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Effect of vagus nerve stimulation on tissue damage and function loss in a mouse myocardial ischemia-reperfusion model

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ABSTRACT

Objectives: In cardiac ischemia, acute inflammatory responses further increase the detrimental effect on myocardial tissue. Since vagus nerve stimulation (VS) attenuates inflammatory responsiveness this study examines the effect of VS on myocardial damage development in a cardiac ischemia-reperfusion (IR) mouse model.
Methods: 54 male C57Bl/6j mice were subjected to an IR procedure with or without prior VS. The effects on inflammatory responsiveness, infarct size, cardiac function, neutrophils, lymphocytes and vascular endothelial growth factor (VEGF) in the infarcted myocardium were measured at 48 h after intervention. Group results were compared with unpaired Mann-Whitney or Kruskall-Wallis test.
Results: A significant decrease in inflammatory responsiveness was not verified by decreased TNFα levels in blood from VS and IR treated mice. The percentage infarct size over area at risk was smaller in the group with VS + IR compared with IR (22.4 ± 10.2% vs 37.6 ± 9.0%, $p = 0.003$). The degree of the reduction in cardiac function was not different between the IR groups with or without VS and no group differences were found in amounts of neutrophils, CD3+ lymphocytes and VEGF in the reperfused mouse heart.
Conclusion: The present study does not provide clear evidence of a reducing role for VS on cardiac function loss. This could mean that VS has a less inhibiting effect on myocardial inflammation than may be expected from the literature.

1. Introduction

The immune system is involved in recognizing and fighting external pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) that originate from injured tissue inside the body (Vajjhala et al., 2017). Simultaneously, immune responses play a role in the repair of damaged tissue and the restoration of organ function (Bianchi et al., 2017; Capobianco et al., 2017). In a healthy physical situation, the immune functions are well balanced since overactivity might lead to unnecessary inflammatory harm while underperformance may give way for infectious disease or illness caused by malfunctioning cells that are not discarded (Tracey, 2007; Sundman and Olofsson, 2014). Health as such depends on a well-regulated and balanced immune system. The autonomic nerve system (ANS) emerges as a major regulator of immune function (Sundman and Olofsson, 2014; Kox and Pickkers, 2015; Pereira and Leite, 2016; Chavan and Tracey, 2017). In the ANS, the sympathetic branch is associated with pro-inflammatory activity while the parasympathetic part is considered to attenuate inflammatory responses. In the last two decades, research yielded a serious amount of proof of attenuation of inflammatory responsiveness by electrical stimulation of the vagus nerve (VS) (Kox and Pickkers, 2015; Borovikova et al., 2000; Mihaylova et al., 2014). Upon

Abbreviations: AAR, Area at Risk; ACh, Acetyl Choline; ANS, Autonomic Nervous System; BW, Body Weight; ChAT, Choline Acetyl Transferase; DAMPs, Damage-associated Molecular Patterns; EDV, End Diastolic Volume; EF, Ejection Fraction; ESV, End Systolic Volume; FDD-mix, Anaesthesia [mg/kg]: Fentanyl 0.05 mg/kg, Domitor 0.5 mg/kg, Dormicum 5.0 mg/kg; Gran, Granulocytes; Hb, Haemoglobin; Hct, Haematocrit; IR, Heart rate; IL, Interleukin; IR, Ischemia-Reperfusion; IS, Infarct size; LAD, Left anterior descending coronary artery; LPS, Lipopolysaccharide; Ly-6G, Antibody for neutrophils; Lym, Lymphocytes; MI, Myocardial infarction; PAMPs, Pathogen-associated Molecular Patterns; PBS, Phosphate buffered saline; RBC, Red Blood Cells; SD, Standard Deviation; SV, Stroke volume; TNFα, Tumour necrosis factor alpha; TTC, Triphenyl tetrazolium chloride; VEGF, Vascular Endothelial Growth Factor; VS, Electrical Vagus Nerve Stimulation; WBC, White blood cells

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vagus activation, acetylcholine binds to α7 nicotinic acetylcholine receptors on inflammatory cells like macrophages and lymphocytes. The release of inflammatory cytokines by these cells is strongly inhibited resulting in an attenuated inflammatory response (Borovikova et al., 2000; Pavlov and Tracey, 2005; Buchholz et al., 2018). Presently VS is, sometimes still experimentally, used as a treatment for inflammation associated ailments like rheumatoid arthritis (Koopman et al., 2014), inflammatory bowel disease (Bonaz et al., 2017) and heart failure (Gold et al., 2016).

In literature, the connection of the ANS with inflammatory processes in atherosclerosis and chronic heart disease is well established (Gidron et al., 2007). Inflammatory cytokines such as IL-1β, IL6 and TNFα are involved in sepsis and chronic inflammatory diseases that have been experimentally treated with VS, but also in cardiovascular diseases such as myocardial ischemia-reperfusion (IR) (Pavlov et al., 2003; Zhao et al., 2012). Upon myocardial infarction (MI) the body starts processes to restore cardiac function and remove debris from the infarcted area. Early reperfusion is needed to limit the damage caused by the ischemic period but reperfusion itself generates additional cardiac tissue damage called reperfusion injury. Reperfusion injury can seriously increase the initial IS which worsens the patient's prognosis (Yellon and Hausenloy, 2007; Simoons et al., 1986). The physiological processes involved are largely based on inflammatory responses.

Recent studies on VS report attenuation of inflammatory cytokine release and therefore better outcome after experimental myocardial IR in rat and pig models (Yellon and Hausenloy, 2007; Uitterdijk et al., 2015). Still, relevant data in human subjects and mice are scarce.

In the present study the hypothesis that VS attenuates the inflammatory processes following myocardial IR and thus reduces the severity of cardiac injury and function loss, was examined in mice. Specifically, the inflammatory processes that are involved in the development of tissue damage and function loss of the infarcted myocardium were studied.

2. Methods

2.1. Animals

Male C57Bl/6j mice from Charles River Laboratories Maastricht, the Netherlands, were used. They were housed in filter-top cages in the university animal facility. Drinking water and regular chow were provided ad libitum and light/dark division was 12 h/12 h. Animal procedures were all performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), with prior approval of the Animal Ethical Experimentation Committee, Utrecht University, the Netherlands and within the applicable European guidelines (2010/63/EU). The required sample size was calculated with G*Power v3.1.9 based on a power of 90% and α = 0,05 and, derived from literature, SD and perioperative mortality (Erdfelder, 2009; Arslan et al., 2010).

2.2. Euthanasia

Forty eight hours after the start of the experiments animals were euthanized by bleeding under general anesthesia with FDD-mix (Fentanyl 0.05 mg/kg, Domitor 0.5 mg/kg, Dormicum 5 mg/kg). The abdomen was opened and a maximum of left ventricular blood was drawn through the diaphragm from the cardiac apex. When this maximum volume of blood was collected, the heart was excised for further examination.

When, during the time span of the experiment, animals reached a humane endpoint as described by the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), they were euthanized in a similar way as described above.

2.3. Experimental setup

A total of 54 mice were weighed and randomly distributed over three experimental groups. These were defined as group IR, undergoing IR together with sham-VS, group VS + IR receiving both VS and IR and group VS with VS and sham-IR. To compare the effects of vagus nerve stimulation, cardiac function was measured together with IS and inflammatory responsiveness. Furthermore, inflammatory cells were counted.

