Neural regulation of inflammatory processes in the heart and in large arteries

Marcel Nederhoff

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Neural regulation of inflammatory processes in the heart and in large arteries

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in het hart en de grote slagaders

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Marcellinus Gijsbertus Jozef Nederhoff

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Promotoren:

Prof. dr. R.L.A.W. Bleys

Prof. dr. G. Pasterkamp

Voor Ellen, Veerle & Rosalie

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CHAPTER 1

General introduction

The immune system supports the body with a wide range of essential functions. It is a primary line of defense against external, non-self-molecules that are expressed on micro-organisms and toxins with the objective to remove waste materials from inside the body. It is also activated to eliminate waste materials from endogenous damaged tissue after injury caused by trauma, chemicals and ischemia and regulates healing processes and restoration of tissue function after such injury¹⁻³.

A specific and very delicate task of the immune system is recognition, selection and removal of aged and damaged cells. Apart from differentiating between self and foreign substances it must be able to distinguish between well-functioning and malfunctioning cells, and subsequently initiate an appropriate response.

Immune cells like macrophages, dendritic cells and neutrophils express pattern recognition receptors (PPRs) in their membranes. They respond to exogenous pathogen associated molecular patterns, PAMPs, and to endogenous damage associated molecular patterns, DAMPs. These are proteins which are part of infective organisms or are released by damaged and necrotic cells respectively. When such protein is recognized as a threat, inflammatory cells invade the place of action and release cytokines and chemokines to further accelerate the inflammatory response and subsequently render the threat harmless, stimulate recovery and remove the resulting debris^{4,5}. Increase of temperature, formation of some edema and neovascularization will often accompany inflammation. In the end of this process cells and cytokines that stop inflammatory activity are released and the tissue will return to its normal state and function. In this process function of some immune cells like neutrophils, macrophages and lymphocytes can change from pro- to anti-inflammatory^{6,7}. Tissue that cannot be healed will be replaced by collagen to form scar tissue.

Since inflammation is involved in degradation as well as in healing, a fine-tuned regulation is necessary to maintain health. Hyperactive inflammatory responsiveness can lead to auto-immune responses or to unnecessary inflammatory damage while underperformance may give way for non-disposal of damaged or malfunctioning cells, leading to serious and even mortal conditions⁸⁻¹¹.

The autonomic nervous system (ANS) is involved in the maintenance of this delicate healthy balance.

The autonomic nervous system and inflammation

The efferent part of the ANS has two functional divisions, the sympathetic and parasympathetic nervous systems. Such a division is not made for the visceral afferent fibers which conduct information from the body to the central nervous system. The sympathetic and parasympathetic systems are presented as counterparts in which the sympathetic part represents “fight, flight or freeze” activity. The sympathetic system becomes active in situations of an acute (potential) danger, in cases when alertness is needed. The major neurotransmitters involved are adrenaline and noradrenaline, mainly produced in different cell clusters of the medulla of the adrenal glands and at the terminals of sympathetic neurons. Noradrenaline is also released by specific immune cells¹².

As the sympathetic nervous system directs activity in moments of vigilance, the parasympathetic nervous system is active in situations of “feed and breed” and “rest and digest”. That means that it is mainly involved in keeping the homeostasis of physical functions. Its main neurotransmitter is acetylcholine (ACh), synthesized by neurons and released in synapses. It can also be produced by specific immune cells¹³.

A third distinct entity within the ANS is the enteric nervous system (ENS), a self-contained system mainly situated in the abdomen that regulates intestinal functions. While it can be influenced by sympathetic and parasympathetic nerve fibers the ENS has its own functions and its own reflexes¹⁴. Strong ENS-immune interactions exist but the ENS will not be discussed further in this thesis.

The sympathetic and parasympathetic nervous systems are often presented as opposing systems since a number of functions do counteract. Still, it would be more appropriate to consider them as complementary since an imbalance may result in disease but a complete absence of one of the two would be lethal.

Apart from the mentioned autonomic neural functions in moments of alertness and during rest, autonomic activity affects crucial processes of the immune system. Many immune cells have adrenergic and cholinergic receptors on their membranes¹⁵ or have the ability to produce these neurotransmitters^{16–19}. Some other immune cells are able to release tyrosine hydroxylase (TH), an enzyme that converts tyrosine to L-DOPA, a precursor of noradrenaline²⁰ while other immune cells can release choline acetyltransferase (ChAT), which is an enzyme necessary to produce acetylcholine. The parasympathetic nervous system has clearly anti-inflammatory influences while the sympathetic nervous system is associated with pro- as well as anti-inflammatory activity. Nerve fibers tend to grow towards their targets initiated by chemoattractants and growth factors, often during vascular development. One of the most important factors is nerve growth factor (NGF) that, next to its nerve growth stimulating function, also has pro- and anti-inflammatory and reparative capacities^{21,22}. NGF, which is produced by a variety of cells through the body can therefore be of importance in the development of inflammation based injury.

The vagus nerve, the tenth cranial nerve, has a large parasympathetic component. It supplies all cervical and thoracic organs and most abdominal organs. Furthermore, the vagus nerve contains many visceral afferent fibers. The last two decades brought increasing evidence for decreased inflammatory responsiveness after electrical or manual stimulation of the vagus nerve (VS). Upon VS an inhibitory signal is sent to the body via vagal or sympathetic efferent fibers. The released acetylcholine binds to the $\alpha 7$ -subunits of nicotinic acetylcholine receptors ($\alpha 7nR$), thereby inhibiting the release of pro-inflammatory cytokines like TNF α and interleukin 1 beta (IL-1 β) upon stimulation of whole blood with lipopolysaccharide (LPS)^{23–26}. The attenuating effect is measurable in blood for approximately 72 hours after VS in rats²⁷. This principle is further defined in the cholinergic anti-inflammatory pathway (CAP) by Tracey and his colleagues²⁸. More recent research has resulted in the therapeutic use of VS for heart failure²⁹, inflammatory bowel disease³⁰ and rheumatoid arthritis³¹. Studies are being undertaken on the benefits of VS in reducing inflammation and edema in patients with severe burning damage³² and to inhibit development of excessive brain damage after stroke^{33,34}.

Figure 1. The Cholinergic Anti-inflammatory Pathway. When a pathogen or injury is recognized by the immune system, an inflammatory process is initiated. This process activates vagal nuclei in the brain stem via the afferent vagus nerve. As a consequence a response is sent by the efferent vagus nerve releasing acetylcholine that binds to $\alpha 7$ AChR-receptors on inflammatory cells which causes a decrease of the release of pro-inflammatory cytokines.

In human patients, the electrode is connected to the left cervical vagus nerve to avoid cardiac complications since the right vagus nerve mainly supplies the sinu-atrial node. The left vagus nerve is thought to innervate the atrioventricular node³⁵. Recent extensive research showed no differences in cardiac response between left and right VS^{36,37}. Minor adverse side effects like hoarseness, tinnitus, nausea and vertigo have been reported during VS but none that persists after stimulation has stopped^{38,39}. In very rare occasions VS caused parasympathetic over-responsiveness resulting in bradycardia followed by cardiac arrest. When stimulation was stopped, no further episodes of asystole occurred^{40,41}.

The implantation procedure is simple but induces a small risk. However, in practice no serious accidents have been reported. The use of VS in experimental and therapeutic settings is therefore generally confirmed.

In summary, the immune system is essential for the maintenance of good health. Given the influence and strength of this system it has to be strictly regulated. The ANS appears to have all the necessary qualifications for a function as such.

Outline of the thesis

Cardiovascular diseases (CVD) account for the most significant inflammatory diseases in the developed countries. Globally, one in every three persons dies from CVD (WHO-facts)⁴², some 18 million victims every year with many more suffering from physical impairment and chronic distress after a non-mortal cardiovascular event. It is therefore important to carry out research to achieve better understanding of the processes involved in initiation and pattern of development of CVD. Understanding inflammatory processes and its control by the ANS is essential. General aims of the studies presented in this thesis are:

- 1: to assess a beneficial effect of vagal stimulation in different manifestations of CVD and;
- 2: to investigate the associations of innervation patterns and local inflammation in the diseased arterial wall.

In **Chapter 2**, the effect of arterial damage and blood sampling on the inflammatory response following a bacterial ligand stimulation were examined in the rat.

Unfortunately, continuous and reproducible in vivo monitoring and influencing of vagal activity and subsequent inflammatory responses is beyond the current technical possibilities. We therefore studied the semi-acute/initial responses of vagal stimulation to myocardial infarction (MI) and ischemia-reperfusion (IR) in the mouse that is described in **Chapters 3 and 4** in this thesis.

In human arterial tissue samples non-interventional studies were performed. In carotid and popliteal artery aneurysms it was studied to what extent these were innervated, regarding general innervation and sympathetic fibers in particular (**Chapter 6**). **Chapter 5** describes a study in human aortic wall samples in which the relation of innervation with inflammatory activity was compared in different developmental stages of atherosclerosis. To enable VS studies over an extended period, an implantable nerve stimulator for small lab animals was developed as described in **Chapter 7**. In the next paragraphs the studies will be outlined in more detail.

Vascular injury

An inflammatory response starts with the recognition of PAMPs or DAMPs by toll like receptors (TLRs) that are located in the membrane of circulating cells. This is followed by the release of inflammatory cytokines in an attempt to mitigate the threat. This principle is broadly confirmed in several animal models. Levels of inflammatory responsivity are determined by stimulation of blood with LPS.

In 2008 Versteeg et al. discovered that percutaneous coronary interventions (PCI) caused a rapid decrease of responsiveness of toll like receptors (TLRs) in the membranes of inflammatory cells which resulted in systemic inflammatory inhibition⁴³. One year later, the same researchers showed that vascular arteriotomy resulted in a decreased expression of tumor necrosis factor alpha (TNF α) by circulating inflammatory cells (D Versteeg et al., 2009). It appeared that small damage to the arterial wall had serious attenuating effects on the inflammatory responsiveness.

When invasive manipulations of arteries are detrimental for innate immune function this can be of great importance for results in (bio-)medical research and medical practice. In

surgical interventions (small) arterial injury is often inevitable and in animal research blood sampling is generally executed by tail clipping in which venous and arterial vessels are injured. Arterial catheterization is also a common procedure to measure blood pressure in animal experimentation. In **Chapter 2** consequences of blood sampling and arterial injury for inflammatory parameters in rats are discussed.

Myocardial Infarction or Ischemia-Reperfusion with or without vagus stimulation

Inflammation also plays an important role in injury and repair after an acute myocardial infarction (MI) and in the processes that are initiated by the reperfusion of infarcted tissues. Myocardial ischemia in the cardiac wall leads to starvation of a large amount of cardiomyocytes which stimulates the release of DAMPs. In a subsequent inflammatory response neutrophils and macrophages invade the infarcted site and with the release of inflammatory cytokines they remove dead myocytes and cell matrix debris. This response is often more than proportional to the initial infarcted area and excessive cytotoxic effects extend the ischemic injury, leading to remodeling of the myocardial wall and ultimately to development of heart failure⁴⁵⁻⁴⁷. Early repression of the inflammatory processes will be beneficial for limiting post MI damage.

Early restoration of the blood flow is also crucial to mitigate the extend of the final damage but reperfusion by itself can generate additional damage called ischemia reperfusion injury⁴⁸. Upon reperfusion inflammatory processes are further stimulated. Release of reactive oxygen species (ROS) by inflammatory cells causes oxidative stress which together with calcium overload and microvascular obstruction leads to secondary injury. As a consequence viable cardiomyocytes die shortly after reperfusion. Recent literature suggests that the processes that lead to reperfusion injury may account for up to 50% of the final infarct size and it has been stated that no effective therapy so far exists that reduces lethal myocardial reperfusion injury⁴⁹. The ANS has been suggested to regulate immune function in cardiovascular diseases (CVD)^{50,51}. In this thesis we present two studies in mice on the inflammation attenuating effect of VS in a myocardial infarction model (**Chapter 3**) and in an ischemia-reperfusion model (**Chapter 4**).

Atherosclerosis

The substrate for MI is atherosclerosis in the coronary arteries. Atherosclerosis is an inflammatory disease of the arterial wall of which the first stages already show at very young age in the aorta^{52,53} while the first fatty streaks in coronary arteries are found ten to fifteen years later⁵⁴. Among the risk factors for atherosclerosis are age, male sex, hypertension, diabetes, hypercholesterolemia and obesity. Smoking and a sedentary lifestyle are very strong risk factors irrespective of age and sex⁵⁵⁻⁵⁷. Atherosclerosis is most likely initiated by a small injury of the endothelium which originates from physical or biochemical forces or immune activity. An inflammatory response is started to heal the injury and to restore the smooth internal vascular lining to provide an easy blood flow and supply to the distal parts of the vascular bed. This response causes the injured endothelium to become permeable for low density lipoproteins (LDL) which accumulate and oxidize in the sub-endothelial layers.

