltetrazolium chloride.

Convention on Animal Care. Locally bred landrace pigs with weight 51 ± 5 kg were stalled and acclimatized 2 weeks before the experiments.

Administration of β-glucan

During the acclimatization period, all pigs were accustomed to a sweet tasting beverage (Calf Mix Supplement, Kalvegodt, Felleskjøpet, Norway), in which β-glucan would later be mixed to make sure that the correct amount was consumed. Pigs in the control group received Calf Mix Supplement only, whereas pigs in the β-glucan group were fed a mixture of Calf Mix Supplement and 50 mg/kg microparticulate β-1,3/1,6-glucan isolated from *Saccharomyces cerevisae* (Biotec Pharmacon, Tromsø, Norway) each day for 10 consecutive days before the experiments.

Anaesthesia

The pigs were premedicated with i.m. atropine (2 mg/kg, Nycomed Pharma, Norway) and ketamine (20 mg/kg, Warner Lambert Nordic, Sweden) before they were transferred to the operating theatre. Following induction of anaesthesia with bolus i.v. injections of pentobarbitone (10 mg/kg, Nycomed Pharma) and fentanyl (0.01 mg/kg, Pharmalink, Sweden), the pigs were tracheostomized and ventilated on a respirator with 50% oxygen. Tidal volume was controlled with repeated blood gas analysis and P_{CO_2} was kept between 4 and 6 kPa. A venous catheter was inserted into the left jugular vein for infusions of continuous anaesthesia with fentanyl (0.02 mg/kg/h, Pharmalink), midazolam (0.3 mg/kg/h, Alpharma, Norway) and pentobarbitone (4 mg/kg/h, Nycomed Pharma) and 10 ml/kg/h glucose enriched sodium chloride as basal fluid replacement (1.25 g glucose/l sodium chloride).

Surgical preparation

Blood pressure was measured in the descending aorta through a cannula in the right femoral artery. Urine was drained through a cystostoma. All pigs received 150 mg amiodarone i.v. as arrhythmia prophylaxis. The heart was exposed through a median sternotomy. The pericardium was incised and a flow probe was placed snugly around the pulmonary artery for continuous measurement of cardiac output. The left anterior descending coronary artery (LAD) was dissected free from the surrounding tissue 6–8 cm from the division of the main left coronary artery to prepare it for non-traumatic clamping. This distance was chosen following pilot experiments, indicating that a substantial number of pigs developed ventricular fibrillation during ischaemia when the LAD was clamped more proximally.

Protocol

An overview of the study is displayed in Fig. 1. Following baseline measurements, the LAD was clamped by a small non-traumatic clamp for 1 h, which provided a clearly detectable infarction zone after tetrazolium staining in pilot experiments. The LAD clamp was removed after 1 h of ischaemia, and the hearts were reperfused for 3 h. A reperfusion period of 3 h was chosen since it has been suggested that reperfusion periods of <3 h are unreliable with the tetrazolium staining method, due to insufficient washout of enzymes (Downey, JM. Measuring infarct size by the tetrazolium method. Available at: http://www.southalabama.edu/ishr/help/ttc/). After 3 h of reperfusion, echocardiographic images were obtained, and the pigs were sacrificed by bolus injections of pentobarbitone. Pigs unable to complete the protocol were excluded. Excluded animals were replaced until a final sample size of seven in each group was reached.

Determination of infarction and risk area

A cardioplegia cannula was inserted in the ascending aorta and the aorta was clamped. The hearts were perfused with 3 l Ringer's acetate with heparin to wash out blood, which would otherwise react with the tetrazolium stain [9]. The LAD was re-occluded and the hearts were perfused with 120 ml of 1% Evans blue with a pressure of 100 mmHg to delineate the area at risk from the non-risk area. The perfusion of Evans blue was kept under these standardized conditions, in order to minimize differences between the groups as a result of staining of the risk zone with Evans blue through collaterals. The hearts were excised and frozen overnight. Following a 24 h freezing period, the hearts were thawed and cut in slices with a thickness of 1 cm from the apex parallel to the atrioventricular groove. The slices were incubated for 15 min in 1% 2,3,5-triphenyltetrazolium chloride (TTC) followed by formaldehyde fixation for 30 min. After formaldehyde fixation, the slices were mounted between two glass sheets and the necrotic area, the viable risk area and the non-at-risk area were delineated by an observer who was blinded for the groups. The drawings were scanned and the areas were measured by the ImageJ software