Two days after the intervention the IR heart is in the stage of acute inflammatory processes. However, TNFα levels will still be low due to the vagus stimulation. For these reasons, after 48 h the animals were terminated as described above under general anesthesia with FDD-mix. Left ventricular blood was drawn from the cardiac apex and the blood samples were collected in anti-coagulated tubes for cell counts and to measure TNF-α levels in the plasma for the determination of inflammatory responsiveness. From 10 animals per group, the heart was collected and preserved in formaldehyde immediately after termination and examined for neutrophils and T-lymphocytes in and around the infarcted area. Another 10 hearts from the IR group and 10 from the VS + IR group were used for determining the IS.

2.4. Echo Cardiography

The mice were weighed and sedated with isoflurane (induction with 4%, maintenance at 1.8% in air:O_2, 2:1) and the medial-right area of the neck, as well as the medial-left area of the thorax was depilated with a chemical depilatory cream (Veet, Benckiser NV. Hoofddorp, Netherlands).

Baseline echocardiography was executed with a Vevo 2100 (Visualsonics, Amsterdam, the Netherlands) at $t = 0$ before surgery and 48 h after surgery, just before termination. The executing lab assistant was blinded for the experimental groups. 3D echoes were recorded from sagittal, coronal and axial orientations of the beating heart. From these recordings end-systolic (ESV) and end-diastolic volumes (EDV) were determined to calculate ejection fraction (EF). EF was calculated and expressed as a percentage of stroke volume (SV) over EDV. The difference per heart between baseline-EF and EF at 48 h after surgery was used as a measure for loss of cardiac function.

2.5. Surgical procedure

After baseline echocardiography, animals were anaesthetized with FDD-mix. They received an additional bolus of 2.5 mg/kg Dormicum for the prolongation of anesthesia after 45 min. The mice were intubated and attached to a mouse ventilator (MiniVent 845, Harvard Apparatus, Holliston, MA, USA) and mechanically ventilated at 175 strokes per minute with a stroke volume of 250 μl. Body temperature was controlled and kept at 38 °C. The animals' extremities were connected to an ECG recorder to measure the heart rate (HR). The ischemia-reperfusion procedure was performed by an experienced surgeon who was unaware of the preceding sham or complete vagus stimulations. The right neck area was sterilized with 70% alcohol and opened to expose the vagus nerve. A bipolar electrode was then placed on the nerve which was then stimulated for 30 s (f = 10 Hz, I = 1 mA, pulse width = 0.5 ms). An acute minimal decrease of 15% in HR was accepted as a proof for effective vagus stimulation. Then, the electrodes of the stimulator and the ECG recorder were removed from the mouse and the skin was closed with Vicryl 4.0.

Subsequently, within 10 min after VS, the thorax was opened left laterally between the fourth and fifth rib and the LAD was exposed and occluded with a braided silk 8.0 ligature over a small piece of polyethylene tubing (ø 0.8 mm). Pale-coloring of the infarcted area and slight arrhythmias were an indication of a solid occlusion. The open thorax was then covered with wet gauze and the occlusion was maintained for 30 min. After this ischemic period, the piece of tubing was

removed and the coronary blood flow was restored. A monofil prolene 8.0 suture was used to close the thorax and the thoracic skin was sutured with Vicryl 4.0. Finally, the anesthesia was antagonized (Antisedan 2.5 mg/kg plus Anexate 0.5 mg/kg) and the animals were administered 0.1 mg/kg buprenorphine (Temgesic®, Reckitt Benckiser, UK) for pain relief. The sham-VS procedure was performed identically but only until the vagus nerve was exposed. The nerve itself was minimally touched to prevent unwanted stimulation. In the sham-IR, the whole procedure was followed and a ligature was pierced underneath the LAD but was not tied up. Both VS and IR operations were executed in one session.

2.6. Blood content, inflammatory responsiveness

Terminal blood was processed through a cell counter (Cell-Dyn Sapphire, Abbott Diagnostics, Lake Forest, Ill., USA) to determine numbers of total white blood cells (WBC), Lymphocytes (Lym), Granulocytes (Gran) and red blood cells (RBC). In the same procedure plasma levels of hemoglobin and hematocrit were determined.

A volume of 100 µl whole blood was stimulated with 100 µl lipopolysaccharide (100 ng/ml LPS, Sigma-Aldrich: cat.nr. L2880) at 37 °C. After 20 h, the blood samples were centrifuged for 5 min at 300G after which the plasma supernatant was transferred into sterile tubes and stored at −80 °C. To determine inflammatory responsiveness, TNFα levels in LPS stimulated plasma samples were determined with ELISA (mouse TNF-alpha Quantikine Kit, R&D systems, cat.nr: MTA00B).

2.7. Infarct size determination

After termination, the hearts of 10 mice were flushed with Phosphate Buffered Saline (PBS) and the LAD was again ligated at the location of the first occlusion. Via the aorta, the myocardium was perfused with Evans Blue to color the whole heart except the area at risk. The hearts were excised and kept at −18 °C for approximately 2 h. Then the hearts were taken from the freezer, cut in 0.9 mm slices and instantly stained with 4% Triphenyltetrazolium chloride solution (TTC, cat.nr:T8877, Sigma-Aldrich, the Netherlands) at 37 °C. This method colors the perfused myocardium blue and the viable tissue red while leaving the infarcted tissue white (Fig. 1). To determine the damage, pictures were taken from the colored slices and subsequently the infarct size (IS) and area at risk (AAR) were determined with the contour tool of ImageJ (Schneider et al., 2012). The ratio "area at risk over left ventricular surface" (AAR/LV) was measured to determine the area of the myocardial tissue that underwent ischemia and reperfusion as a part of the total left ventricular area observed. The ratio IS/AAR is a measure to determine IS within the endangered myocardium and is the primary endpoint from which the efficacy of treatment is addressed. Since the area at risk in all hearts was located in the lower 3 heart slices, only these slices were included in the calculation of the total IS and the three were averaged per heart.

2.8. Immunohistochemistry

The hearts for immunohistochemistry were perfused with PBS to wash out the remaining blood and fixed for ca. 90 min in 4% formaldehyde where after they were stored in PBS with 15% sucrose. They were embedded in paraffin and cut in 5 µm sections, perpendicular to the apex to aortic base axis. Antigen retrieval was performed in 2.94 g/l Trisodium citrate $2H_2O$ (pH = 6) for 20 min at 96 °C and 5 µm sections from 2 mm above the apex were stained for inflammatory cells and for vascular endothelial growth factor (VEGF). The number of neutrophils, T-lymphocytes and the amount of VEGF was determined with Ly6G (BioLegend, Cat.nr:108402), anti-CD3 (DAKO, ref.:A0452) and anti-VEGFA (Abcam, Cat.nr:ab46154) respectively. Finally, they were stained with Liquid Permanent Red (LPR, K0640, Dako Agilent, Amstelveen the Netherlands). Pictures of the stained sections of 1.25 × 0.94 mm were taken at 200× magnification and analyzed with ImageJ (Schneider et al., 2012). Transverse sections of the heart were divided into four distinct areas.

Inflammatory cells and VEGF in the transverse slices were measured in 4 separate locations as shown in Fig. 1. Remote area (septum) was defined as a control area and compared with the IS and the combined ventral and dorsal border areas. Results were recalculated and expressed as a number of stained pixels per mm^2 of total measured tissue area.