Aggregated oxidized LDL (oxLDL) is then being phagocytized by resident dendritic cells and by invading macrophages. In these phagocytes, oxLDL is esterified and the overly filled phagocytes turn into foam cells which all together contribute to a lipid pool formation. While in principle the first fatty deposits can still be cleared from the vessel wall, further developmental stages will have a more permanent character. Foam cells induce cytokine and chemokine production that recruit additional circulating immune cells, further propelling the inflammatory response. These inflammatory cytokines include interleukin (IL)-1 α and IL-1 β , interferon (IFN)- γ , MCP-1. Smooth muscle cells (SMCs) in the tunica media can change their phenotype when exposed to oxLDL and cytokines and develop phagocytic properties with macrophage like markers in their membrane and support the macrophages in their effort to scavenge oxLDL⁵⁸. The overly filled foam cells eventually die and in the ongoing process, however, active clearance of those deceased cells is impaired and apoptotic debris accumulates in the atherosclerotic plaque. This causes a further impulse for inflammation and can ultimately lead to instability and rupture of the plaque with infarction as a consequence. To summarize we can state that inflammation is a determining factor in the onset, the development and the progress of atherosclerosis. The significance of the damage is highly dependent on inflammatory activity in the atherosclerotic process. In **Chapter 5** a study on the association of inflammatory cells and innervation in atherosclerosis in the human abdominal aortic wall will be presented.

Aneurysms

An aneurysm is a balloon-like bulging that develops progressively at a weakened site of the arterial wall⁵⁹. Pathogenesis is multifactorial and includes inflammation, extracellular matrix degeneration and apoptosis of smooth muscle cells^{60,61}. This process causes a continuous weakening and further dilatation of the arterial wall until it ruptures. Rupturing of an aneurysm is a dangerous event which can cause major damage and function loss with a reported mortality of over 50%^{62–64}. Aneurysms are generally asymptomatic for a long period and mostly appear when they cause physical problems and by that time have developed to detectable proportions. They can occur anywhere in the arterial vasculature but most often near or in the Circle of Willis and the abdominal aorta at branching points. Other locations are the aortic arch and carotid and popliteal arteries. Aneurysms occur in several shapes but cerebral aneurysms are often “saccular” or “berry shaped” while most abdominal aneurysms are “fusiform”.

The underlying cause of aneurysm formation is not clear. Aneurysms have long been thought of being caused by atherosclerosis. Although aneurysms are often found in patients that also show atherosclerosis, this is not always the case. They do share a number of risk factors like age, gender, family history, high cholesterol, hypertension, obesity and coronary artery disease. Diabetes, however, an important risk factor in most arterial diseases, shows to be not or even negatively correlated with the presence of aneurysms^{65,66}. Genetic risk factors and biochemical mechanisms are also different.

The causes, however, may show topographical heterogeneity since there are differences in embryologic origin and tissue composition of the arterial wall at different locations⁶⁷. They

may therefore respond differently to mechanic forces, biochemical substances and cytokines. Genetic studies in families with an aneurysm sensitivity demonstrate that most families have either thoracic or abdominal aneurysms but not both⁶⁸ which can be explained by the different embryonic development of aortic sections.

Aneurysms and atherosclerosis may better be considered to be different manifestations of the response to a small arterial wall injury caused by mechanical forces, biochemical or infection⁶⁷. This small injury then causes an inflammatory response to heal the damage and in the process of aneurysm formation, causes weakening of the vessel wall matrix and loss of elasticity. The threatened wall together with strong pulsatile pressure causes further injury and tiny ruptures arise in the stretched wall prolonging the inflammatory activity that leads to irreversible dilatation of the affected part of the vessel. Sometimes the tiny ruptures give the blood access to the compromised layers of the wall where it forms balloon shaped out-bulgings. Collagen formation follows in an effort to withstand the pressure and protect the wall from rupturing which further reduces the flexibility of the artery. The iterative inflammatory process continues to weaken the arterial wall and if not treated, it becomes unable to withstand the forces of the luminal blood pressure resulting in progressive dilatation and rupture. Further research into the etiology and the developmental course of different types of aneurysms is required, with more emphasis on ways of attenuating the inflammatory processes involved. **Chapter 6** addresses inflammatory parameters in coexistence with the nerve supply of human abdominal aneurysms to find a possible relation between inflammation and innervation.

Vagus nerve stimulator

Inflammatory responsiveness is at least partially regulated by the ANS. Although a lot of new knowledge on the topic has been published in recent years it has also elicited many new questions. With the acceptance of vagus nerve stimulation as a useful way to trigger the CAP and suppress inflammatory responsiveness a means for extensive study of inflammatory processes has become available.

In biomedical research small laboratory rodents are frequently used. Inbred animals are explicitly useful for studies on a wide range of inflammation related diseases. Today, with modern genetic manipulation techniques, the numbers of available small animal strains for disease models have dramatically expanded. Mouse, but to a lesser extend rat models are developed for inflammatory diseases like rheumatoid arthritis, atherosclerosis, diabetes, inflammatory bowel disease, obesity and many others.

Many studies have been published about single dose VS in small animals⁶⁹⁻⁷² but research on repeated VS over a longer period is still scarce. For reliable results of immune parameters animals should be kept free moving and free from stress.

In **Chapter 7** the first experiences with an, in our lab developed, implantable vagus nerve stimulating device will be presented. The device is small enough for implantation in a mouse, it has a long work span and it produces no or minimal stress for the animal.

Research topics that were previously not possible such as long-term and teratologic effects of VS in immune related processes, combined effects of medication and VS, VS effect on responses and final outcome after surgical operations, all become in reach with such device.

In **Chapter 8** the main conclusions of all investigations described are reviewed and discussed, and future perspectives are considered.

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CHAPTER 2

Minor vascular injury has a major influence on the innate immune response in a rodent animal model

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M.G.J. Nederhoff^{1,3}
E.H.A.M. Elsenberg¹
I.E. Höfer²
M. de Goeij^{1,3}
J. van Hengstum¹
R.L.A.W. Bleys³
G. Pasterkamp².

¹ Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands

² Department of Clinical Chemistry, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands

³ Department of Anatomy, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands

ABSTRACT

Introduction

In biomedical animal research, protocolled vascular interventions such as regular blood sampling or monitoring of organ functions are common practice. Vascular interventions and punctures are applied on large scale with concomitant injury to the arterial wall. In human cardiovascular patients, percutaneous coronary intervention or surgical vascular arteriotomy elicits a rapid decrease of the innate immune response in whole blood samples, shown by a down toning of TNF α expression after incubation with lipopolysaccharides. Significant changes in innate immune responsiveness could influence outcomes in animal experimental research when arterial trauma is part of the study protocol.

Methods

In the present study, we assessed the effect of vascular trauma that is regularly applied in routine animal experimental studies on the innate immune response. We damaged the rat femoral arterial wall or executed a regularly practiced tail cut for blood withdrawal. Subsequently we examined the responsiveness of whole blood upon stimulation with a TLR4 ligand. Since anaesthetics are known to have an immunosuppressive effect, a volatile and an injectable anaesthetic were also compared.

Results

We found a strong inhibition of the innate immune response following vascular trauma in the rat. Blood sampling can already provoke a strong down toning of the immune responsiveness, irrespectively of sample volume or duration under anaesthesia.

Conclusions

Our results stress that significant inhibition of immune responsiveness should be considered when intravascular interventions or blood punctures are applied in *in vivo* experimental research. It merits consideration that this effect can have an impact on the outcome of studies in experimental animal models.

INTRODUCTION

Animal models are widely used to study cardiovascular or other chronic inflammatory diseases and in many cases vascular interventions are part of the experimental protocol. These interventions, inevitably, will cause injury to the arterial wall.

It was previously reported that vascular arteriotomy resulted in a blunted expression of tumour necrosis factor alpha (TNF α) by circulating cells in response to incubation with lipopolysaccharide (LPS) ¹. In addition, percutaneous coronary interventions (PCI) resulted in a fast inhibition of the responsiveness of the Toll Like Receptors (TLRs) on circulating cells. This down toning of the innate immune response occurred within minutes after percutaneous vascular intervention in patients with a coronary stenosis ².

TLRs recognize pathogens or damage associated proteins and respond with activation of the innate immune system by translocation of nuclear factor kappa B ^{3,4}. As a consequence, interleukins and cytokines are expressed and secreted which will further accelerate the inflammatory process.

In experimental research, animals are often subjected to interventions that require vascular catheterisation, blood sampling or even surgical exploration of organs. In these animal models the pathogenesis of diseases is studied, diseases, often considered to have an inflammatory origin. It is assumed that the experimental protocol does not influence the readout parameter since also sham operated animals undergo most of these interventions. It is unknown to what extent vascular experimental procedures do affect the innate immune system. Still, this is relevant for the extrapolation of research results since many inflammation based readout parameters considered in these animal models, could be directly accelerated or aggravated by the experimental procedure. The experimental procedure could elicit an unwanted side effect that limits the utilisation of the experimental outcome.

Blood sampling is a routinely performed procedure in animal research. The quality of the sampling varies per method. The experience of the technician is undoubtedly essential for small variance ⁵ and sampling methods come with different (dis-) advantages. In that respect, haemolysis, differences in packed cell volume, in white blood cell count, haemoglobin and corticosterone levels have been reported ^{6,7}. In rodent models tail tip clipping is regularly applied. This method yields a mixed arterial and venous sample of variable quality. In this method the blood is often contaminated with tissue products while "milking" the tail causes additional damage to the tissue ⁸. The method with least contamination, however, is the method of cardiac puncture producing a left ventricular blood sample. This method is advised to be used only once, in a terminal procedure.

In the current study we investigated to what extent vascular damage, among which induced by routinely executed vascular interventions, could influence the acute inflammatory responsiveness of circulating white blood cells.

In the rat model we studied inflammatory responsiveness upon arterial damage with a special attention to blood sampling. *Tail tip clipping* and *intra cardiac sampling* are used as means for blood collection.

We report that vascular injury in a rodent model elicits a strong acute down toning of the immune responsiveness of circulating blood cells. The same effect is observed following successive blood sampling. Our results support the view that a significant effect of experimental procedures on immune responsiveness may influence experimental outcomes and should be taken into account when results are extrapolated to human disease.

METHODS

Surgical and experimental preparation

For all experiments male Lewis rats were used, 320g $\pm$ 20g. (Lew/Crl, Charles River Europe). Anaesthetics are known to influence immune responses ^{9,10}. The use of the agents isoflurane, fentanyl and medetomidin is widespread. Isoflurane has been identified as a suppressor of inflammation after stimulation with endotoxin by attenuating the release of inflammatory cytokines ^{11,12}. This could explain an effect on immune responsiveness following vascular trauma. For these reasons, we have anesthetized rats with either isoflurane or an injectable Fentanyl/Medetomidin mixture.

In the procedure for repeated blood sampling, animals of all groups except B were anesthetized with ca. 2.0 % isoflurane in air:O₂ (2:1), using a nose cuff. Group B, however, received an intra-peritoneal injection with a mixture of Fentanyl 200 μ g/kg, (Bipharma, Hameln, Germany), and Medetomidin 300 μ g/kg, (Produlab Pharma, Raamsdonksveer, Netherlands), (FD mix). Body temperature was controlled and kept at 38°C. All experiments were approved by the Animal Ethical Experimentation Committee of the University Hospital.

In the procedure for inducing vascular injury the skin of the right inside leg was opened and the femoral artery was carefully exposed. Then, the artery was lifted up and clamped over approximately 16mm between the corrugated jaws of a needle holder. After 30 seconds, the needle holder was removed and the blood flow was restored. A needle holder provided a reliable and reproducible injury. The resulting damage ("crush") was equal in size and severity in all treated vessels and was found in the tissues of all the layers of the arterial wall (Picture 1).

Vascular injury

The vascular injury experiments consisted of three groups. Next to the arterial injury animals the "crush" group, N=7, we introduced a sham operated group of rats, N=6, that underwent the whole procedure but without lifting and crushing the femoral artery. Thereby, care was taken that the artery was kept fully undissected and uninjured. The third group served as a control that did not undergo any treatment except for a single blood sampling at the end of the same experimental period, N=5.

Picture 1. Cross sections of hematoxylin-Eosin stained femoral arteries. B: artery subjected to the crush procedure. Compared to A, a healthy control artery, the crushed vessel shows serious dilatation and damage to the intimal, medial and adventitial layers. A considerable haemorrhage is seen in the media. The lumen in B is filled with erythrocytes.

Table 1. Vascular injury, group definitions.