(ImageJ 1.14, National Institutes of Health, Bethesda, MD, USA). For comparison with strain derived from STE, each slice was geometrically divided into an 18-segment model using the papillary muscles and the inferior and anterior insertion of the right ventricle to the left ventricle as markers. Each slice was divided into six segments according to Cerqueira *et al.* [10]. Apical, middle and basal necrosis were defined as the sum of the necrosis measured in the two most apical, middle and basal slices, respectively.

Speckle tracking echocardiography

Two-dimensional left ventricular short- and long-axis images were recorded following ischaemia and 3 h of reperfusion. Echocardiographic images were recorded during a short period of apnoea using a Philips IE33 ultrasound scanner (Philips Medical Systems, Andover, MA, USA) with a 5 MHz transoesophageal probe that was positioned epicardially. The thoracic cavity was filled with 38°C of saline, in order to provide air-free insonation of the left ventricle. Apical, middle and basal short-axis views were taken from the right ventricular side in order to depict the left ventricle at a lower sector-angle. Apical views were taken also from an apical right ventricular approach for four- and three-chamber views, while imaging loops of the anterior and posterior wall were acquired positioning the probe towards the posterior apical and anterior apical segment respectively. By reducing imaging depth and insonation angle without reducing spatial resolution, the technique provided images with a frame rate of 77 ± 11 Hz.

Echocardiographic analysis

All images were analysed off-line using dedicated software (Syngo® Velocity Vector Imaging, Siemens, Germany). Three cardiac cycles were acquired and averaged by the software. An 18-segment model was used with six segments at each level [10]. The LAD vascular territory was defined as follows: apical anterior, apical antero-septal, apical infero-septal, mid-anterior, mid-antero-septal, basal anterior and basal antero-septal. Ejection time was derived from measurements of aortic valve closure time and opening time as determined by Doppler acquisitions. Strain values were acquired according to their maximal positive or negative peak values during the ejection time. The correct tracking of border-zones was visually controlled and manually corrected by an observer who was blinded for the groups. Quality was assessed on 2D images only and segmental analyses discarded when reverberations or missing signals were present.

Statistics

Data are presented as group means and standard deviation, unless otherwise noted. The small sample size in this study led to non-normal distribution of data and consequently non-parametric statistical methods were used. Within group comparisons were made by Wilcoxon signed ranks test. Comparisons between groups were done by Mann–Whitney *U*-test. All statistical analyses were performed with the SPSS 16.0 software programme (Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Table 1: Haemodynamic parameters and temperature

Index	Group	Baseline	3 h reperfusion	$P_{between}$
MAP (mmHg)	Control	96 ± 5	76 ± 16*	1.0
	β-Glucan	93 ± 10	74 ± 8*	
HR (beat/min)	Control	86 ± 10	147 ± 19*	0.80
	β-Glucan	79 ± 12	137 ± 36*	
CO (l/min)	Control	3.7 ± 0.5	2.9 ± 0.6*	0.89
	β-Glucan	3.4 ± 0.4	2.7 ± 0.8*	
SV (ml)	Control	44 ± 8	20.3 ± 4.6*	1.0
	β-Glucan	44 ± 7	21.3 ± 11.1*	
CVP (mmHg)	Control	6.3 ± 1.5	7.9 ± 1.2*	0.60
	β-Glucan	7.8 ± 0.8	9.0 ± 1.1*	
Temperature (°C)	Control	38.3 ± 0.6	39.0 ± 0.4*	0.52
	β-Glucan	38.2 ± 1.0	39.0 ± 0.6*	

Data are presented as mean ± standard deviation.
*$P < 0.05$ from baseline (Wilcoxon signed ranks test). *P* between refers to mean change from baseline between the groups (Mann–Whitney *U*-test). CO: cardiac output; CVP: central venous pressure; HR: heart rate; MAP: mean arterial pressure; SV: stroke volume.