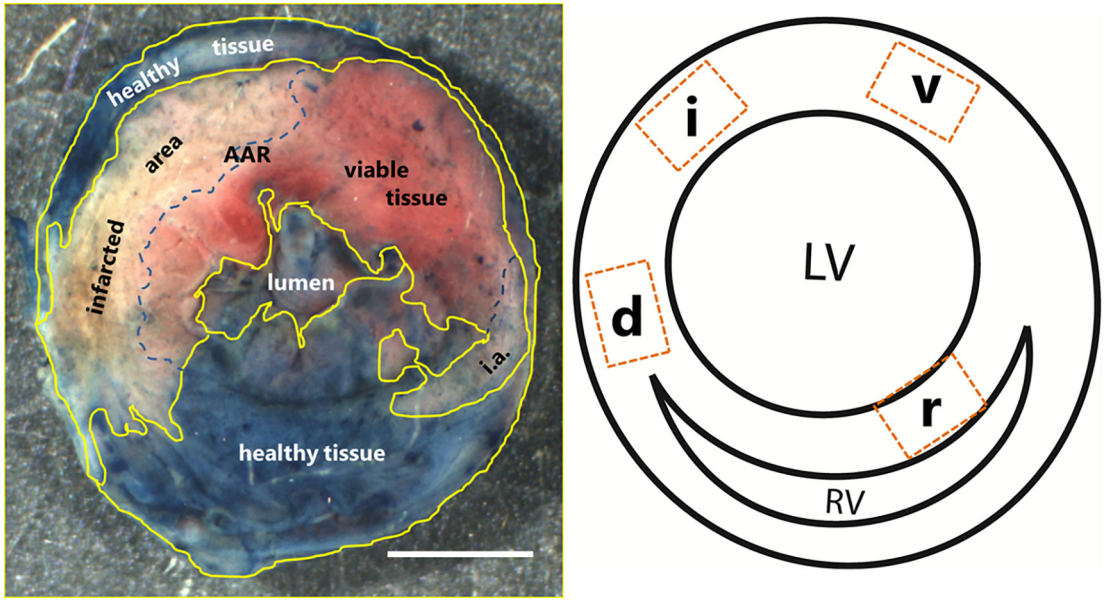

Fig. 1. Areas in stained transversal sections of the ischemic heart. Left: Example of 0.9mm slice of the IR mouse heart. Red (TTC-viable), white (infarcted) and blue (healthy perfused) tissue contours drawn with ImageJ. Scale bar = 2mm Right: Distinctive areas in transversal heart sections. Dorsal (d) and ventral (v) border areas are adjacent to the infarcted (i) area while the septal area is reflecting a remote area (r).

2.9. Data processing

Quantification of TTC-areas, VEGF and inflammatory cells was performed in ImageJ by a blinded assistant (Schneider et al., 2012). Between groups, comparisons were conducted with an unpaired Mann-Whitney or Kruskall-Wallis test in SPSS-22 (SPSS 22; IBM Corp, n.d.) (IBM, Statistics for Windows, 2013) and Prism-7 (GraphPad Prism Version 7 for Windows, n.d.) (GraphPad Prism 7 Software, La Jolla California USA) with no corrections for multiple groups. Grubbs outlier detection, a built-in analysis in Prism-7 was used to detect and exclude significantly deviating values, $p = 0.05$ (Grubbs, 1969). The level of significance in this study is kept at 0.05, two-tailed.

3. Results

3.1. Animal details

The 54 C57Bl/6j mice weighed 26 ± 1.5 g. Overall mortality due to surgery was 13%, resulting in 47 included animals distributed over three groups: group IR ($N = 19$) receiving sham VS plus IR, group VS + IR ($N = 18$) treated with both VS and IR, and group VS ($N = 10$) that underwent VS plus sham IR. A loss of body weight after 48 h was found in all groups, 6.5% and 5.9% in groups IR and VS respectively and 11.5% in group VS + IR. At LAD-occlusion all IR treated animals showed a pale coloring of the ischemic area and some minor arrhythmias. Anesthesia of the animals already caused a small decrease in HR. The included VS treated mice all met the criteria for effective vagus nerve stimulation, i.e. a decrease of minimal 15% was achieved from the resting rate as it was immediately before vagus stimulation (range 15%–75%). This decrease was immediately restored when the VS was stopped.

3.2. Blood content

Terminal blood parameters were measured with the Sapphire Analyzer. Cell values at $t = 48$ after surgery are presented as 10^9 cells/l, hemoglobin in mmol/l and hematocrit as l/l.

Total white blood cell (WBC) as well as red blood cell (RBC) numbers did not significantly differ among the groups. Nor was a significant difference found between levels of hematocrit. Amounts of lymphocytes in VS + IR were significantly different from that in the VS group, 3.09 ± 3.31 vs 5.48 ± 3.40 $*10^9$ cells/l respectively ($p = 0.020$). In the IR group, granulocytes values were significantly higher than in the VS group, (2.52 ± 2.32 vs 1.18 ± 1.50, $p = 0.049$). Granulocytes did not reach significance for the difference between IR and VS + IR (1.51 ± 1.78 $*10^9$ cells/l, $p = 0.069$). Hemoglobin was slightly but significantly higher in VS + IR, 8.86 ± 0.70, compared to the level of 8.33 ± 0.48 mmol/l in the IR group ($p = 0.012$) (Table 1).

3.3. Cardiac function

The EF, as a measure for cardiac function, is shown as stroke volume as a percentage of end-diastolic volume (SV/EDV $*100$). At the time of termination, after 48 h, the EF in the IR group decreased from 59.9 ± 5.3% to 44.2 ± 6.7% ($p = 0.0001$) and in the VS + IR group from 57.4 ± 5.3% to 44.6 ± 6.8% ($p = 0.012$). As expected, the EF in the VS group did not change (Fig. 2). Although the mean decrease in the VS + IR was slightly bigger than that in the IR group, the EF-decreases in both groups were not significantly different.

Table 1
Blood plasma values at t = 48h. White blood cells (N = 10), Lymphocytes (N = 10), Granulocytes (N = 10) and Red blood cells (N = 4), presented as numbers $*10^9$ cells/l. Hemoglobin (N = 10) as mmol/l and hematocrit (N = 4) as l/l. a, b and c are significantly different pairs (Kruskall-Wallis).

	WBC	Lym	Gran	RBC	Hb	Hc
IR	7.21	4.04	2.52[b]	8.96	8.33[c]	0.42
IR + VS	5.15	3.09[a]	1.51	9.27	8.86[c]	0.44
VS	7.45	5.48[a]	1.18[b]	8.48	8.36	0.40

3.4. Infarct size determination

The mean IS in the red-white-blue stained heart slices was bigger in the IR than in the VS + IR group while the AAR was bigger in the VS + IR group (ns). The IS/AAR showed a significant bigger ratio for the IR group 37.6 ± 9.0% vs. VS + IR: 22.4 ± 10.2% $p = 0.003$. (Fig. 2).

3.5. Inflammatory responsiveness

TNFα-levels in terminal whole blood samples were measured to show the level of inflammatory responsiveness of white blood cells upon stimulation. The mean of the IR group (70.0 ± 100.3 pg/ml) did not significantly differ from VS + IR (33.7 ± 33.5 pg/ml) or VS (28.2 ± 33.9 pg/ml). (Fig. 3).

3.6. Inflammatory cells in the heart

The presence of inflammatory cells was quantified in transverse sections of the heart. The remote area (septum) was defined as the control area and was compared with the infarcted area and the combined ventral and dorsal border areas (see Fig. 1). Amounts of cells are presented as stained area in pixels $*0.01$ per mm^2. The number of neutrophils in the IR group ($N = 8$) did not significantly differ from the VS + IR group ($N = 6$). Within the IR group, more neutrophils were detected in the infarcted and border areas compared to the remote area ($p = 0.022$ and $p < 0.000$ resp.). The neutrophils in the remote areas in the IR and VS + IR groups were equal to those in the VS group ($N = 6$) without IR. In IR most neutrophils were found in the border zones. No significant differences were found within the groups of VS + IR and VS. As depicted in Fig. 4 with values in Table 2, both infarcted areas of the IR and the VS + IR group were significantly higher than the VS group ($p = 0.008$ and $p = 0.028$ resp.) and the same is found for the border areas of IR and VS + IR which are also bigger than values in the group with VS alone ($p = 0.006$ and $p = 0.036$ resp.) (see Fig. 5).