Group	Intervention
Control	No intervention
Sham	Sham operated
Crush	Arterial damage

From the three vascular injury groups only one terminal intra cardiac blood sample per experiment was drawn at the end of the procedure, 10 minutes after the time point of femoral injury. Using the cardiac puncture method, a 24G needle on a 1ml syringe was put through the thoracic wall into the left ventricle of the heart. Blood was then drawn up in the syringe and gently transfused into a heparinized 1ml vial (Multivette 600, Sarstedt, Nümbrecht, Germany).

Table 2. Repeated blood sampling, group definitions.

Blood sampling					
group nr	N	anaesthesia	frequency	volume µl	time points minutes
A	5	isoflurane	repetitive	750	0, 25, 50
B	5	FD-mix	repetitive	750	0, 25, 50
C	4	isoflurane	repetitive	350	25, 50
D	5	isoflurane	single	750	0
E	4	isoflurane	single	750	50

Repetitive blood sampling

Five animal groups were formed to examine the influence of successive blood drawings on inflammatory responsiveness. These groups did not undergo any other procedure than sedation and blood sampling. Group size: A= 5, B= 5, C= 4, D= 5 and E= 4 rats.

In all groups whole blood samples were taken by tail tip clipping. In this method, a piece of 1mm maximum was cut from the tail tip with a scalpel blade yielding a mixed sample of venous and arterial blood. A sample volume of 0.75ml or 0.35ml was collected directly after induction of anaesthesia, t=0, and at time points of t=25 and t=50 minutes thereafter. In one group the smaller volume of 0.35ml of blood was drawn to examine whether the size of the sample was responsible for a change in immune responsiveness. All samples were collected in heparinized Multivette vials and processed within 2 hours.

All groups were anesthetized with isoflurane except for the one group that received anaesthesia by intra peritoneal injection (i.p.) of FD-mix.

We also varied the sampling frequency between groups to examine the effect of repetitive blood sampling.

LPS challenge

Subsequently, whole blood samples were stimulated with 100 ng/ml lipopolysaccharide (LPS; E.coli 055-B5, Sigma-Aldrich, St. Louis, USA) for 20h at 37°C and 5% CO₂. Phosphate buffered solution (PBS) stimulation served as a control to determine baseline expression. Subsequently samples were centrifuged for 5 minutes at 300 x G and the resulting supernatant stored at -80°C until further analysis. As a surrogate for TLR4 response, TNF α levels were measured in the supernatants of all samples using an ELISA (Rat TNF α Quantikine immunoassay, R&D systems, Minneapolis, USA).

Statistics.

All statistics were performed using SPSS 20.0 (Chicago, IL, USA). Mann-Whitney U test was used to compare differences between two groups. Comparison between more than 2 groups was performed in a Kruskal-Wallis test. All values are presented as mean $\pm$ SEM and p-values <0.05 were regarded as significant.

RESULTS

Vascular injury induces down-regulation of TLR-4 response in rats

With respect to the inflammatory responsiveness, we observed that the anesthetized sham-operated animals showed lower levels of TNF α compared to a control group that only received anaesthesia. That means that just preparation of the femoral artery already caused a minor but not significant decrease of 31% in TNF α levels following LPS incubation (Figure 1A).

Comparison of rats subjected to femoral artery damage (crush) and rats that underwent sham surgery showed that vascular injury resulted in a major down-regulation of TLR-

induced TNF α release (sham operated = set to 100% versus crush = 36% (Figure 1B). Thus, TNF α levels in the crush group decreased to 25% compared to the healthy controls.

Figure 1. Panel **A**: TLR-induced TNF α release in control rats (n=5) that were only anesthetized and in rats that underwent sham surgery (n=5). Control mean TNF α is set at 100%, sham TNF α is 69% of controls. Panel **B**: TNF α release in crush group (n=7), compared to sham operated animals (n=6). Sham group mean is set at 100%, crush group TNF α is 36% of sham group.

Repeated blood sampling

Next, we studied the effect of repeated samplings on whole blood TLR-4 induced TNF α release. Three blood samples were sequentially taken by tail tip clipping with intervals of 25 minutes, during initiation of anaesthesia, following preparation of the femoral artery and at termination time. Inevitably, in these further unharmed animals, some vascular damage has been induced during the tail tip clipping. Comparison of TNF α levels in the sequential blood samples shows that TLR response decreases in every next sample in the same rat. (Figure 2, panel A) (t=0: 100%, t=25min: 65%, t=50min: 27%).

Effect of blood sampling on TLR response with a different anaesthetic

As a control for inhibiting effects of isoflurane as an anaesthetic we used FD-mix by i.p. injection in group B. Rats were anesthetized and blood was drawn at the same time points as in group A and no further intervention was performed. Thus, using a different anaesthetic without any arterial damage we observed a similar gradual decrease of the immune responsiveness following repeated blood sampling (t=0min: 100%, t=25min: 55%, t=50min: 35%) (Figure 2, panel B).

Sample volume effect

Larger blood samples reduce the total blood volume to such an extent that it changes the physiological environment of the circulating cells. In the former experiments ± 750 μ l was drawn at each time-point. To examine whether our results could be explained by large sample volumes, smaller volumes of only 350 μ l were drawn from an isoflurane anesthetized group at t=25 minutes and t=50 minutes.

As a result we found that smaller quantities of blood withdrawal caused a similar down-regulation in TLR-induced TNF α as bigger volumes did (t=50min: 52% of t=25min) (Figure 3).

Figure 2. TNF α release in blood samples of 750 μ l drawn by tail tip clipping. Mean TNF α levels at t=0 are set at 100%. Group **A** (n=5), effect of repeated blood sampling on TNF α levels in isoflurane anesthetized rats. Compared to t=0: t=25min: 65%, t=50min: 27%. Group **B** (n=5), influence of used anaesthetic on TNF α release in repeated blood sampling in FD-mix anesthetized rats. Compared to t=0: t=25min: 55%, t=50min: 35%.

Figure 3. Group **C** (n=4). Influence of sample volume in repeated blood sampling on TNF α release in smaller blood samples of 350 μ l at t=25 and t=50 minutes after induction of anaesthesia. Mean level of t=25min measurements is set at 100%. Mean TNF α level at t=50min resulted in 52% of t=25min.

Effect on TLR response of single blood sampling at different time points

The duration of the time under anaesthesia could also explain the gradual down tuning of the innate immune response. For that reason only one blood sample per animal was drawn from two groups at different time points, group D at t=0 and group E at t=50 minutes after induction of anaesthesia. Again, no arterial intervention was performed. Now, no differences in TLR-induced TNF α release were observed between both time points, which implies that the duration of isoflurane anaesthesia has no influence on TLR response and that blood sampling in itself was likely to be responsible for the down-regulation in TLR response (Figure 4).

Figure 4. Groups D (n=5) and E (n=4). Effect of period under anaesthesia on TNF α release. Single blood samples of 750 μ l at two different time points after induction of anaesthesia. Mean TNF α level of t=0 (D) is set at 100%, t=50min (E): 103%.

DISCUSSION

Vascular injury

These data show that vascular injury results in a rapid down tuning of LPS induced TNF α release. Even minor tissue handling around the artery causes some effect, regardless of the chosen anaesthetic or time span under sedation. Previously, a similar down tuning of the innate immune response was observed following stenting procedures or vascular surgery in humans ^{2,1} and our current results show that the rat can serve as an appropriate model to study vascular injury induced changes in immune responsiveness. The results reveal the strong sensitivity of the innate immune system for vascular trauma.

The Toll Like Receptors are the gatekeepers of the innate immune system that recognize exogenous and endogenous ligands that are expressed by bacteria and viruses. They are also involved in chronic and acute inflammatory diseases. Rodent models are often applied to study the role of the immune system in many different disease entities. In cardiovascular disease rats and mice have been used to study the effect of the innate immune system on development of atherosclerosis, ischemia reperfusion injury and heart failure ¹³⁻¹⁵. Vascular interventions are often an obligatory part of these animal models among which the placement of carotid cuffs or inducing myocardial infarction by thoracotomy and coronary ligation. These experimental procedures as well as blood sampling, necessarily implicate vascular injury and our results demonstrate that serious effects on immunologic parameters should be taken into account.

Specifically the effect of a minor injury from blood withdrawal is impressive since this is an inherent part of any experimental procedure. As a consequence of the results on repeated blood withdrawal we recommend to limit the number of blood samplings as much as possible and preferably to only one blood sample during termination.

The choice of our method for vascular injury may be criticized. In the setup of the rat model we also tested an alternative method of scratching the intima. In that method the arterial wall was perforated with a suture needle and the intima was scratched with the tip of the needle to induce arterial injury. That method yielded similar but more varying results and additional unwanted bleeding. Standardization of the extent of the damage in this way is difficult. Therefore, we chose for the crush method instead of an intra-vascular way of injuring.

Damage of other than arterial tissue could have an effect on the immune system as well. In our sham procedure, preparation of the femoral artery caused a minor but non-significant down regulation, possibly caused by damage to small arterial branches in the operation site. This suggests that injury to the vessel is accelerating the observed effects but that minimal other tissue trauma may also yield some effect on the innate immune responses. Other supportive literature states that in a porcine myocardial infarction model, the infarct size is reduced when the infarction is preceded by a medial sternotomy. Animals with the same treatment but in a closed chest model showed bigger infarct sizes than the ones that underwent this invasive surgery ¹⁶.

A period of 20 hours LPS stimulation is quite long and beyond the response maximum. However, TNF α levels after such a period are still increased and measurable and differences are clear to see. For logistic reasons in our experimental schedule it was not possible to choose for a shorter period.

It is not entirely clear whether the mentioned injury after PCI was a result of the stretch of the vascular wall, caused by the inflation of the PCI-balloon, or an effect of the short ischemia-reperfusion period. The authors ² state, however, that the found inflammatory attenuation might serve as a protection against an excess of cytokine release, which can be seriously harmful to the body. What the bio-functionality of the reflex can be is still to be investigated.

The effect of trauma on the immune system is fast and occurs within minutes. Therefore, it has been suggested that the autonomic nerve system could be the principal system of action behind the resulting inflammatory modulation ². Other publications support the evidence for a specific attenuating role of the parasympathetic nervous system. LPS-induced abdominal inflammation can be inhibited by electrical or mechanical stimulation of the vagus nerve in rats ^{17,18}. More recently, vagal nerve stimulation was found to attenuate resulting damage while promoting functional recovery in a mouse model for cerebral ischemia-reperfusion ¹⁹.

Tracey and colleagues found proof for the existence of a "cholinergic anti-inflammatory pathway" (CAP), that suppresses inflammatory activity through $\alpha 7$ nicotinic receptors ($\alpha 7 n R$) on basic circulating immune cells like macrophages. This pathway should function as to balance the inflammatory responsiveness and prevent the body from additional damage after an infection ²⁰⁻²². Yet, it is not clear which types of immune cells and cytokines are

involved in the described processes, nor do we know exactly how and where these cells are triggered by the autonomic nerve system.

In this rat model, the subject of injury was arterial. In future experimentations, however, more attention should be paid to study differences between the effect of arterial and venous injuries. Also the location of the injured vessel might be of specific interest since autonomic effects upon identical stimuli might differ in central compared to more peripheral arteries. Anatomical differences in autonomic innervation of specific vessels may play a role in the immunologic responsiveness to small injury.

Although parasympathetic activity is very likely, the inflammatory attenuation described in this paper might not be controlled by the vagus nerve. Anatomically, no direct autonomic nervous connection is present between the femoral area and the vagus nerve. This suggests a role for a short reflex via interposition of parasympathetic ganglia in the sacral region or via a systemic way.

We emphasize the need for further research after the anatomic and immunologic principles involved in the responses to vascular damage. Of specific interest are the autonomic innervation of the arterial wall and the location and function of cytokine releasing cells like monocytes and mast cells. Anatomical differences in autonomic innervation of specific vessels may play a role in the immunologic responsiveness to small injury.

The effects of vascular trauma on the immune responsiveness may be temporarily. Therefore our results may have stronger implications for acute experimental animal models and may have less impact on chronic inflammatory disease models.

Blood withdrawal

We found that successive blood withdrawal has a negative effect on inflammatory responsiveness. This effect was not caused by a longer period under anaesthesia as the data in figure 5 shows. The type of anaesthetic could not explain the down toning since changing from isoflurane to injectable FD-mix did not abolish the effect. Although there might be other anaesthetics that may have different influence on immunologic parameters, we expect the down toning to remain evident.

Changing the blood sample volume did not alter our results either. Possibly, the sample volume might still have been too large since a maximum amount of 1% of the circulating blood volume per 24 hour is advised by the NIH guidelines⁸. The normal blood parameters may need this period to get restored. The data in this article suggests that when studying immune responses in rat models only one time point of blood withdrawal should be used. In the tail tip clipping, however, a minor vascular injury was instigated. That injury might have caused the measured inflammatory inhibition.

Thus, the sampling method itself, inevitably accompanied by vascular damage, can be of serious influence. Since we only examined TLR response, measured by TNF α release, further research should be done to seek out to what extent the influence upon vascular injury is measurable.