RESULTS

Feasibility of the protocol

One pig in the β-glucan group and one pig in the control group developed ventricular fibrillation during ischaemia that was resistant to conversion by repeated epicardial defibrillator shocks. Another pig in the control group suffered from a major lung embolus, which lead to premature cardiac arrest. These three animals were excluded and replaced, since they were unable to complete the protocol according to exclusion criteria. The mean distance from the division of the left main coronary artery to the location of the LAD clamp was 7.0 ± 0 cm in the β-glucan group compared with 6.7 ± 0.9 cm in the control group (ns).

Haemodynamic parameters and temperature

Haemodynamic parameters and rectal temperature are listed in Table 1. Mean arterial pressure, cardiac output and stroke volume dropped significantly following ischaemia and 3 h of reperfusion. A significant increase was found with respect to heart rate, central venous pressure and temperature. However, no differences between the groups were observed.

Infarction size and viable myocardium at risk

Results from the measurements of infarction size are demonstrated in Fig. 2. Representative sections from the two groups are shown in Fig. 3. The risk area was exclusively confined to the middle and apical parts of the left ventricle. The area at risk in per cent of the left ventricle was 12.3 ± 4.0% in the control group compared with 12.5 ± 4.3% ($P = 0.70$). The infarction area in per

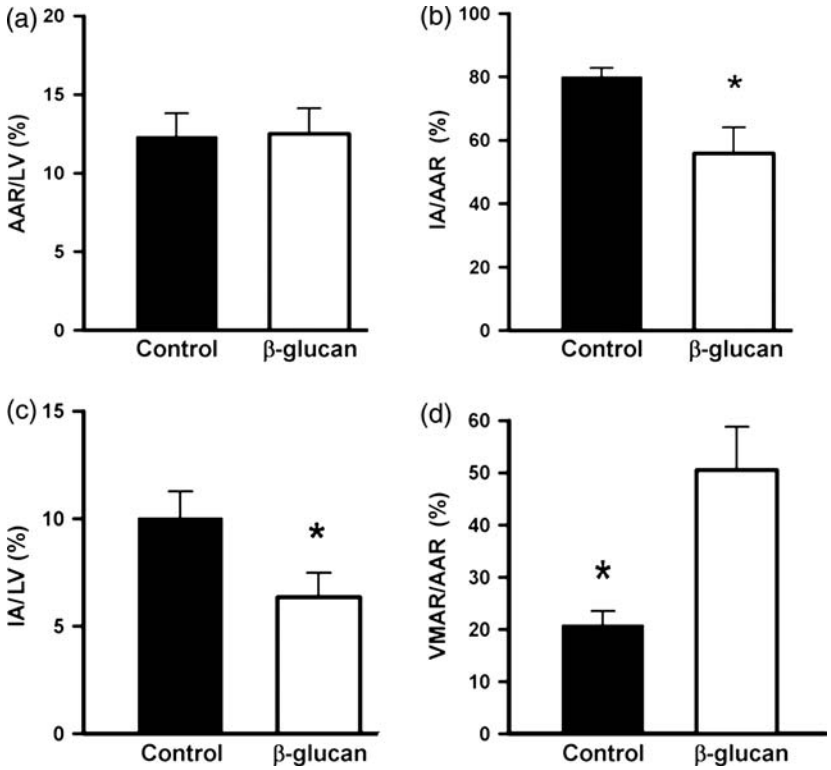

Figure 2: Measurements of infarction size and area at risk. Area at risk was similar in pigs treated with β-glucan and control (**a**). β-Glucan pretreatment reduced infarct size as expressed by lower infarct area in percent of area at risk (**b**), lower infarct area in percent of left ventricular area (**c**) and more viable myocardium at risk (**d**). *$P < 0.05$ as determined by Mann–Whitney *U*-test. AAR: area at risk; IA: infarct area; LV: left ventricle; VMAR: viable myocardium at risk.

cent of left ventricular area was 37% smaller in the β-glucan group compared with the control group ($P < 0.05$). The ratio between infarct area and area at risk was 36% lower in the β-glucan group compared with controls ($P < 0.05$), corresponding to 30% more viable myocardium in the risk zone in the β-glucan group ($P < 0.05$).