Numbers of $CD3^+$ T-lymphocytes in the IR group border area were higher than in the IR remote area ($p = 0.031$) and the border area of the VS-group ($p = 0.036$). The IR infarcted area also contained significantly more T lymphocytes than the same area in the VS group ($p = 0.026$) (Fig. 6). See Table 3 for values.

3.7. Vascular endothelial growth factor

VEGF in the reperfused heart was measured in the expectation that the anoxic myocardium would strive for revascularization. The mean amounts of VEGF over all cardiac areas together were highest in the IR group but yet the difference was not significant between mice in the IR and VS + IR groups. In all groups VEGF levels were highest in the border areas but this was only significant in the VS + IR group (Fig. 7).

4. Discussion

There is no doubt that inflammatory processes are important for recovery after cardiac ischemia and reperfusion but the same inflammation can also cause additional damage to the already threatened myocardial tissue. Vagal activity is described as having an attenuating effect on inflammatory responsiveness.

The goal of this study was to examine whether electrical VS could attenuate the severity of the inflammatory response following IR.

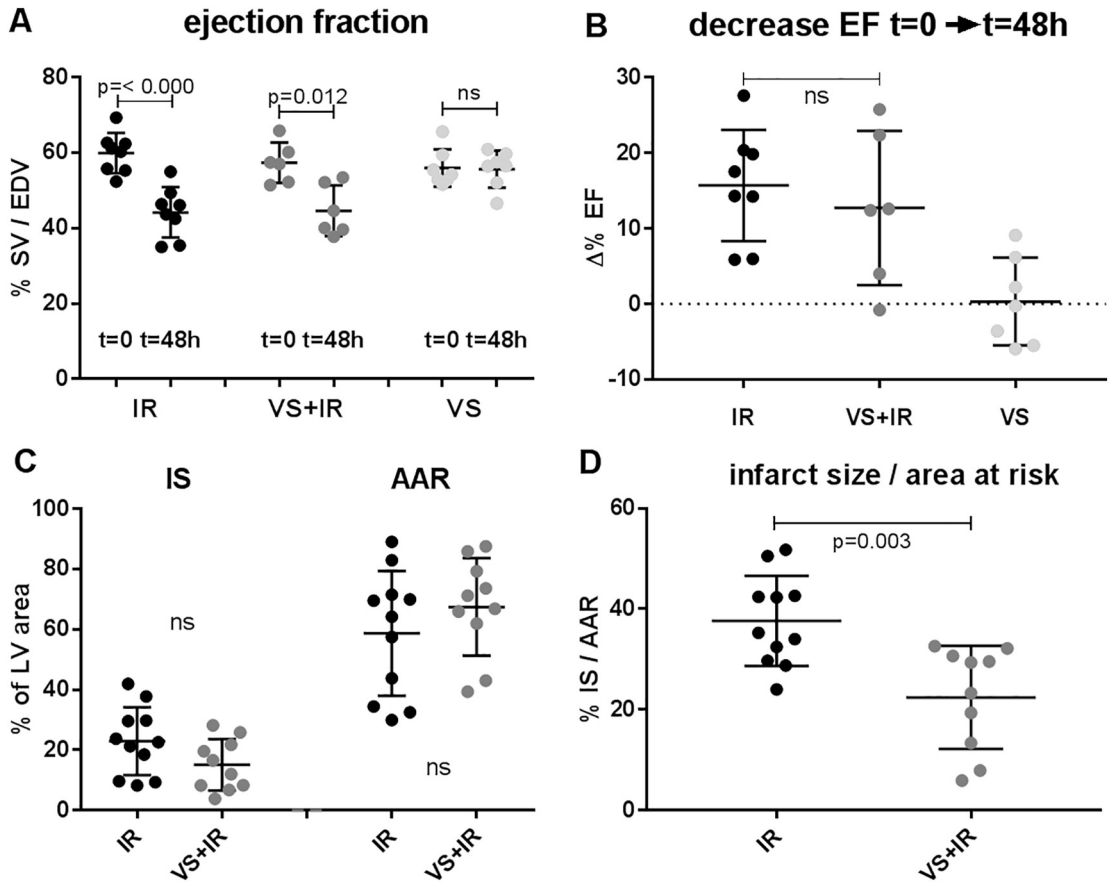

Fig. 2. Ejection fraction, infarct size and area at risk. Ejection fraction (EF) presented as percentage of stroke volume over end-diastolic volume (% SV/EDV) from baseline to 48 hours after surgery. Infarct size (IS) and area at risk (AAR) as a percentage of the left ventricular area (%LV). (Means ± SD). A: The EF of the VS group (N = 7) does not change from baseline to t = 48h. The EF in IR (N = 8) and VS + IR (N = 6) decreases from 59.9 ± 5.3 to 44.2 ± 6.7 (p = < 0.000) and 57.4 ± 5.3 to 44.6 ± 6.8% (p = 0.012) respectively.B: Decrease in EF per group, t = 0 minus t = 48h values for IR: 15.7 ± 7.4%, VS + MI: 12.7 ± 10.2% and VS: 0.3 ± 5.8%. IR vs VS + IR differences are not significant. IR and VS + IR differ significantly from VS: p = 0.004 and p = 0,041 respectively. C: IS values, IR (N = 11): 23.0 ± 11.3%, VS + IR (N = 10): 15.1 ± 8.5%. AAR values, IR: 58.7 ± 20.7%, VS + IR: 67.5 ± 16.2%. D: Ratio Infarct size over Area at risk. Group IR: 37.6 ± 9.0% and VS + IR: 22.4 ± 10.2%. IR vs VS + IR: p = 0.003. (Panels A and B: Kruskall-Wallis, panels C and D: Mann-Whitney).

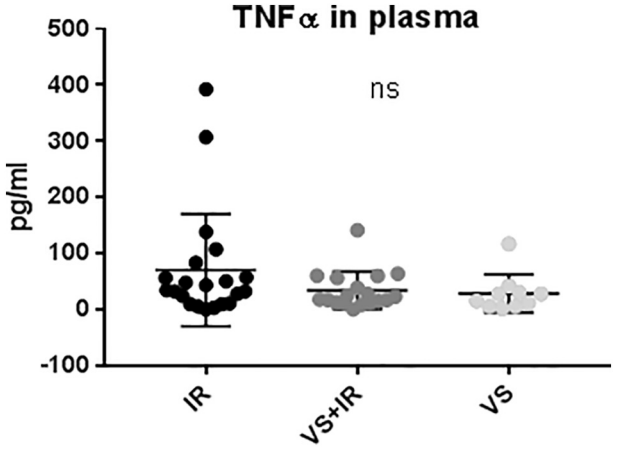

Fig. 3. Levels of TNFα in pg/ml in whole blood at t = 48h. Mean ± SD of IR (N = 21): 70.0 ± 100.3 pg/ml, VS + IR (N = 18): 33.7 ± 33.5 pg/ml and VS (N = 10): 28.2 ± 33.9 pg/ml. No significant group differences (Kruskall-Wallis).

VS prior to IR resulted in a smaller IS/AAR ratio. Although a tendency was observed, strong evidence that VS inhibits inflammatory responsiveness could not be demonstrated in this study.