CONCLUSIONS

Blood sampling as well as vascular injury has serious effects on the reactivity of the innate immune system as measured by TLR induced TNF α release. This effect sustains regardless of the period under anaesthesia. The sample volume, at the same time, does not seem to be of influence on the results either.

The procedure described in this article may function as a model, mimicking the inflammatory down toning after arterial injury in human patients.

Future research is required for a better insight by which pathways, and to what extent vascular injury influences the innate immune system.

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CHAPTER 3

Effect of Vagus Nerve Stimulation on tissue damage and function loss in a mouse myocardial infarction model

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M.G.J. Nederhoff¹
S.A.M.W. Verlinde¹
T. van Zuuren¹
G. Pasterkamp²
R.L.A.W. Bleys¹

¹ Department of Anatomy, University Medical Center Utrecht, Utrecht University, The Netherlands.

² Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht University, The Netherlands.

ABSTRACT

Introduction

Autonomic regulation of immune functions has increasingly been studied in the last decades. The attenuating effect of the parasympathetic nerve system on inflammatory processes is demonstrated in many studies on inflammatory disease like rheumatoid arthritis, chronic bowel disease and heart failure. Similar inflammatory processes play a role in the development of damage to the heart following myocardial infarction. In this study, the effect of vagus nerve stimulation was measured on the development of tissue damage and function loss after myocardial infarction in the mouse heart.

Methods

Anaesthetized male C57Bl6 mice were treated with or without 30 seconds of vagus nerve stimulation (VS) prior to experimental myocardial infarction (MI). Baseline and terminal heart function was measured. After 48 hours the animals were sacrificed and infarct size and presence of inflammatory cells in different locations in the infarcted heart were determined. Also, inflammatory responsiveness and inflammatory cells in the blood were measured.

Results

The differences in ejection fraction and infarct size were found to be not significant. Counts of CD3 T-lymphocytes and macrophages were equal on baseline and at termination. Neutrophil counts were higher in the area remote from the infarction in the MI group compared to that in the VS+MI group. Amounts of VEGF were not different in the infarcted hearts of MI as compared to the hearts of VS+MI treated mice.

Conclusions

Ejection fraction decreased at 48 hours after MI, less in the VS+MI group than in MI alone. However, this decrease was not significantly different between these groups. Although values of infarct size and cardiac function come close to significant reduction, this study does not show clear proof for an inhibiting effect of VS on MI injury or function loss. Since the limited time period after MI could be of influence on the variation in the data, an additional measurement could provide essential information on the development of tissue damage and functional reduction.

INTRODUCTION

Myocardial ischemia (MI) triggers an inflammatory response in the peri-infarct area with the aim of repairing injured tissue and removing cellular debris. In many cases however, patients recovering from MI remain in a delicate situation because of the development of ventricular remodelling and heart failure.

Inflammatory activity plays a crucial role in both the development of cardiovascular disease (CVD) and in function loss in later stages of CVD^{1,2}. This inflammatory activity itself, however, appears to be balanced by the autonomic nerve system (ANS). Accumulating evidence shows the modulating effects of the ANS in which sympathetic innervation is generally associated with an increased, and the parasympathetic with an attenuated inflammatory response^{3,4}. As a consequence, the risk for CVD increases with an enhanced sympathetic and a lowered parasympathetic tone. Keeping an autonomic balance is essential in cardiac health maintenance, especially when a serious threat like a MI elicits a strong inflammatory response.

Electrical stimulation of the vagus nerve (VS) increases parasympathetic activity, while, at the same time, it decreases the sympathetic tone^{5,6}. As found in recent studies, VS strongly attenuates inflammatory responsiveness in experimental sepsis⁷ inflammatory bowel disease⁸, heart failure⁹ and rheumatoid arthritis¹⁰. Apart from this effect, VS attenuates development of edema¹¹ and stimulates vasodilation and angiogenesis¹².

Upon VS, a clear decrease was found in plasma levels of inflammatory cytokines like TNF α , IL-1 β and IL6, the same cytokines that play an important role in atherogenesis and MI. It is largely unknown how parasympathetic nerve stimulation influences the acute inflammatory response following myocardial inflammation.

In the effectiveness of VS, the main factor is the release of acetylcholine and therefore the process is named the Cholinergic Anti-inflammatory Pathway. This pathway describes how cytokines are released when a physical threat, like a pathogen, an injury or ischemia, initiates an inflammatory response. This activity is sensed by afferent vagal branches that transport a signal to vagal nuclei in the brain. In those nuclei the signal is processed and an inhibiting signal is sent back through the efferent vagus nerve. Upon this signal, acetylcholine (ACh) is released, blocking the $\alpha 7$ -subunits of nicotinic ACh-receptors ($\alpha 7$ -nAChR) on inflammatory cells. This block inhibits inflammatory cytokine release and thus prevents excessive inflammation^{13,14,15}. We hypothesize that VS has the potency to attenuate the inflammatory response that occurs after MI which may lead to a better recovery of the heart function.

So far, patients surviving an initial MI are often still in the danger zone, caused by ventricular arrhythmia, remodelling of the left ventricle and development of chronic heart failure. Obviously, an effective therapy to prevent excessive loss of viable myocardial tissue is necessary. In this respect, the parasympathetic nerve system could be a means to prevent

excessive damage after MI but crucial information about mutual ANS vs. inflammation influence is still lacking.

We hypothesized that VS reduces excessive tissue damage and cardiac function loss after MI. The increase of vagal tone, causing vasodilation and angiogenesis, combined with a decrease of inflammation and reduction of edema formation, can be of great therapeutic value for patients recovering from MI. We therefore examined cardiac function and the size of the infarcted area in mice that underwent VS or sham-VS prior to permanent occlusion of the left anterior descending coronary artery (LAD). Additionally we studied the presence of inflammatory cells and the amount of vascular endothelial growth factor (VEGF) in the infarcted heart.

METHODS

Animals

Sample size was calculated with G*power¹⁶. Forty five male C57Bl/6j mice were used (Charles River Laboratories Maastricht, the Netherlands). Average weight was 27 ± 1 gram and they received regular chow as well as water ad libitum. Animal procedures were all performed in accordance with the Guide for the Care and Use of Laboratory Animals, with prior approval by the Animal Ethical Experimentation Committee, Utrecht University, the Netherlands.

Euthanasia

After 48 hours animals were euthanized by bleeding under general anaesthesia 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.

Animals that reached a humane endpoint during the experiment were also sacrificed using the same method.

Experimental setup

Three experimental groups were defined. The first group MI, underwent both MI and sham-VS while a second group VS+MI received MI preceded by VS. A third group VS was administered VS plus sham-MI. The mice were randomly assigned to one of the groups in order that the MI, VS+MI and VS contained 17, 17 and 11 animals respectively. To examine the effect of vagus nerve stimulation, cardiac function was measured as well as infarct size and present inflammatory cells. All animals were terminated 48 hours after surgery, by bleeding, under complete anaesthesia. The time point of 48 hours was chosen because the first response of neutrophils should still be visible, just like the inhibited inflammatory response as shown by plasma TNF α levels. Blood was collected in anti-coagulated tubes to be measured for determination of inflammatory responsiveness by plasma levels of TNF- α .

A small volume of blood was used for circulating cell counts. The hearts from 9 mice of both MI and VS+MI and 6 from the VS group were excised to be examined for inflammatory cells and VEGF. Eight hearts of the MI and VS+MI and 5 hearts of the VS were prepared to assess the infarct size.

Echo Cardiography

The mice were weighed and sedated with isoflurane (induction with 4%, maintenance at 1.8% in air:O₂, 2:1) and the medial-right area of the neck, as well as the medial-left area of the thorax was chemically depilated (Veet, Benckiser NV. Hoofddorp, Netherlands).

Base line echo cardiography was executed at t=0 before surgery and at termination 48 hours thereafter with a Vevo 2100 (Visualsonics, Amsterdam, the Netherlands). The executing lab assistant was blinded for the experimental groups. Three views were recorded in a sagittal, coronal and axial orientation, from which end systolic and end diastolic volumes (EDV) were determined to calculate ejection fraction (EF). EF is expressed as a percentage of stroke volume (SV) over EDV. Or put simply as: $EF = ((EDV - ESV)/EDV) * 100$. The difference per heart between baseline-EF and EF at 48 hours after surgery is used as a measure for loss of cardiac function.

Surgical procedure: MI and VS

For surgery, the animals were anaesthetized via intraperitoneal injection with FDD-mix (Fentanyl 0.05 mg/kg, Medetomidine 0.5 mg/kg, Dormicum 5 mg/kg). 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 250µl. Body temperature controlled and kept at 38 °C. Both operations were executed in one session.

The extremities of the mice were connected to ECG electrodes. The neck was sterilized with 70% alcohol and opened to expose the right vagus nerve. A bipolar electrode, connected to an external stimulator (TTi, type TGP110) was placed on the nerve and the nerve was stimulated for 30 seconds (f=10Hz, I=1mA, pulse width=0.5ms). A minimal decrease of 20% in heart rate during VS was accepted as proof for an effective vagus stimulation since A-fibers in the vagus nerve that inhibit inflammatory responsivity are easier to stimulate than B-fibers that trigger heart rate to slow down^{16,17,18}. Then, the electrodes of the stimulator and the ECG recorder were removed and the skin was closed with Vicryl 4.0.

Subsequently, the thorax was opened left laterally between the fourth and fifth rib and the LAD was occluded with a braided silk 8.0 ligature. Pale colouring of the infarcted area and slight arrhythmias were taken as an indication of a solid occlusion. The thorax was closed with monofil prolene 8.0 and the thoracic skin was sutured with Vicryl 4.0. Finally, the anaesthesia was antagonized with Antisedan (Pfizer) 2,5mg/kg and Anexate (Roche) 0,5mg/kg. The sham-VS procedure was performed identically but only until exposure of the vagus nerve. The nerve as such was minimally touched to prevent unwanted effects. In the sham-MI the whole procedure was followed, a ligature was pierced underneath the LAD but was not tied up. Immediately after surgery, the animals received 0.1 mg/kg buprenorphine (Temgesic®, Reckitt Benckiser, UK)

Immunohistochemistry

The hearts for immunohistochemistry were flushed with Phosphate Buffered Saline (PBS), fixed in 4% formaldehyde for ca. 90 minutes and stored in PBS with 15% sucrose. They were embedded in paraffin and cut in 5µm sections. Sections from ca. 2mm above the apex were used for inflammatory cell staining. Antigen retrieval was performed in 2.94g/l Tri-sodium-citrate • 2H₂O (pH=6) for 20 minutes at 96 °C and the sections were stained for inflammatory cells. The amounts of neutrophils, T-lymphocytes and macrophages were determined with Ly6G antibody (BioLegend, Cat.nr:108402), anti-CD3 (DAKO, ref:A0452) and MAC-3 (BD Biosciences, cat.nr:553322) respectively and all stained with Liquid Permanent Red (LPR). Vascular Endothelial Growth Factor (VEGF) was determined with anti-VEGFA (Abcam, Cat. nr:ab46154). Pictures of the stained sections were taken at 200x magnification and analysed for relative amounts of stained area with the colour selection tool of ImageJ²⁰. CD3+ lymphocytes were manually counted by a blinded co-worker. The left ventricular myocardium was divided in 4 separate locations. (Figure 1). Zone 3 is the infarcted area, 1 and 2 are border zones, ventral and dorsal respectively, and zone 4 represents the septal area, most remote from the infarction. The zones 1 and 2 were taken together as one border zone to be compared with the remote and infarcted areas. The measurements were recalculated to counts or stained area per mm².

Figure 1. Section of the heart with infarcted area (3), remote area (4) and border area's (1 and 2). LV and RV: left and right ventricle.

Figure 2. Transverse section of the TTC-stained heart. LV: left ventricular lumen, RV: right ventricular lumen, IA: infarcted area.

Blood content, inflammatory responsiveness.

Terminal blood was processed through a cell counter (Cell-Dyn Sapphire, Abbott Diagnostics, Lake Forest, Ill, USA) to define numbers of total white blood cells (WBC), Lymphocytes, Granulocytes and red blood cells (RBC). In the same procedure plasma levels of haemoglobin 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 hours incubation, the blood samples were centrifuged for 5 minutes 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 examined with ELISA (mouse TNF-alpha Quantikine Kit, R&D systems, cat.nr: MTA00B).