Regional myocardial function

Results from STE measurements of the middle and apical segments of the LAD territory are summarized in Table 2. In the anterior apical segment, longitudinal strain and circumferential strain were significantly better preserved in the β-glucan group compared with controls ($P < 0.05$). A tendency towards better post-ischaemic preservation of radial strain was found in the same segment, but this difference failed to reach statistical significance. No differences were found with respect to longitudinal strain, circumferential strain or radial strain in the mid-anterior, mid-anteroseptal or apical septal segments of the left ventricle. Neither were any significant differences found with respect to myocardial function in the basal segments supplied by the LAD or in segments supplied by the circumflex or right coronary arteries (data not shown).

DISCUSSION

The present study demonstrates that oral β-glucan pretreatment attenuates infarction size and improves post-ischaemic regional segmental function in a porcine ischaemia/reperfusion model. The results support our previous report, demonstrating that CABG patients treated with 1400 mg β-glucan a day, 5 days before surgery, had lower postoperative levels of creatine kinase myocardial bound and troponin T compared with untreated control patients [6].

Despite significant differences with respect to infarction area and the infarction area-to-area at risk ratio between pigs treated with β-glucan and controls, these differences were not accompanied by post-ischaemic improvement in any of the measured haemodynamic parameters. This is probably due to the relatively small infarctions that resulted from clamping the LAD 7 cm from the division of the left main coronary artery. In pilot experiments, a substantial number of pigs were unable to complete the protocol because of ventricular fibrillation when the LAD was clamped at a more proximal level. Our experiences from these pilot studies are in accordance with previous reports from our laboratory, demonstrating a low tolerance to ischaemia in porcine hearts [11, 12].

The LAD territory was defined by seven segments in this study; two basal, two in the middle and three apical. Because of the relatively small infarctions that were induced, we chose to demonstrate strain values from the mid-ventricular and apical parts of the left ventricle only, since the area at risk did not extend into any of the basal segments. From the remaining five segments, strain was only found to be better preserved in the apical anterior segment in the β-glucan group compared with controls, reflecting the difference between the groups with respect to necrosis, which was most pronounced in this segment.

Interestingly, the anterior apical segment in the control group was found to exhibit a positive mean longitudinal strain value,