VS in this model caused a smaller IS and a slightly bigger AAR in the VS + IR group than in the IR group. Together these results caused a significantly smaller IS/AAR ratio for the VS + IR treated animals ($p = 0.003$).

This could mean that the threatening inflammatory processes have become less severe and are distributed over a larger area thereby spreading the risk. VS can also stimulate vasodilation (Nilsson, 1996; Sheng and Zhu, 2018) and the combination of the mentioned effects could hypothetically slow down the development of damage and generate time for revascularization and formation of collateral blood vessels and thus prevent unnecessary necrotic loss. More tissue might be saved if treated early and effectively.

The findings are in accordance with the literature (Uitterdijk et al., 2015; Yi et al., 2016; Kelly et al., 2017) and support the hypothesis that VS attenuates the processes that result in ischemia/reperfusion damage.

Wurfel et al. found that among human subjects there are high and low responders to LPS (Wurfel et al., 2005). Recently, in our laboratory we have found evidence that the same occurs in mice (unpublished data). This may be a reason for the large variation in the observed AAR in this study.

In human patients, treated with VS, the electrode is connected to the left vagus nerve to avoid cardiac complications since the right vagus nerve is the main supply for the sinoatrial node, while the left vagus nerve innervates the atrioventricular node (Randall et al., 1986). However, more recent research showed no differences in cardiac response or inflammatory responsiveness between left and right VS (Coote, 2013; Yamakawa et al., 2014; Kwan et al., 2016). Choosing for left or right VS should therefore have no advantage or disadvantage. In

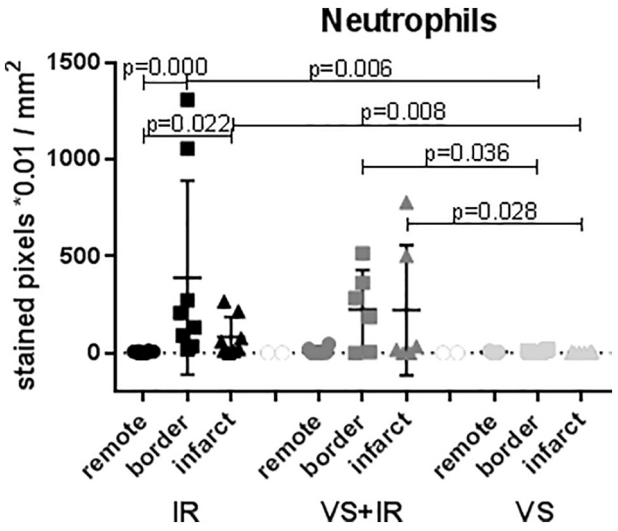

Fig. 4. Neutrophils are shown as the stained area in pixels*0.01 per mm^2. Within the IR group (N = 8) a significantly bigger amount of neutrophils is found in the border area than in the remote (p = < 0.000) or infarcted area (p = 0.022). Within the VS + IR (N = 6) and VS (N = 6) groups and between the remote areas are no differences found. Infarcted areas of IR and VS + IR show both more neutrophils than the infarcted area in the VS group, p = 0.008 and p = 0.028 respectively. The border areas in the IR and the VS + IR groups show also a bigger amount of neutrophils than the border area in the VS group, p = 0.006 and p = 0.036 respectively (Kruskall-Wallis).

Table 2
Neutrophil values in transversal sections from treated hearts presented as mean stained area in pixels*0.01 per mm2. Means ± SD. a t/m f are significantly different pairs (Kruskall-Wallis).

Mean (SD)	IR	VS + IR	VS
Remote	6.0 (4.8)^{ab}	12.4 (18.9)	4.0 (4.2)
Border	390.5 (501.4)^{ae}	225.3 (203.9)^{f}	7.8 (8.2)^{ef}
Infarct	83.3 (102.1)^{bc}	222.7 (336.3)^{d}	1.7 (2.6)^{cd}

this study the choice for stimulating the right cervical vagus nerve has no specific physiological reason.

VNS would, in particular, be beneficial when administered pre-ischemic but this is only relevant in cases of planned interventions like cardiac surgery. Still, VS applied shortly after the onset of an infarction but before reperfusion can reduce tissue loss (Shinlapawittayatorn et al., 2014).

Thus far it is unclear whether additional immune functions like the disposal of waste material and stimulation of tissue repair are also decreased after VS. Although this is very interesting it is beyond the scope of this 48 h study and has to be examined in follow up research. Better understanding of the inflammatory processes involved may help in prevention and in finding new therapeutics for cardiovascular and other inflammatory diseases.

While we were looking for a protective role of vagal activity, BW loss after surgery in the VS + IR group (−11.5%) is nearly twice that of the other groups, IR (−6.5%) as well as VS (−5.9%). The animals showed some discomfort and refused to eat and drink and had to be administered additional wet food. Apparently, the combination of VS plus IR worsens the condition of the mice compared to just IR or VS or it could have otherwise reduced the appetite of the animals.

WBC and lymphocytes in the circulation at $t = 48$ h are found to be lower in the VS + IR group than in the IR group (Table 1). Although these values do not differ significantly, lower numbers support the hypothesis that VS attenuates inflammatory responsiveness. In that respect, it is remarkable that the VS + IR group-means for WBC and lymphocytes are also lower than those of the VS group. As previously found in our institute, arterial injury is also able to cause inhibition of inflammatory responsiveness in rodents as well as in human patients (Nederhoff et al., 2017; Versteeg et al., 2009). This principle could be responsible for the relatively low values in plasma levels of WBC and lymphocytes in terminal blood since the LAD-occlusion may have left a small but significant arterial wall injury. This, in addition to VS could have caused an accumulated inhibitory effect on levels of circulating inflammatory cells.

The mean decrease in SV in time from $t = 0$ to $t = 48$ h is less severe for the VS + IR group than for the IR group. However, this difference is not significant (Fig. 7). The same applies to the EF where no significant effect between groups is found that was caused by VS.

Other pro-inflammatory cytokines like IL-1β and IL-6 are known to be produced by the body in response to a local ischemic period. TNFα however, is a potent cytokine and one of the most early to respond to an inflammatory stimulus and we therefore accepted TNFα levels as a good measure for inflammatory responsiveness.

In some other studies longer periods up to 150 min of VS have been used to achieve a reduced inflammatory response (Shinlapawittayatorn et al., 2014; Katare et al., 2009). Huston however, initiated an inhibitory effect on inflammatory responsiveness in the levels of TNFα in LPS stimulated mouse blood that was detectable for almost 72 h. This was initiated by only 30 s of effective vagus stimulation (Huston et al., 2007). To determine TNFα in the present study, terminal whole blood at 48 h after VS and IR was incubated with LPS for 20 h. This incubation period is quite long and beyond the response maximum. However, TNFα levels after such a period should still be increased and measurable and actual differences would be obvious (Huston et al., 2007; Xing and Remick, 2003). However, for future studies, we would recommend the use of an incubation time close to the optimum of 6 h to yield more explicit results.

The mean TNFα level (Fig. 3) was highest for the IR group (70.0 ± 100.3 pg/ml) compared to the VS + IR (33.7 ± pg/ml) and the VS group (28.2 ± 33.9 pg/ml) but this was mainly caused by a small number of extreme values. Although differences in TNFα levels should be visible after 48 h, inflammatory responsiveness could already be halfway back to baseline and therefore less distinguishable. Previously we assessed the acute inhibitory response on whole blood LPS responsiveness in a femoral artery injury model and observed a significant decrease of the TNFα release which supports the view that the delay of 48 h may have diluted the effect of VS on LPS induced TNFα release (Nederhoff et al., 2017) (data not shown).