Infarct size

For measurement of the infarct size, the hearts were flushed with saline, excised and put in a -20 °C freezer for ca. 60 minutes. Then, the hearts were sliced in 0.9mm sections, perpendicular to the long axis. These sections were incubated for 15 minutes in 4% 37 °C Triphenyl tetrazolium chloride (TTC-) solution (2,3,5-Triphenyl-tetrazolium chloride, Sigma-Aldrich, cat.nr: T8877). In TTC, viable tissue stains red while infarcted tissue remains white. After staining, slices were preserved in 4% formaldehyde for 60 minutes and then transported into phosphate buffered saline with 15% sucrose and 0,05% Na-azide. Pictures at equal distance were taken immediately after preparation and infarct size was determined as a percentage of the whole section with the contour tool in ImageJ²⁰ (Figure 2). Since the infarcted area in all hearts was located in the lower transversal heart sections, only these four sections closest to the apex were included in the calculation of the total infarct size and the four were averaged per heart.

Data processing

Quantification of TTC-area's, cardiac volumes and inflammatory cells was performed by blinded co-workers. Statistics were performed in Prism-7 (Graphpad Prism 7 Software, La Jolla California USA). Significant outlier identification was performed with Grubbs outlier detection. Differences were determined by ANOVA-Kruskal-Wallis or t-test-Mann-Whitney, both non-parametric, and all results are presented as means (M) with standard deviation (SD). Results were considered significant when p-values were smaller than 0.05.

RESULTS

Experimental details

In the 2 days following surgery, the animals decreased in body weight, VS: 4%, VS+MI: 4.4% and MI: 4.9% (ns). The mortality after MI was 11% in 48 hours leaving 15 mice in the MI group, 14 in the VS+MI and 11 in the VS group. After termination, two animals were excluded from the MI group because no infarction, shown as a pale area under the ligature, was observed on the heart.

With respect to the VS procedure, all the subjects met the criteria for reliable stimulation. An average decrease of 63% in heart rate was achieved (range 40% - 75%) which was immediately restored when the VS was stopped.

Terminal blood values

One value from the VS+MI group and from the MI group two values are missing because the condition of the animal was not good enough to draw a sufficient amount of blood. Amounts of blood cells in values $\times 10^9$ cells/L and haemoglobin in mmol/L at t=48h are shown in (Table 1). Although means of the VS+MI (N=13) group are highest for all blood cells, the values did not significantly differ from the MI (N=12) group. VS (N=11) appears to cause lower levels of total WBC than VS+MI: 5.36 ± 3.52 vs 9.02 ± 4.21 counts per liter blood ($p=0.040$).

Table 1. Group averages of plasma content.

Mean $\pm$ (SD)	WBC	Lym	Gran	Hb
MI	7.09 (4.12)	4.85 (2.96)	0.45 (0.38)	8.00 (1.05)
VS+MI	9.02 (4.21)	6.06 (2.60)	1.02 (0.82)	8.55 (0.89)
VS	5.36 (3.52)	3.76 (2.54)	0.54 (0.57)	8.59 (0.70)

White blood cells (WBC), lymphocytes (Lym) and granulocytes (Gran) as 10^9 cells/L, and haemoglobin (Hb) in mmol/L 48h after intervention. Differences between MI and VS+MI are not significant. Mean $\pm$ (SD), Kruskal-Wallis of One Way ANOVA.

Cardiac function

In the MI and VS+MI groups a total of 12 echographies had to be excluded because of technical problems at the moment of termination.

The EF, a measure for cardiac function, is shown as stroke volume as a percentage of the EDV. Baseline and terminal group values of EF at t=0 and t=48h are depicted in (Figure 3). Compared to t=0 the terminal EF decreased in both the MI and the VS+MI groups. The t=48h EF of the MI group decreased to $28.5 \pm 10.6\%$ compared to the baseline value of $58.2 \pm 4.2\%$ ($p=0.0001$). In the VS+MI group, the t=0 EF of $60.7 \pm 6.5\%$ decreased to $44.5 \pm 11.0\%$ at t=48h ($p=0.0019$). The group that only received VS did not change in cardiac function.

The average decrease in EF of the MI group (N=9), however, did not significantly differ from the decrease of the VS+MI group (N=7), $30.7 \pm 10.1\%$ vs $16.0 \pm 8.8\%$ ($p=0.075$) respectively. As expected, the EF in the VS group (N=11) did not change in the 48 hours after surgery. T=0 vs t=48h change in EF of the VS group was $3.4 \pm 5.1\%$ (ns), Means $\pm$ SD (Figure 4).

Infarct size.

From the MI group one mouse was excluded from analysis because no effective MI was verified. The infarct size from the TTC-staining was measured with ImageJ and depicted as percentage of total slice area (Figure 5). Although not significant, group averages show smaller infarctions in the VS+MI compared to the MI group ($p=0.100$). Group means with SD of infarct size are respectively MI: $22.8 \pm 7.3\%$ (N=7), VS+MI: $15.7 \pm 4.7\%$ (N=8) and VS: $2.2 \pm 1.5\%$ (N=5).

Figure 3. Ejection fraction (EF) from baseline to 48 hours after surgery in % SV/EDV. Both groups MI and VS+MI differ significantly from VS. A stronger decrease after 48 hours is detected in the MI than in the VS+MI group. MI (N=9): $58.2 \pm 4.2\%$ vs $28.5 \pm 10.6\%$, VS+MI (N=7): $60.7 \pm 6.5\%$ vs $44.5 \pm 11.0\%$, VS (N=11): $61.2 \pm 5.3\%$ vs $57.8 \pm 5.6\%$ (Mean $\pm$ SD).

Figure 4. Mean decrease in EF per group. t=0 minus t=48h values for MI (N=9): $30.7 \pm 10.1\%$, VS+MI (N=7): $16.0 \pm 8.8\%$ and VS (N=11): $3.4 \pm 5.1\%$ (Mean $\pm$ SD)

Figure 5. Difference in % infarcted area 48h after intervention. The mean infarcted area of MI (N=7): $22.8 \pm 7.3\%$ is not significantly different from VS+MI (N=8): $15.7 \pm 4.7\%$ (ns, Kruskal-Wallis of One Way ANOVA). VS group (N=5): $2.2 \pm 1.5\%$ (Mean $\pm$ SD)

Figure 6. Plasma levels of TNFα at 48h. MI (N=11): $33.7 \pm 27.4\text{pg/ml}$, VS+MI (N=13): $27.9 \pm 18.6\text{pg/ml}$ and VS (N=10): $14.2 \pm 12.9\text{pg/ml}$ (Mean $\pm$ SD, Kruskal-Wallis of One Way ANOVA)

Inflammatory responsiveness.

TNFα levels in LPS stimulated blood were determined with ELISA. TNFα levels between MI and VS+MI did not show a significant difference (Figure 6). Both groups MI (N=11) and VS+MI (N=13) significantly differed from the VS (N=10) group, respectively 33.7 ± 27.4 ($p=0.039$) and 27.9 ± 18.6 ($p=0.040$) vs $14.2 \pm 12.9\text{pg/ml}$ plasma. (N=10).

Inflammatory cells.

In preserved and stained transversal slices of the infarcted heart at t=48h, amounts of neutrophils, T-lymphocytes and macrophages were quantified. One value from the VS+MI and two from the VS group were detected as significant outliers by Grubbs outliers test and therefore excluded.

Figure 7. Neutrophils (Ly6G+) stained with LPR in the infarcted, remote and border areas. Scale bar = 50µm

Neutrophils, presented as stained pixels $\times 10^2$ per mm^2 , in the infarcted areas appeared to be less present in the VS+MI group than in the MI group (ns). On the other hand, in the remote areas, the VS+MI group shows significantly higher values than the MI group ($p=0.040$). Within the MI group the presence of neutrophils is bigger in the infarcted area (ns) than in the remote area ($p=0.024$). The values in the VS group are close to zero and show no internal difference between areas. The combined results are shown in (Figure 7 and 8).

The results of the present CD3^+ T-lymphocytes in the heart at $t=48\text{h}$ did not exhibit significant differences between the MI and VS+MI group. On the average, slightly more CD3^+ T-lymphocytes are found in the VS+MI as compared to the MI group, specifically in the remote areas (MI 4.29 ± 4.42 vs VS+MI 6.83 ± 6.37 pixels $\times 10^2/\text{mm}^2$, ns) (Figure 9). The values of present macrophages in the stained sections of the MI group and VS+MI group did not differ between groups. Virtually no macrophages were found in the vagus stimulated hearts (Figure 10).

Figure 8. Neutrophils in the infarcted, border and remote areas of the heart at 48h after surgery. MI (N=7): remote 217 ± 278 , border 373 ± 250 , infarcted 855 ± 624 , VS+MI (N=6): remote 695 ± 501 , border 452 ± 217 , infarcted 530 ± 563 , and VS (N=6): remote 5.67 ± 3.88 , border 3.80 ± 3.77 and infarcted area 2.60 ± 1.67 (pixels $\times 10^2/\text{mm}^2$, Mean $\pm$ SD).

Figure 9. Counts of CD3^+ positive T-lymphocytes through the groups at 48h after surgery. The VS areas were all significantly different from those in the MI and VS+MI group. No significant differences were found between the MI and VS+MI group. MI (N=7): remote 4.29 ± 4.42 , border 6.43 ± 3.16 , infarcted 5.14 ± 4.26 , VS+MI (N=6): remote 6.83 ± 6.37 , border 7.50 ± 4.97 , infarcted 5.67 ± 3.78 , and VS (N=6): remote 0.83 ± 1.17 , border 0.17 ± 0.41 and infarcted area 0.17 ± 0.41 (pixels $\times 10^2/\text{mm}^2$, Mean $\pm$ SD).

VEGF

Amounts of Vascular Endothelial Growth Factor (VEGF) in the different areas of the infarcted heart are depicted in figure 10 as stained pixels per mm^2 . In values for VEGF stained area there are no significant differences to report between the groups. A big variation is found through both MI and VS+MI groups (Figure 11).

Figure 10. Macrophages presented as pixels*10²/mm², at 48h after surgery. The VS (N=6) values were all significantly different from those in the MI (N=7) and VS+MI (N=6) group. No significant differences were found between the MI and VS+MI group.

Figure 11. VEGF as stained area / mm² in the infarcted heart as stained area per mm², 48h after surgery. No significant differences between the MI (N=6) and VS+MI (N=6) groups. VS group N=6.

VS-MI-DISCUSSION

Inflammatory processes play a crucial role in tissue repair and recovery after MI. The ANS modulates inflammation and the parasympathetic branch of the ANS serves as an attenuating player in this connection, inhibiting the inflammatory response^{21,22}. This study explored whether parasympathetic activation, by electrical stimulation of the vagus nerve could reduce tissue damage and cardiac function loss after MI.

To measure a potential protective effect, mice were subject to experimental MI with or without electrical VS and the effects were studied at 48 hours after surgery and defined in difference of heart function, ischemic area and inflammatory activity.

Cardiac function

The EF values and averages at t=0 as well as 48h after intervention are consistent with existing literature^{22,23,24}. Moreover, EF in the MI group at t=48h is significantly lower than baseline values (28.46±12.07 vs 58.46±4.50%) a decrease of 51% from baseline (p=<0.000) while the reduction in EF of the VS+MI group is only 37% from t=0 (39.38±15.42 vs 62.48±5.36%, p=0.002). However both groups show a decreased EF, the reduction in the VS+MI group is seriously less than in the MI group. The difference between MI and VS+MI comes close to significance (p=0.075), such that it reinforces our basic assumption that VS reduces the development of function loss in MI.

Infarct Size

Infarcted area as measured with TTC staining yielded a non-significant difference between MI and VS+MI (p=0.100). However, after 48h the exact infarct borders are not yet fully outlined and sometimes difficult to define. The shape of the infarction can be irregular and slices of 0.9mm could show a different ischemic pattern on each side of the slice. Nevertheless, quantification of all caudal/superior surfaces or of the cranial/inferior surfaces yielded equal results for infarcted area.

Inflammatory responsiveness

Inflammatory responsiveness was measured as TNF α levels in LPS stimulated blood 48 hours after MI and VS. A period of 20 hours LPS stimulation is quite long and beyond the response maximum. However, TNF α levels after such a period are still increased and measurable and differences are clear to see. For logistic reasons in our experimental schedule it was not possible to choose for a shorter incubation period.

The TNF α levels in the MI and VS+MI groups are not found to be significantly different. Both groups however, are significantly higher than the VS group. This could indicate that a MI serves as a major inflammatory trigger, causing an immune reaction to such an extent that the influence of 30 seconds of VS on TNF-levels is overruled within the recovery period of 48 hours.

Inflammatory cells

Neutrophils, T-lymphocytes and Macrophages were quantified in transverse sections of the infarcted heart. Resulting values in the VS group were for all subsets close to zero indicating that the scores in the other two groups were caused by the MI procedure.

Neutrophils belong to the first responders after MI. They are attracted to the infarcted area and reach a peak between one and three days after intervention^{25,26,27}. However, an increase in neutrophils was not found in this study. While within the MI group a clear difference is found between the remote and infarcted area ($p=0.024$), this is not the case in the VS+MI group. The remote area is considered to be a control area, being the same type of tissue but not affected by the MI process. Nevertheless, while in the VS no cells were found, serious amounts of neutrophils were located in the remote areas of the other groups. Moreover, significantly more neutrophils were found in the remote area of the VS+MI than in the remote area of the MI group ($p=0.040$). This is contrary to the expectations because high numbers of neutrophils are generally associated with increased injury. Through the border and infarcted areas however, no significant difference was found between the MI and VS+MI groups.