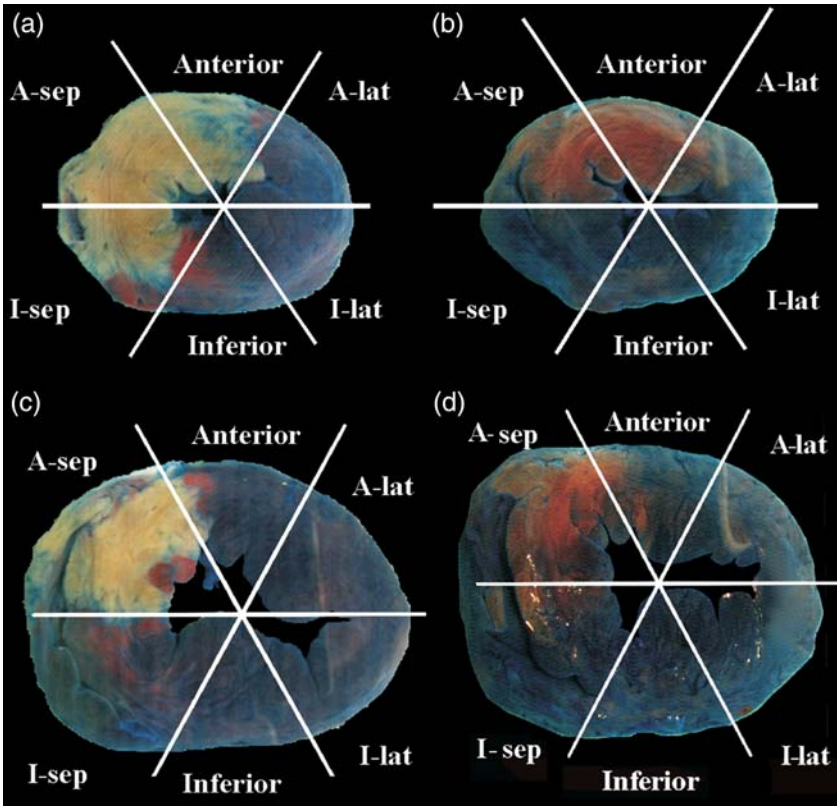

Figure 3: Cross sections illustrating differences between the groups. Cross sections of porcine hearts pretreated with placebo (**a**) and (**b**) or β-glucan (**b**) and (**d**). (a) and (b) represent apical sections. (c) and (d) represent the middle. Pale regions indicate necrotic myocardium, while viable myocardium at risk appears red. Blue areas represent area non-at-risk. The slices have been oriented with the right ventricle to the left. White lines indicate borders separating the left ventricular segments from which strain values are reported. The risk zone (pale and red areas) is predominantly confined to LAD territory (apical anterior, apical antero-septum, apical infero-septum, mid-anterior and mid-antero-septum).

Table 2: Necrosis and strain in pigs pretreated with β-glucan ($n = 7$) or placebo ($n = 7$) following myocardial infarction

	Group	LAD territorial segment				
		Middle		Apex		
		Anterior	Antero-sep	Anterior	Antero-sep	Infero-sep
Necrosis (%)	Control	7 ± 7	48 ± 22	61 ± 31*	77 ± 17	18 ± 30
	β-Glucan	8 ± 11	33 ± 20	27 ± 23	58 ± 26	15 ± 13
LS (%)	Control	−12.5 ± 6.5	−0.5 ± 10.9	5.9 ± 6.1*	5.7 ± 6.9	−3.0 ± 6.9
	β-Glucan	−8.4 ± 5.4	−5.8 ± 6.7	−4.7 ± 9.4	2.5 ± 6.5	1.8 ± 5.4
CS (%)	Control	−15.0 ± 2.5	−12.4 ± 1.9	−7.7 ± 4.3*	2.7 ± 18.1	−13.1 ± 11.2
	β-Glucan	−17.4 ± 9.2	−12.3 ± 4.8	−14.7 ± 6.6	−4.7 ± 9.6	−10.6 ± 11.9
RS (%)	Control	24.7 ± 3.7	17.6 ± 4.5	7.1 ± 11.8	1.0 ± 13.7	9.5 ± 14.2
	β-Glucan	24.3 ± 10.6	11.2 ± 10.8	15.1 ± 7.7	5.4 ± 14.2	17.2 ± 11

*$P < 0.05$ refers to differences between the control group and the β-glucan group as determined by Mann–Whitney *U*-test. CS: circumferential strain; LS: longitudinal strain; RS: radial strain.

indicating stretching of the tissue during systole. In contrast, mean circumferential strain remained negative and mean radial strain remained positive in the same segment in this group. There are two reasons why ischaemic myocardial segments may be more prone to exhibit positive longitudinal strain, indicating paradox stretching of the tissue during systole, while circumferential strain in the same segment remains negative and radial strain remains positive. First and perhaps most importantly, longitudinal fibres, especially in the apex, are predominantly located in the subendocardium [13, 14], which is most susceptible to ischaemia. These fibres may therefore more easily succumb to the abnormal stretching pattern during systole, reflected by a shift from negative to positive mean longitudinal strain. Secondly, longitudinal fibres exhibit a relatively longer

radius of the curvature, when compared with the radius of the curvature in the short-axis view. According to the law of Laplace, wall stress increases with increasing radius of the curvature. Longitudinal fibres may therefore be exposed to higher wall stress compared with the wall stress afforded by the relatively shorter radius of the short-axis curvatures, which are decisive for circumferential and radial strain [15].