As expected, most neutrophils and T-lymphocytes were found in the infarcted and border areas of the IR myocardium (ns), more in the borders than in the infarcted areas. The numbers of inflammatory cells in the infarcted heart may not be at their peak level after 48 h. Neutrophils tend to peak at 24 h and are already decreasing on day 2 after IR while T-lymphocytes reach their maximum around day 3 and could still be at a poor detection level at the time of termination (Arslan et al., 2010; Yan et al., 2013). Therefore, further studies should examine separately periods of 24 h and 7 days to cover both the first inflammatory onset and the more developed situation.

Cholinergic activation stimulates the release of Choline Acetyl Transferase (ChAT) (Kakinuma et al., 2013) which is associated with VEGF production, neovascularisation and collateral formation in dogs (Meesmann and Schulz, 1970). Also, ACh-esterase inhibitor pyridostigmine increases VEGF-production in the left ventricle after MI, suggesting myocardial angiogenesis (Lataro et al., 2013) caused by an elevated ACh presence. VEGF levels, as well as microvessel density, were also higher in the penumbra of experimental cerebral ischemia in rats after VS (Jiang et al., 2016). These data suggest a relationship between the protective role of the parasympathetic nervous system and elevated VEGF levels in the area at risk after an ischemic period. In this study, some higher values lift the mean VEGF amount to a higher level in the border area as compared to the remote area ($p = 0.067$). In total, the mean of the IR group is bigger than VS + IR (ns) or VS ($p = 0.039$)

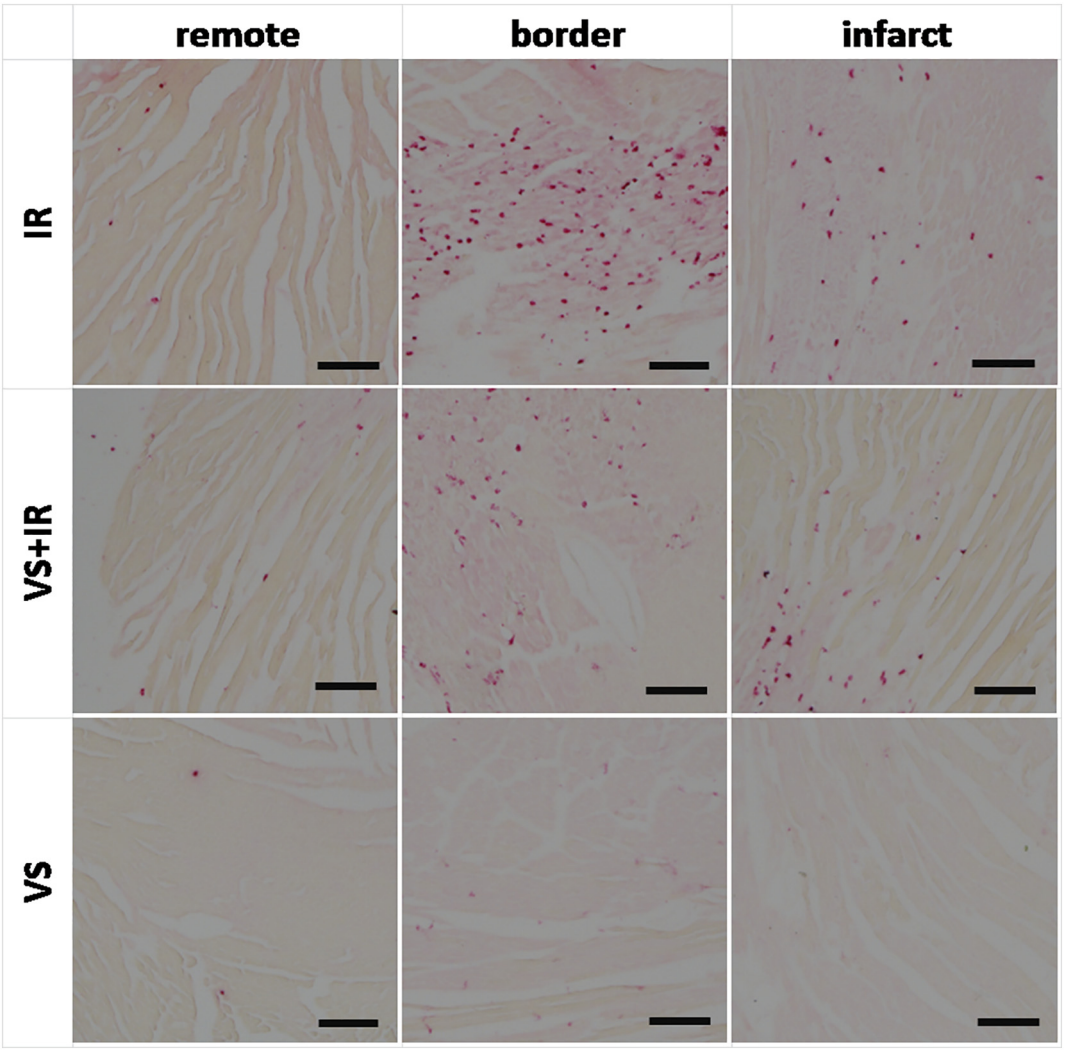

Fig. 5. LPR stained neutrophils in transversal heart sections. (100x). Scale bar = 100 μm.

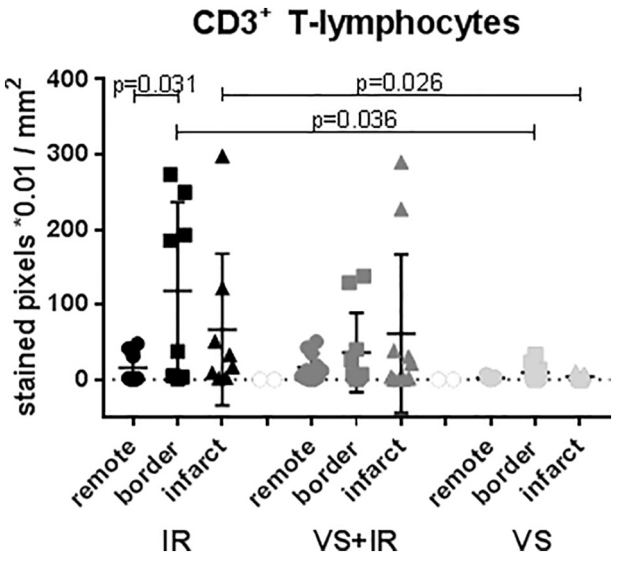

Fig. 6. CD3$^+$ lymphocytes in transversal sections are shown as the stained area in pixels*0.01 per mm2. In the IR group (N = 8), the border area shows more CD3+ lymphocytes than the remote area (p = 0.031) and the VS group (N = 9) border area (p = 0.036). The infarcted area in the IR group contains more CD3$^+$ lymphocytes than the VS group does (p = 0.026) (Kruskall-Wallis).

Table 3
CD3$^+$ lymphocytes values in transversal sections from treated hearts presented as mean stained area in pixels*0.01 per mm2, Means ± SD. a, b and c are significantly different pairs (Kruskall-Wallis).

Mean (SD)	IR	VS + IR	VS
Remote	15.9 (20.8)[a]	16.5 (18.3)	2.1 (2.0)
Border	118.6 (118.1)[ab]	36.0 (53.0)	9.4 (11.2)[b]
Infarct	66.8 (101.3)[c]	61.5 (105.8)	4.2 (3.7)[c]

see Fig. 8.