In the VS+MI group, neutrophil counts in the infarcted area did not differ from the counts in the remote or border area. Concerning neutrophils, VS prior to MI does not seem to have a great additional effect on the infarcted heart, although small differences were found in different locations. In the VS+MI group most neutrophils were found in the remote area while in the MI group the highest concentrations were located in the infarcted area.

The reported average increase of CD3⁺ lymphocytes after two days of MI varies in literature from none to a small increase. A stronger visible increase was found four days after surgery^{25,26,27}. In the present study no differences were found in counts of CD3⁺ T-lymphocytes between different areas and groups at $t=48h$. Macrophages are essential for tissue repair after infarction²⁹. They accumulate and a clear increase in the infarcted heart can be found after three days^{28,29}. In the present study no significant differences were found in resident macrophages in areas of the ischemic heart, the levels at termination were found to be almost identical in both MI and VS+MI groups. Unchanged levels of macrophages can also

be explained by the phenotypical plasticity of macrophages. M1 macrophages produce pro-inflammatory cytokines, while M2 macrophages provide anti-inflammatory functions and are involved in wound healing and fibrosis^{30,31}. Upon certain triggers they can easily change from type M1 to type M2. In a mouse study on VS in renal ischemia-reperfusion injury, the numbers of infiltrating macrophages did not change upon VS but the phenotype of those cells did change from M1 to M2³². Another study showed an increase of M1 in the first 3 days after I-R, where after a decrease started. For M2 the opposite occurred by a decrease until day 3 followed by a gradual increase²⁸. In future studies separate determination of M1 and M2 could shed some new light on the inflammatory and repairing processes involved.

VEGF

Hypoxia, as caused by MI urges dying myocardial cells to produce VEGF in an attempt to re-vascularize the threatened tissue^{33,34}.

Levels of VEGF in the infarcted heart were not found to be different between MI and VS+MI treated mice which means that a positive effect of VS to save the infarcted myocardium by stimulation of new vessel growth is not demonstrated.

Limitations

The physical condition of some of the animals was not good enough to draw a sufficient amount of blood at the moment of termination. Also, some of the echocardiographic data at termination time were difficult to analyse. The unstable position of the heart without an intact pericardium often caused blurred recordings, some of which didn't yield reliable values and had to be excluded.

The results of some of the parameters do not reach significance because of a large SD. This can partly be explained by the relatively short time of two days between the surgical intervention and the moment of termination. After a recovery period of 48 hours ongoing migratory processes of inflammatory cells like neutrophils may not yet be stable and cause fluctuations in the results. Small differences in the recovery speed may be reflected in substantial variation in experimental outcomes.

For cells like lymphocytes and macrophages 48 hours could be too short of a period when some changes have to develop and will only become detectable after a longer time. A second measurement point, later on in the process, would provide extra information on the course of development of damage and function loss. Preferably around day 5 when the inflammatory processes are more stabilized.

CONCLUSION

VS+MI treated mice showed less reduction of ejection fraction compared to MI alone and a close to significant smaller infarct size. The degree of this reduction is not found to be significantly different in MI as compared to VS+MI mice. Circulating cells and resident

inflammatory cells in the infarcted heart were not different in MI compared to the VS+MI group and the same applies for levels of VEGF in the heart. Inflammatory responsiveness as measured with TNF α was not significantly influenced by VS in MI treated mice 48 hours after surgery. In conclusion, this study did not show clear proof for an inhibiting effect of VS on development of MI injury.

Future studies on this topic should include an extra measurement step to examine the longer term effects of vagus nerve stimulation on injury on day 5 after MI.

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CHAPTER 4

Effect of Vagus Nerve Stimulation on tissue damage and function loss in a mouse myocardial ischemia-reperfusion model

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M.G.J. Nederhoff¹*

M.A.D. Brans²

D.E. Fransen¹

S.A.M.W. Verlinde¹

G. Pasterkamp²

R.L.A.W. Bleys¹

¹ Department of Anatomy, Division Surgical Specialties, University Medical Center Utrecht

² Experimental Cardiology Laboratory, Division Surgical Specialties, University Medical Center Utrecht The Netherlands

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 hours after intervention. Group results were compared with One-way ANOVA or Student's T-test.

Results

A significant decrease in inflammatory responsiveness was not verified by decreased TNF α 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 ($22.4\pm10.2\%$ vs $37.6\pm9.0\%$, $p=0.003$). 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.

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 ¹. Simultaneously, immune responses play a role in the repair of damaged tissue and the restoration of organ function ^{2,3}. 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 ^{4,5}. 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 ⁵⁻⁸. 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) ^{6,9,10}. Upon vagus activation, acetylcholine binds to $\alpha 7$ 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 ^{9,11,12}. Presently VS is, sometimes still experimentally, used as a treatment for inflammation associated ailments like rheumatoid arthritis ¹³, inflammatory bowel disease ¹⁴ and heart failure ¹⁵.

In literature, the connection of the ANS with inflammatory processes in atherosclerosis and chronic heart disease is well established ¹⁶. Inflammatory cytokines such as IL-1 β , IL6 and TNF α 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) ^{17,18}. 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 ^{19,20}. 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 ^{19,21}. 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.

METHODS

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 hours/12 hours. Animal procedures were all performed in accordance with the Guide for the Care and Use of Laboratory Animals²², 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 $\alpha = 0,05$ and, derived from literature, SD and perioperative mortality^{23,24}.

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 Animals²², they were euthanized in a similar way as described above.

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, TNF α levels will still be low due to the vagus stimulation. For these reasons, after 48 hours 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.

Echo Cardiography

The mice were weighed and sedated with isoflurane (induction with 4%, maintenance at 1.8% in air:O₂, 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 hours 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 hours after surgery was used as a measure for loss of cardiac function.

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 minutes. 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 μ l. 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 seconds ($f=10\text{Hz}$, $I=1\text{mA}$, pulse width=0.5ms). 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 minutes 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 ($\varnothing 0.8\text{mm}$). 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 minutes. 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 2.5mg/kg plus Anexate 0.5mg/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.

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

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 two hours. 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 (Figure 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 ImageJ²⁵. 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.

Immunohistochemistry

The hearts for immunohistochemistry were perfused with PBS to wash out the remaining blood and fixed for ca. 90 minutes in 4% formaldehyde where after they were stored in PBS with 15% sucrose. They were embedded in paraffin and cut in 5 μ m sections, perpendicular to the apex to aortic base axis. Antigen retrieval was performed in 2.94g/l Tri-sodium-citrate•2H₂O (pH=6) for 20 minutes at 96 °C and 5 μ m sections from 2mm 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 1.25x0.94mm were taken at 200x magnification and analyzed with ImageJ²⁵. 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 Figure 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 mm² of total measured tissue area.

Figure 1. Areas in stained 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).

Data processing

Quantification of TTC-areas, VEGF and inflammatory cells was performed in ImageJ by a blinded assistant²⁵. Between groups, comparisons were conducted with an unpaired Mann-Whitney or Kruskal-Wallis test in SPSS-22 47 (IBM, Statistics for Windows, 2013) and Prism-7 48 (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, $p=0.05$ ²⁶. The level of significance in this study is kept at 0.05, two-tailed.

RESULTS

Animal details

The 54 C57Bl/6j mice weighed 26 ± 1.5 grams. 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 48h 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.

Blood content

Terminal blood parameters were measured with the Sapphire Analyzer. Cell values at t=48 after surgery are presented as 10⁹ 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*10⁹ 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*10⁹ 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).

Table 1. Group averages of plasma content at 48h after intervention.

	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

White blood cells (N=10), Lymphocytes (N=10), Granulocytes (N=10) and Red blood cells (N=4), presented as numbers *10⁹ cells/L. Hemoglobin (N=10) as mmol/L and hematocrit (N=4) as L/L. ^a, ^b and ^c are significantly different pairs (Kruskal-Wallis).

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 hours, 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 (Figure 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.

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. (Figure 2).

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

Figure 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+IR: 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: Kruskal-Wallis, panels C and D: Mann-Whitney).

group (70.0±100.3pg/ml) did not significantly differ from VS+IR (33.7±33.5 pg/ml) or VS (28.2±33.9 pg/ml). (Figure 3).

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 Figure .1). Amounts of cells are presented as stained area in pixels*0.01 per mm². 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,

Figure 3. TNF α levels blood plasma at t=48 hours. Levels of TNF α in pg/ml in whole blood at t=48h. Mean $\pm$ SD of IR (N=21): 70.0 $\pm$ 100.3 pg/ml, VS+IR (N=18): 33.7 $\pm$ 33.5 pg/ml and VS (N=10): 28.2 $\pm$ 33.9 pg/ml. No significant group differences (Kruskal-Wallis).

Figure 4. Neutrophils in transversal sections from IR treated hearts. Neutrophils are shown as the stained area in pixels*0.01 per mm². 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. (Kruskal-Wallis).

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 Figure 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 Figure 5).

Table 2. Neutrophils per area in transverse sections from IR treated hearts.

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}

Values are presented as mean stained area in pixels*0.01 per mm², Means $\pm$ (SD). ^a t/m ^f are significantly different pairs. (Kruskall-Wallis).

Figure 5. LPR stained neutrophils in mouse heart sections. (100x). Scale bar = 100 μ m.

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 ($p=0.026$) (Figure 6). See table 3 for values.

Figure 6. CD3⁺ lymphocytes in transversal sections from treated hearts.

CD3⁺ lymphocytes 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 ($p=0.026$). (Kruskal-Wallis).

Table 3. CD3⁺ lymphocytes per area in transverse sections from IR treated hearts.

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

Values are presented as mean stained area in pixels*0.01 per mm², Means $\pm$ (SD). ^a, ^b and ^c are significantly different pairs. (Kruskal-Wallis).

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 (Figure 7).

Figure 7. Stained VEGF in heart sections and stroke volume by cardiac echo.

A: Amounts of Vascular Endothelial Growth Factor (VEGF) in 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, Kruskal-Wallis).

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.

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^{27,28} 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^{21,29,30} 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 LPS³¹. 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 node³². However, more recent research showed no differences in cardiac response or inflammatory responsiveness between left and right VS³³⁻³⁵. Choosing for left or right VS should therefore have no advantage or disadvantage. In this study the choice for stimulating the right cervical vagus nerve has no specific physiological reason.

VS 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 loss³⁶.

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 hour 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=48h 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^{37,38}. 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 $t=48$ hours is less severe for the VS+IR group than for the IR group. However, this difference is not significant (Figure 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-1 β and IL-6 are known to be produced by the body in response to a local ischemic period. TNF α however, is a potent cytokine and one of the most early to respond to an inflammatory stimulus and we therefore accepted TNF α levels as a good measure for inflammatory responsiveness.

In some other studies longer periods up to 150 minutes of VS have been used to achieve a reduced inflammatory response^{36,39}. Huston however, initiated an inhibitory effect on inflammatory responsiveness in the levels of TNF α in LPS stimulated mouse blood that was detectable for almost 72 hours. This was initiated by only 30 seconds of effective vagus stimulation⁴⁰. To determine TNF α in the present study, terminal whole blood at 48 hours after VS and IR was incubated with LPS for 20 hours. This incubation period is quite long and beyond the response maximum. However, TNF α levels after such a period should still be increased and measurable and actual differences would be obvious^{40,41}. However, for future studies, we would recommend the use of an incubation time close to the optimum of 6 hours to yield more explicit results.

The mean TNF α level (Figure 3) was highest for the IR group (70.0 ± 100.3 pg/ml) compared to the VS+IR ($33.7 \pm$ 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 TNF α levels should be visible after 48 hours, 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 TNF α release which supports the view that the delay of 48 hours may have diluted the effect of VS on LPS induced TNF α release³⁷ (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 hours. Neutrophils tend to peak at 24 hours 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^{24,42}. Therefore, further studies should examine separately periods of 24 hours 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)⁴³ which is associated with VEGF production, neovascularisation and collateral formation in dogs⁴⁴. Also, ACh-esterase inhibitor pyridostigmine increases VEGF-production in the left ventricle after MI, suggesting myocardial angiogenesis⁴⁵ caused by an elevated ACh presence. VEGF levels, as well as micro-vessel density, were also higher in the penumbra of experimental cerebral ischemia in rats after VS⁴⁶. 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 ($p=0.039$) see Figure 8.

Figure 8. VEGF group and area means in heart sections. Differences in vascular endothelial growth factor (VEGF) levels through the groups and three areas of the reperfed 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, Kruskal-Wallis).