Although this study reports attenuation of myocardial ischaemia/reperfusion injury by oral β-glucan pretreatment, the mechanisms behind this effect remain elusive. Pretreatment with the water soluble β-1,3-glucan, glucan phosphate, has previously been shown to protect against ischaemia/reperfusion injury in rodents through the ip. administration route in a study by Li *et al*. In these experiments, the cardioprotection afforded by β-glucan was found to be mediated by stimulation of the phosphoinositide 3-kinase (PI3K)/Akt pathway and inhibition of the toll-like receptor/MyD88-dependent nuclear factor kappa B pathway. The protection from ischaemia/reperfusion injury by ip. soluble β-glucan as reported by Li *et al*. failed to induce cardioprotection in a porcine CPB model with global ischaemia and reperfusion in a study conducted by our group [16]. A possible explanation to the contradictory findings by Li *et al*. and our previous study include structural differences between the glucan phosphate in the study by Li *et al*. compared with the 1,3/1,6-β-glucan utilized in our previous study. In addition, Li *et al*. demonstrated a stimulation of the PI3K/Akt pathway, which was not reproduced by our previous experiments, and finally there were important differences between the ischaemia/reperfusion models that were used.

The microparticulate β-glucan used in the present study and previously in patients undergoing CABG is not water soluble, and is therefore difficult to measure in the systemic circulation following oral ingestion. Interestingly, Rice *et al*. [17] reported that plasma IL-6 was elevated 8 h after oral administration of microparticulate β-glucan in rodents without detectable levels of β-glucan in the systemic circulation. This observation suggests that β-glucan is recognized by cell surface receptors in the gut, possibly Dectin-1, eliciting a signal cascade to other immune competent cells, which in turn effectuate the response. This theory is supported by the findings of Beier and Gebert [18], who demonstrated transcytosis of yeast particles from *S. cerevisiae* by small intestinal M cells in <1 h in pigs. A putative protective effect of β-glucan pretreatment against reactive oxygen species (ROS) is also suggested. β-Glucans have been shown to increase levels of anti-oxidants in *in vitro* experiments [19, 20], following ip. administration [21, 22] and recently also following oral administration [5, 23–25]. Our findings may therefore be related to increased levels of endogenous-free scavengers, which in turn leads to less oxidative damage created by ROS during ischaemia and reperfusion. These theories are likely to be pursued by future studies.

In contrast to the sudden and without warning onset of a myocardial infarction, the clinical setting with planned cardiac surgery is amenable to a pretreatment strategy, since the planned procedure requests a cardioprotective therapy being initiated prior to the ischaemia/reperfusion injury. Thus, pretreatment with β-glucan may be utilized to attenuate the global ischaemia/reperfusion injury associated with cardiac surgery on CPB. Future clinical randomized trials may determine whether such a strategy may improve the outcome in patients with limited cardiac reserves undergoing complex procedures with long cross-clamping times.

Limitations of the study

The aim of the present study was to examine whether a cardioprotective effect of pretreatment with β-glucan reported in patients undergoing CABG could be reproduced in a standardized experimental model. Although the results from the present study are in agreement with the findings from our previous clinical study, the importance of the results is limited by the different models being used. Regional ischaemia is different from cardioplegia-induced global ischaemia associated with cardiac surgery on CPB. Regional myocardial function is more likely to be affected in the present study, whereas global myocardial function is depressed following cardiac surgery. Accordingly, one should be cautious to translate these results to the clinical situation of cardiac surgery on CPB, although both studies demonstrate a reduced ischaemia/reperfusion injury. Moreover, the pig as an experimental model is another limitation due to the poor ischaemic tolerance in this animal which restricted us to induce relatively small infarctions.

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Conflict of interest: none declared.

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