While baseline echoes were easy to perform and yielded clear results, in the 48-hour measurements exact orientation of the heart in the thorax was difficult to define. This could have been due to the short period between the intervention and termination. During LAD-occlusion the pericardium is damaged and after 48 h the position of the heart in the thorax is probably not yet stable. This could have led to cardiac echo recordings of poor quality and subsequently a big variation in the results.

Some terminal blood data were missing because of technical problems. This has happened to the data of one mouse from the IR group, three from the VS + IR group and two mice from the VS group. Despite this fact, group numbers were still large enough for analysis.

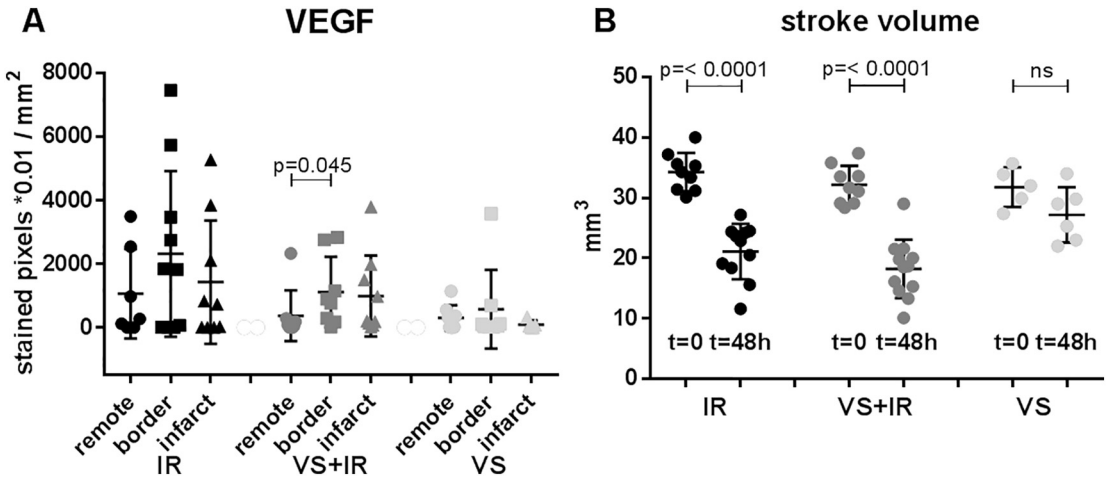

Fig. 7. Amounts of Vascular Endothelial Growth Factor (VEGF) in transversal sections of the heart shown as stained area in pixels*0.01 per mm2. Apart from a higher value in the border area as compared to the remote area in the VS + IR group, no significant differences are detected. IR: N = 9, VS + IR: N = 8, VS: N = 8. B: Stroke volumes (SV) at t = 0 to 48 hours after surgery in mm3. Groups IR (N = 10) and VS + IR (N = 10) show significantly decreased values at t = 48h compared to baseline. IR baseline 24.3 ± 3.2mm3 to t = 48h: 21.1 ± 4.6mm3 (p = < 0.0001), VS + IR baseline 32.2 ± 3.1mm3 to t = 48h: 18.2 ± 4.9mm3 (p = < 0.0001). The VS group (N = 6) shows no significant difference (Mean ± SD, Kruskall-Wallis).

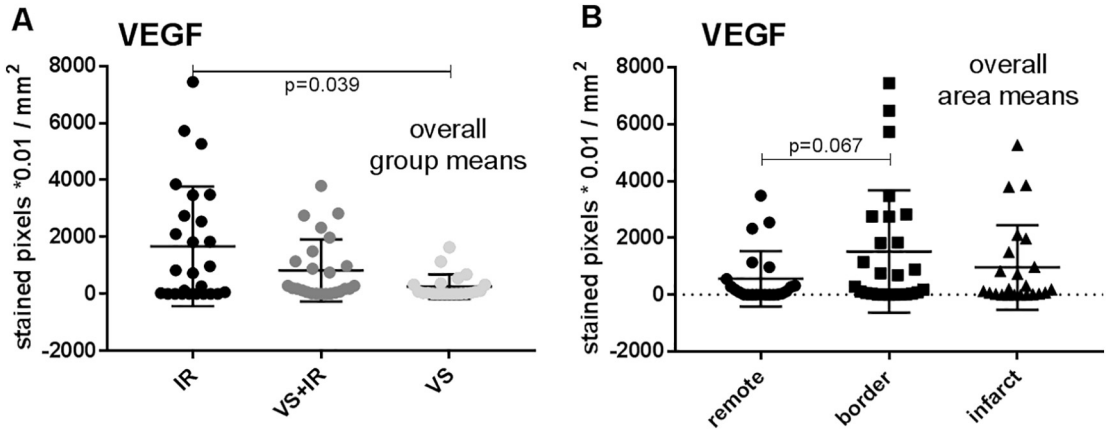

Fig. 8. Differences in vascular endothelial growth factor (VEGF) levels through the groups and three areas of transversal sections of the reperfused heart at 48h. Values presented in stained area *0.01 pixels per mm2. A: IR group, N = 26: 1667 ± 2100, VS + IR group, N = 25: 826.3 ± 1090 and VS group, N = 21: 260.5 ± 430.6. B: Remote area, N = 22: 562.9 ± 975.3, Border area, N = 26: 1518.0 ± 2150.0 and Infarcted area, N = 23: 803.8 ± 1168.0 (Mean ± SD, Kruskall-Wallis).

5. Conclusions

Vagus nerve stimulation, prior to an ischemia-reperfusion protocol in mice decreases the infarct size over area at risk ratio at 48 h after intervention. No difference is detected in the decrease of ejection fraction or numbers of neutrophils or lymphocytes in hearts of vagally stimulated versus not stimulated mice that underwent an ischemia-reperfusion treatment. Solid evidence for an inflammatory attenuation caused by vagus nerve stimulation is not found in this study. This could imply that VS has a less inhibiting effect on inflammatory responsiveness than may be expected from literature.

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Authors' individual contributions statement

M.G.J. Nederhoff[1]* was in charge of the setup and execution of the study and wrote the manuscript.

S.A.M.W. Verlinde[1] and D.E. Fransen[1] assisted in surgical interventions. They were also involved in tissue preparation and carried out histochemical analysis.

G. Pasterkamp[2] and R.L.A.W. Bleys[1] supervised and financed the project and took part in final editing of the manuscript.

M.A.D. Brans performed the majority of the echo cardiographies and all of the ischemia-reperfusion surgery.

Declaration of competing interest

None declared.

References

Arslan, F., et al., 2010. Myocardial ischemia/reperfusion injury is mediated by leukocytic toll-like receptor-2 and reduced by systemic administration of a novel anti-toll-like receptor-2 antibody. Circulation 121, 80–90.

Bianchi, M.E., et al., 2017. High-mobility group box 1 protein orchestrates responses to tissue damage via inflammation, innate and adaptive immunity, and tissue repair. Immunol. Rev. 280, 74–82.

Bonaz, B., Sinniger, V., Pellissier, S., 2017. Vagus nerve stimulation: a new promising therapeutic tool in inflammatory bowel disease. J. Intern. Med. https://doi.org/10.1111/joim.12611.

Borovikova, L.V., et al., 2000. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405, 458–462.