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 hours 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.

CONCLUSIONS

Vagus nerve stimulation, prior to an ischemia-reperfusion protocol in mice decreases the infarct size over area at risk ratio at 48 hours 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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Conflict of interest

None declared.

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CHAPTER 5

Innervation and inflammation in atherosclerotic abdominal aortas

Under submission.

M.G.J. Nederhoff¹

G. Pasterkamp²

J.H.N. Lindeman³

R.A. van Dijk³

D.R. Cuppen¹

S.A.M.W. Verlinde¹

R.L.A.W. Bleys¹

¹ Department of Anatomy, Division Surgical Specialties, University Medical Center Utrecht, the Netherlands.

² Experimental Cardiology Laboratory, Division of Heart and Lungs, University Medical Center Utrecht, the Netherlands.

³ Department of Vascular Surgery, Leiden University Medical Center, Leiden, the Netherlands.

ABSTRACT

Introduction

Atherosclerosis has long been accepted as an inflammatory disease. Although inflammation is at least partially regulated by the autonomic nervous system direct proof for autonomic involvement in human atherosclerotic plaque development is scarce.

Methods

In this study 69 samples of abdominal aortas were allocated to five stages of atherosclerotic development: healthy samples, non-progressive intimal lesions, progressive lesions, vulnerable lesions and stabilized lesions. They were examined for numbers of CD3⁺ (total T-cells), CD4⁺ (T-helper cells), CD8⁺ (cytotoxic T- cells), CD20⁺ (total B-cells) and CD138⁺ (plasma cells). Neural parameters PGP 9.5⁺ (total innervation), TH⁺ (sympathetic fibers) and ChAT⁺ (cholinergic activity) were also quantified and compared with lymphocyte counts.

Results

Numbers of all T-lymphocytes show low values in healthy vessels, non-progressive and stabilized lesions but a gradual increase through further atherosclerotic development. Numbers of total B-cells increase identically but are even higher in stabilized lesions. Plasma cell numbers show a dip in vulnerable plaque but do increase to highest values in the stabilized lesions. As a result of a disproportionate increase of cytotoxic T-cells, the CD4:CD8 ratio shows a decreasing curve. Total and sympathetic innervation follow the same increasing pattern as the T-cells until progressive lesions and then show a small drop in vulnerable lesions. Like T-lymphocytes, this neural presence decreases further in stabilized lesions. Cholinergic activity shows a reversed pattern until vulnerable lesions but like total and sympathetic innervation and T-cells, values turn to low again in stabilized lesions. A strong positive correlation of total fibers with all measured T-cells and total B-cells is found, just like a correlation of sympathetic fibers with total T-cells and T-helper cells. Cholinergic staining correlates with T-helper cells.

Conclusions

Similar patterns suggest a relation between numbers of present lymphocytes and neural activity through developing atherosclerotic stages. Positive correlations of total and autonomic nerve counts with total t-cells and T-helper cells strengthen that suggestion. As compared to counts of sympathetic fibers, the reversed values of cholinergic activity show the balancing character of the autonomic nervous system. Slightly lower values of total and sympathetic fibers but still climbing numbers in lymphocytes in vulnerable lesions could indicate that a decrease in neural presence precedes a decline of inflammatory activity in atherosclerotic lesions.

INTRODUCTION

In the healthy body the autonomic nervous system (ANS) and immune system collaborate as a well-balanced system that monitors and regulates essential physiological functions. Among these functions are the defense against invaders from outside, removal of waste materials and the repair of tissues after injury¹⁻⁴. In this system inflammation is a strong and necessary instrument that has to be properly regulated⁵. However, alterations in inflammatory responsiveness may disturb the balance with devastating local and systemic consequences^{6,7}. Overactivity elicits serious damage by inflammatory disease while underperformance could be destructive when decrepit and bad functioning cells are not being disposed. In general, in the regulating role of the autonomic nervous system the parasympathetic division functions as anti-inflammatory while the sympathetic part can act both ways, pro- and anti-inflammatory^{8,9}. When the vagus nerve is manually or electrically stimulated, acetylcholine stimulates the $\alpha 7$ subunits of the nicotinic acetylcholine receptors ($\alpha 7$ nAChR) on inflammatory cells, thus inhibiting the production of inflammatory cytokines^{6,10}. One of the most common inflammatory diseases is atherosclerosis, being the initiator of serious arterial disorders that often lead to life threatening conditions including myocardial infarction, heart failure and stroke. Upon a small injury of the intimal layer, a cascade of immune reactions cause an influx of neutrophils and macrophages initially meant to repair the damage but simultaneously causing accumulation of fatty deposits in the arterial wall and leading to vessel narrowing, insufficiently perfused tissues and organs and ultimately ending in tissue and function loss. Inflammation is involved in the onset as well as the progression of atherosclerotic plaque formation. In recent studies the relation between atherosclerotic development and inflammatory cells is shown^{11,12}. The autonomic nervous system has a regulatory function in inflammatory processes^{3,13} but little is known about its influence in the development of atherosclerosis. Vascular innervation is heterogeneous and therefore each vascular bed has specific nerve types and nerve densities. Moreover it has the capacity to adapt to altering functional demands as present during ageing and disease¹⁴. Local changes of nerve fiber density are highly suggestive for interactions between the nerves and their targets. Therefore the aim of the present study is to determine nerve densities and inflammatory cells in aortic samples with different stages of atherosclerotic development.

METHODS

Sample collection and preparation

Aortic samples were generously provided by the department of Vascular Surgery of the Leiden University Medical Center, the Netherlands. These samples were obtained from cadaveric donors during organ transplantation, two centimeters proximal and distal from the renal artery ostium. All donors met the criteria set by The Eurotransplant Foundation (<https://www.eurotransplant.org>). Sample collection and handling was performed in

accordance with the guidelines of the Medical and Ethical Committee in Leiden, Netherlands and the code of conduct of the Dutch Federation of Biomedical Scientific Societies. Detailed information of donor population and preparation of the samples were previously described by van Dijk et al¹⁵.

Samples were excised and fixed by 4% formaldehyde for 6 or 7 days after which they were decalcified (Kristensens solution and formic acid). After dehydration and embedding in paraffin they were cut in 5µm sections. As a standard procedure all sections were treated for antigen retrieval by heating them for 20 minutes in a 95 °C, 0.01M citrate solution with pH=6. Subsequently they were Movat and Haematoxylin and Eosin (H&E) stained and classified for atherosclerotic stage of development according to the modified American Heart Association (AHA) classification as proposed by Virmani et al¹⁶. Five classes were formed: normal aortic tissue (N); non-progressive intimal lesions with intimal thickening and xanthoma (NIL); progressive atherosclerotic lesions with a pathological intimal thickening and fibroatheroma (PAL); vulnerable atherosclerotic lesions with thin cap fibro-atheroma and plaque rupture (VAL) and stabilized lesions with fibrotic calcified plaque (SL).

Immunohistochemistry

Sections were immunohistochemically (IHC) stained for total B-lymphocytes (CD20⁺), plasma cells (CD138⁺), total T-lymphocytes (CD3⁺), T-helper cells (CD4⁺) and T-cytotoxic cells (CD8⁺). Sections were also labelled for nerve fibers.

Anti-Protein Gene Product 9.5 (PGP 9.5⁺) was used as a general neural marker to determine the total amount of all types of nerves. Anti-Tyrosine Hydroxylase (TH⁺) was used to determine sympathetic nerve fibers and antibodies against Choline acetyl transferase (ChAT⁺) were used to visualize parasympathetic nerve fibers.

T and B lymphocytes were stained with antibodies as shown in table 1. All sections were developed with LPR (Liquid Permanent Red, Dako, Glostrup, Denmark, cat.nr: K5355) and counterstained with haematoxylin 5% for 10sec.

Table 1. Measured parameters and corresponding antibodies.

target	antibody	source
total B-lymphocytes	CD20	DAKO M0755
Plasma cells	CD138	DAKO M7228
total T-lymphocytes	CD3	DAKO A0452
T-helper cells	CD4	DAKO M7310
cytotoxic T-cells	CD8	DAKO M7103
Overall nerve fibers	PGP 9.5	DAKO Z5116
Sympathetic fibers	TH	Pel Freez P40101
Cholinergic activity	ChAT	Millipore AB144P

Figure 1. Complete slice of atherosclerotic aortic tissue (tile scan, CD3 stained from PAL-group) with detailed media-adventitial (ca. 20%-80%) selections for quantification (1.26x0.94mm). M=media, A=adventitia, VV=vas vasorum.

At 100x magnification on light microscopy, sections were digitized as field of view (1.26x0.94mm) images of the adventitial-medial border with a micro camera (Leica DFC 700T associated with Leica microscope DM6 B). This location is chosen for the reason that virtually all innervation is found in the adventitia. The media-adventitia ratio in the images was set ca. 20% over 80% respectively and every second image was used for quantification [fig1]. The number of quantified images therefore depended on the size of the sample [see table 1].

Quantification

Neural and inflammatory cell parameters in digitized images were quantified in ImageJ^{17,18} by a blinded assistant. Lymphocyte counts were determined with ImageJ as numbers of cells per mm². Nerve fibers were identified and manually counted as bundles or as solitary fibers. Some solitary fibers were found in close vicinity of the vasa vasorum. During quantification any cluster of nerve fibers, separated from the surrounding tissue by an epineurium, was counted and scored as one bundle. Any group of nerve fibers closely surrounding the vasa vasorum was also scored as one. The remaining nerve fibers, not organized in a bundle or near a vas vasorum, were considered solitary fibers and received one count per ten individual fibers. The three resulting values were added per image and averaged per sample. For statistical analysis sample scores were averaged per group and recalculated to counts per mm². This manual quantification method was validated by comparing quantification by two different observers to assess inter- and intra-observer correlations.

Table 2. Group characteristics, Atherosclerotic stages and numbers of subjects per sex.

group	Atherosclerotic stage	including	female		male	
			N	age	N	age
N	Normal aorta		7	10.0	7	23.8
NIL	Non-progressive intimal lesions	adaptive intimal thickening and intimal xanthoma	7	32.5	7	38.9
PAL	Progressive atherosclerotic lesions	pathological intimal thickening and fibroatheroma	7	50.8	7	50.5
VAL	Vulnerable atherosclerotic lesions	thin-cap fibroatheroma and plaque rupture	7	53.0	6	57.1
SL	Stabilizing lesions	fibrotic calcified plaque	6	55.1	8	60.7

Statistical analysis

Between groups, comparisons were conducted with an unpaired Kruskal-Wallis test in Prism-8 (GraphPad Prism 8 Software, La Jolla California USA) non-parametric and without corrections for multiple groups. Grubbs outlier detection, a built-in analysis in Prism-8 was used to detect and exclude significantly deviating values at $p=0.05$ ¹⁹. The Spearman's r analysis that was used to determine inter group correlations yields the best result when a linear progression is expected through the successive groups. The groups from N to VAL are expected to increase in inflammatory activity while the Stabilized Lesions group will have returned to basic numbers of inflammatory cells and innervation and therefore disturb the linearity. The SL group was therefore excluded from the correlation analysis. The level of significance in this study is kept at 0.05, two-tailed.

RESULTS

Of the 69 included aortic samples 35 were male. Average age was 49.4 ± 14.3 and 43.8 ± 17.3 for males and females respectively. Numbers and distribution over sexes is shown in table 2. Immunohistochemistry yielded satisfying specific results from PGP 9.5, TH and ChAT as well as CD3, CD4, CD8, CD20 and CD138 staining procedures (Figure 1). Images were clear and well suited for quantification.

The manual quantification method for nerve tissues was validated by comparing in duplo quantification by two different observers to provide inter- and intra-observer correlations. With power=1.00000 and $\alpha=0.05$ together with a large number of subjects, an intraclass correlation was achieved of 0.80 resulting in an acceptable bias for the inter-observer correlation of maximum 0.13 for counted fibers ($M=1.09$) quantification and -0.06 for the intra-observer correlation^{20,21}.

Kruskal Wallis comparisons of innervation and lymphocyte values in atherosclerotic stages were analyzed and depicted in table 3. Correlations between nervous and lymphocyte parameters are shown in table 4.

Figure 2. Exemplary detailed pictures of IHC stained nerve fibers and lymphocytes.

Table 3. Means of lymphocyte counts per mm² in atherosclerotic stages.

Kruskall Wallis					
means	N	NIL	PAL	VAL	SL
CD20	0.21	0.64	1.62	8.42**	21.0***
CD138	0.21	0.12	1.55*#	0.73	2.15***#
CD3	1.46	3.59	7.68*	12.7***	3.85
CD4	3.21	4.93	4.57	6.39*	3.71
CD8	0.29	1.57	6.50*	7.55***	3.07*
CD4:CD8	11.7	6.25	2.59*	2.15**	4.38*
PGP	1.36	2.06	3.51***#	2.91**	1.76
TH	1.76	2.04	2.91*	2.56	0.95
ChAT	1.57	1.41	1.09	1.35	0.98

Significant difference * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ from N. Sign. Diff. # $p < 0.05$ from prior atherosclerotic stage.