Buchholz, B., et al., 2018. Vagal stimulation mimics preconditioning and post-conditioning of ischemic myocardium in mice by activating different protection mechanisms. Am. J. Physiol. Circ. Physiol. 314, H1289–H1297.
Capobianco, A., Cottone, L., Monno, A., Manfredi, A.A., Rovere-Querini, P., 2017. The peritoneum: healing, immunity, and diseases. J. Pathol. 137–147. https://doi.org/10.1002/path.4942.
Chavan, S.S., Tracey, K.J., 2017. Essential neuroscience in immunology. J. Immunol. 198, 3389–3397.
Coote, J.H., 2013. Myths and realities of the cardiac vagus. J. Physiol. 591, 4073–4085.
Erdfelder, E., 2009. Statistical Power Analyses Using G* Power 3. 1: Tests for Correlation and Regression Analyses. vol. 41. pp. 1149–1160.
Gidron, Y., Kupper, N., Kwaijtaal, M., Winter, J., Denollet, J., 2007. Vagus – brain communication in atherosclerosis-related inflammation: a neuroimmunomodulation perspective of CAD. Atherosclerosis 195, e1–e9.
Gold, M.R., et al., 2016. Vagus nerve stimulation for the treatment of heart failure: the INOVATE-HF trial. J. Am. Coll. Cardiol. 68, 149–158.
GraphPad Prism Version 7 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com
Grubbs, F.E., 1969. Procedures for detecting outlying observations in samples. Technometrics 11, 1–21.
Huston, J.M., et al., 2007. Transcutaneous vagus nerve stimulation reduces serum high mobility group box 1 levels and improves survival in murine sepsis. Crit. Care Med. 35, 2762–2768.
Jiang, Y., et al., 2016. Auricular vagus nerve stimulation promotes functional recovery and enhances the post-ischemic angiogenic response in an ischemia/reperfusion rat model. Neurochem. Int. 97, 73–82.
Kakinuma, Y., et al., 2013. Heart-specific overexpression of choline acetyltransferase gene protects murine heart against ischemia through hypoxia-inducible factor-1?-related defense mechanisms. J. Am. Heart Assoc. 2, 1–14.
Katare, R.G., et al., 2009. Vagal nerve stimulation prevents reperfusion injury through inhibition of opening of mitochondrial permeability transition pore independent of the bradycardiac effect. J. Thorac. Cardiovasc. Surg. 137, 223–231.
Kelly, J., Cicale, E.M., Buchholz, B., Casanova, V.C., Gelpi, R.J., 2017. Myocardial protection by vagal stimulation in ischemia-reperfusion injury in mice Protección miocárdica por estimulación vagal en la injuria por isquemia y reperfusión en ratones. Rev. Argent. Cardiol. 85, 6–12.
Koopman, F.A., Schuurman, P.R., Vervoordeldonk, M.J., Tak, P.P., 2014. Vagus nerve stimulation: a new bioelectronics approach to treat rheumatoid arthritis? Best Pract. Res. Clin. Rheumatol. 28, 625–635.
Kox, M., Pickkers, P., 2015. Modulation of the innate immune response through the vagus nerve. Nephron 131, 79–84.
Kwan, H., et al., 2016. Vagus nerve stimulation for treatment of inflammation: systematic review of animal models and clinical studies. Bioelectron. Med. 3, 1–6.
Lataro, R.M., et al., 2013. Increase in parasympathetic tone by pyridostigmine prevents ventricular dysfunction during the onset of heart failure. AJP Regul. Integr. Comp. Physiol. 305, R908–R916.
Meesmann W, Schulz FW, Schley G, Adolphsen, P. Survival rate after acute experimental coronary occlusion as related to the spontaneous collateral vessels in hearts. Z Gesamte Exp Med. 153, 246–264 (1970).
Mihaylova, S., Schweighöfer, H., Hackstein, H., Rosengarten, B., 2014. Effects of anti-inflammatory vagus nerve stimulation in endotoxemic rats on blood and spleen lymphocyte subsets. Inflamm. Res. 63, 683–690.
National Research Council, 2011. Guide for the Care and Use of Laboratory Animals, Eighth edition. the National Academic Press.
Nederhoff, M.G.J., et al., 2017. Minor Vascular Injury has a Major Influence on the Innate Immune Response in a Rodent Animal Model. vol. 4. pp. 5–10.
Nilsson, S.F.E., 1996. Nitric oxide as a mediator of parasympathetic vasodilation in ocular and extraocular tissues in the rabbit. Investig. Ophthalmol. Vis. Sci. 37, 2110–2119.
Pavlov, V. a, Tracey, K.J., 2005. The cholinergic anti-inflammatory pathway. Brain Behav. Immun. 19, 493–499.
Pavlov, V. a, Wang, H., Czura, C.J., Friedman, S.G., Tracey, K.J., 2003. The cholinergic anti-inflammatory pathway: a missing link in neuroimmunomodulation. Mol. Med. 9, 125–134.
Pereira, M.R., Leite, P.E.C., 2016. The involvement of parasympathetic and sympathetic nerve in the inflammatory reflex. J. Cell. Physiol. 231, 1862–1869.
Randall, W.C., Milosavljevic, M., Wurster, R.D., Geis, G.S., Ardell, J.L., 1986. Selective vagal innervation of the heart. Ann. Clin. Lab. Sci. 16, 198–208.
Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675.
Sheng, Y., Zhu, L., 2018. The crosstalk between autonomic nervous system and blood vessels. Int J Physiol Pathophysiol Pharmacol 10, 17–28.
Shinlapawittayatorn, K., et al., 2014. Vagus nerve stimulation initiated late during ischemia, but not reperfusion, exerts cardioprotection via amelioration of cardiac mitochondrial dysfunction. Heart Rhythm. 11, 2278–2287.
Simoons, M.L., et al., 1986. Early thrombolysis in acute myocardial infarction: limitation of infarct size and improved survival. J. Am. Coll. Cardiol. 7, 717–728.
SPSS 22; IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.
Sundman, E., Olofsson, P.S., 2014. Neural control of the immune system. AJP Adv. Physiol. Educ. 38, 135–139.
Tracey, K.J., 2007. Physiology and immunology of the cholinergic antiinflammatory pathway. J. Clin. Invest. 117, 289–296.
Uitterdijk, A., et al., 2015. Vagal nerve stimulation started just prior to reperfusion limits infarct size and no-reflow. Basic Res. Cardiol. 110.
Vajjhala, P.R., Ve, T., Bentham, A., Stacey, K.J., Kobe, B., 2017. The molecular mechanisms of signaling by cooperative assembly formation in innate immunity pathways. Mol. Immunol. 86, 23–37.
Versteeg, D., et al., 2009. Toll-like receptor 2 and 4 response and expression on monocytes decrease rapidly in patients undergoing arterial surgery and are related to preoperative smoking. Shock 31, 21–27.
Wurfel, M.M., et al., 2005. Identification of high and low responders to lipopolysaccharide in normal subjects: an unbiased approach to identify modulators of innate immunity. J. Immunol. 175, 2570–2578.
Xing, L., Remick, D.G., 2003. Relative cytokine and cytokine inhibitor production by mononuclear cells and neutrophils. Shock 20, 10–16.
Yamakawa, K., et al., 2014. Electrophysiological effects of right and left vagal nerve stimulation on the ventricular myocardium. Am. J. Physiol. Circ. Physiol. 307, H722–H731.
Yan, X., et al., 2013. Temporal dynamics of cardiac immune cell accumulation following acute myocardial infarction. J. Mol. Cell. Cardiol. 62, 24–35.
Yellon, Derek M., Hausenloy, D.J., 2007. Myocardial reperfusion injury. N. Engl. J. Med. 357, 1121–1135.
Yi, C., et al., 2016. Vagus nerve stimulation attenuates myocardial ischemia/reperfusion injury by inhibiting the expression of interleukin-17A. Exp. Ther. Med. 11, 171–176.
Zhao, M., et al., 2012. Vagal nerve modulation: a promising new therapeutic approach for cardiovascular diseases. Clin. Exp. Pharmacol. Physiol. 39, 701–705.