Lymphocytes and atherosclerotic stages

Table C shows lymphocyte counts expressed as numbers per mm² for the atherosclerotic stages. Stated p-values refer to differences from the N-group unless separately specified. All cellular parameters were low in the N (normal) group. Low or non-significantly increased numbers were found in the NIL group. CD20⁺ total B-cells were somewhat higher in the PAL group (n.s.) but significantly increased in VAL (8.42±9.51, $p = 0.001$) and were even further increased in SL (21.0±19.7, $p < 0.001$). Plasma cells, CD138⁺, showed with 1.55±2.23 ($p = 0.010$) significantly higher counts in PAL and were also increased in the SL group (2.15±2.08, $p < 0.001$). CD3⁺ total T-lymphocyte counts were raised in the PAL group to 7.68±9.29 ($p = 0.016$) and further to 12.65±13.38 ($p < 0.001$) in the VAL group. A decrease was found in the SL group (3.85±4.01, n.s.). CD4⁺ T-helper cells were also increased through the atherosclerotic stages to 6.39±4.09 ($p = 0.028$) in the VAL group and a lower value of 3.71±2.43 (n.s.) in the SL. Cytotoxic T-lymphocytes (CD8⁺) were increased to 6.50±11.6 in the PAL ($p = 0.025$) and reached its peak level in the VAL group at 7.55±8.38, $p < 0.001$. In the SL group the counts went down again to 3.07±3.43 ($p = 0.011$). As a consequence of the T-helper and cytotoxic cell counts CD4:CD8 ratio for the normal healthy (N) samples was relatively high with 11.69±13.4 and lower in the other groups, NIL 6.25±8.7 (n.s.), PAL 2.59±3.5 ($p = 0.010$), VAL 2.15±3.6 ($p = 0.002$) and slightly increased in SL to 4.38±8.5 ($p = 0.016$).

Neural parameters and atherosclerotic stages

Total nervous tissue as stained with PGP-9.5 showed increasing levels of counts per mm² from the N group (1.36±1.68) to the NIL group (2.06±1.15, n.s.) and PAL group (3.51±1.70, $p < 0.001$). PGP-9.5⁺ in the group with VAL (2.91±1.76, $p = 0.004$) was slightly lower than PAL but still significantly higher than Normal. In the SL group PGP was again lower and close to normal values (1.76±1.04, n.s.). Resulting values for sympathetic nerves (TH⁺) followed the same pattern as total nervous tissue with increasing values from N (1.76±2.57) to NIL (2.04±1.40, n.s.) and PAL (2.91±2.54, $p = 0.028$) and somewhat decreased in the VAL group

Table 4. Correlations of lymphocytes with innervation in atherosclerotic stages and numbers of lymphocytes. SL group not included. Significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Spearman r				
	Athero-stage	PGP	TH	ChAT
		0.44***	0.22	0.03
CD3	0.47***	0.42**	0.43**	0.11
CD4	0.26	0.39**	0.28*	0.17
CD8	0.53***	0.35*	0.23	0.17
CD20	0.52***	0.40**	0.32*	-0.03
CD138	0.28*	0.19	0.25	-0.10
CD4:CD8	-0.50***	0.07	-0.03	0.02

(2.56 ± 2.52 , n.s.). The counts in the SL group were down to even below normal levels (0.94 ± 0.59 , n.s.).

The averages of the cholinergic counts (ChAT⁺) through the atherosclerotic stages did not increase, see table 3. Although the differences in the Cholinergic ChAT counts in further stages were not significantly different from N (1.57 ± 1.71), the pattern found from N to VAL was contrary to total and sympathetic nervous fibers which showed the opposite curve. However, after a small increase in ChAT values in the VAL group (1.35 ± 0.89 , n.s.) counts lowered again in the stabilized group (0.98 ± 0.52 , n.s.). This decrease in the SL was also found in the total and sympathetic innervation and T-lymphocytes.

Correlations of Lymphocytes with innervation

Table 4 shows the Spearman-r correlations between the B- and T- lymphocytes and the presence of PGP 9.5, TH- and ChAT-positive fibers in atherosclerotic developmental stages. As explained in the methods section the SL-group is not included in the atherosclerotic stages.

The stages correlated strongly with total innervation ($p<0.001$) but not with sympathetic or cholinergic tissues. They also showed a correlation with CD3⁺ total T-lymphocytes ($p<0.001$), CD8⁺ cytotoxic T- lymphocytes ($p<0.0001$), CD138 Plasma cells ($p=0.042$) and CD20⁺ total B-lymphocytes ($p<0.001$) and a negative correlation with the CD4 over CD8 ratio ($p<0.001$). CD4⁺ T-helper cells just did not reach significant values to correlate with developing atherosclerotic stages ($p=0.051$).

Counts of total innervation (PGP-9.5⁺) correlated strongly with sympathetic fibers ($p<0.0001$) but not with ChAT⁺ cholinergic activity. They did also correlate with numbers of total CD3⁺ T-lymphocytes ($p<0.002$), CD4⁺ T-helper Cells ($p=0.003$) CD8⁺ Cytotoxic T-cells ($p=0.011$) and CD20⁺ total B-cells ($p=0.003$). Plasma cells and CD4:CD8 ratio did not correlate with total innervation.

Sympathetic fibers did show a positive correlation of $r=0.36$ with ChAT⁺ fibers ($p=0.008$). At the same time they showed a correlation with total T-lymphocytes ($p<0.002$), T-helper cells

($p=0.043$) and total B-lymphocytes ($p=0.023$). No correlations were found of sympathetic fibers with Cytotoxic T-cells, Plasma cells or CD4:CD8 ratio.

ChAT⁺ fibers did only significantly correlate with sympathetic fibers ($p=0.008$) and not with total nerve fibers or any of the lymphocyte subsets.

DISCUSSION

This study aimed to define to what extent autonomic innervation is related to inflammatory activity as measured by lymphocyte presence in abdominal aortic samples with different stages of atherosclerosis.

The found lymphocyte numbers follow broadly the same patterns through the atherosclerotic stages as previously published by van Dijk et al.¹¹. These patterns show increasing numbers of cells from normal healthy arteries through non-progressive and progressive intimal lesions to vulnerable lesions and plaque rupture. This supports the expectation of more inflammatory activity in further stages of atherosclerosis. In the stabilized lesions group the T-cell numbers return back to somewhat above normal levels while plasma cells remain high in the SL group and total B-cells show even higher numbers, see table 3 and figure 3. Counts of total and sympathetic nerve fibers are also elevated until the PAL stage but show a small decrease in the VAL group that continues in the SL group. Compared to normal, the counts of ChAT fibers show a strong decrease in the PAL stage but are back to normal in the VAL stage and decrease again to normal values in the SL stage.

In the IHC staining method different antibodies were used for the different subsets. Those antibodies bind to different sites in the tissue and some stain more intense or are less specific than others. Consequently, comparison of the amount of stained tissue is only valid when the same method is used, in identical tissues and with identical antibodies, preferably from the same batch. In quantification, differences can be determined between groups within one subset but quantitative differentiation between subsets cannot be made. This may generate confusion since the sympathetic or parasympathetic nerves are by definition a part of total innervation but still can show values higher than total innervation. For determination of differences between subsets we used a method of manual quantification which was validated and was performed by two independent and blinded co-workers.

ChAT antibody does not only bind to neural antigens but labels cellular ChAT as well²². The same applies for TH since TH can also be produced by immune cells²²⁻²⁴. It may therefore well be that some structures have erroneously been recognized and scored as neural fibers. However, the tissue stained positive for choline acetyltransferase or tyrosine hydroxylase positive and therefore represents cholinergic or adrenergic activity respectively.

The CD4:CD8 ratio is often used as a measure for resistance to infections and for inflammatory responsiveness^{25,26} and this is usually done in blood samples. In this study,

the CD4 and CD8 content could only be measured in the available sample tissue which resulted in a value of 11.69 ± 13.4 for the normal group. Significantly lower values are found in next stages from NIL to VAL. Higher lymphocyte levels generally indicate stronger inflammatory activity. In further developed atherosclerotic plaque in this study higher counts of both CD4 and CD8 lymphocytes result in lower CD4:CD8 values. This is mainly caused by a stronger increase of CD8 cytotoxic lymphocytes. Recent findings in atherosclerosis research show that CD8⁺ cells can promote atherosclerotic development and vulnerability of plaque ²⁷ but other publications report, despite the name cytotoxic, anti- as well as pro-atherogenic functions of CD8⁺ T-cells ^{28,29}. Whether they are the cause for the atherosclerotic development up to vulnerable plaque levels, or the onset of the sudden decrease of T-cell activity towards stabilization, is still to be examined. They may be the reason for both processes.

Figure 3. Lymphocyte patterns through atherosclerotic stages : N=normal, NIL=non-progressive intimal lesions, PAL=Progressive atherosclerotic lesions, VAL=Vulnerable atherosclerotic lesions, SL=Stabilized lesions. Cell numbers per mm², significant difference from N-group: * p<0.05, **p<0.01, ***p<0.001.

Both total nerve population and specifically sympathetic nerve fibers show a strong correlation with counts of CD3⁺, CD4⁺, CD8⁺ and CD20⁺ lymphocytes. Values increase in atherosclerotic development up to the stage of progressive intimal lesions. But while the developing stages of atherosclerosis show further increasing numbers of lymphocytes up to the VAL group, counts of total and sympathetic innervation show a drop from the PAL to the VAL group. At the same time, ChAT activity increases and a rise in parasympathetic together with a drop in sympathetic is indicative for an impulse for decreasing inflammatory activity. Regarding the correlation between numbers of inflammatory cells and nerve densities it is difficult to distinguish cause and consequence. These findings could mean that decreasing total and sympathetic innervation together with increasing ChAT activity precedes inflammatory inhibition, decreased numbers of lymphocytes and plaque stabilization. Increased nerve density is the result of growth of nerve collaterals and requires the presence and neuronal uptake of neurotrophic factors such as Nerve Growth Factor (NGF)¹⁴. NGF can be synthesized by present neurons, smooth muscle cells and inflammatory cells and is found to be enhanced in inflamed tissues³⁰⁻³³. Apart from stimulation of nerve growth, NGF is able

Figure 4. Nerve fiber count patterns through atherosclerotic stages : N=normal, NIL=non-progressive intimal lesions, PAL=Progressive atherosclerotic lesions, VAL=Vulnerable atherosclerotic lesions, SL=Stabilized lesions. Counts per mm², significant difference from N-group: * p<0.05, **p<0.01, ***p<0.001.

to act pro- as well as anti-inflammatory and it can stimulate the release of acetylcholine as well as the synthesis of tyrosine hydroxylase i.e. the enzyme that regulates noradrenaline release^{34,35}. NGF is therefore suggested to play an important role in the regulation of inflammatory activity^{36,37}. It may contribute to increased innervation and the exaggerated inflammatory response upon a minor vascular wall damage in the development of atherosclerosis. However, it may also be an attenuating force when a plaque stabilizes and decrease of innervation and inflammation takes place.

The origin of NGF and its potency to regulate nerve growth as well as acting pro- and anti-inflammatorily makes it an interesting target for further atherosclerosis research.

ChAT⁺ activity does not follow the pattern of the total nerve population and sympathetic fibers but rather shows a tendency for an inverted pattern until the VAL stage. Interestingly, the lower levels of total and sympathetic fibers in the VAL group are accompanied by a small raise of cholinergic nerve fibers emphasizing a move of the autonomic balance towards a lower inflammatory responsiveness. This is also supported by a decreasing curve of the CD4:CD8 ratio from the PAL to the VAL stage identically indicating lower inflammatory activity. These findings are indicative of the balancing character of the autonomic nervous system.

Inevitably development of atherosclerosis is strongly related to age, and age as such is directly of influence on immunity and innervation. Interestingly, numbers of blood and tissue T and B lymphocytes generally decrease with age^{38,39} which is contrary to the findings in this study.

CONCLUSIONS

The results indicate a positive correlation between total and sympathetic nerve population and inflammatory activity as shown by numbers of present CD3⁺, CD4⁺, CD8⁺ and CD20⁺ lymphocytes, through developing atherosclerotic lesions. The autonomic nervous system may be involved in enhancing inflammatory processes in beginning atherosclerotic lesions but may also provide an inhibiting force in further stages.

Slightly lower values of total and sympathetic fibers together with increasing cholinergic activity while numbers of lymphocytes still increase from progressive to vulnerable lesions could mean that neuronal decrease precedes a decline of inflammatory activity in atherosclerotic lesions, ultimately leading to stabilization of these lesions.

As compared to counts of sympathetic fibers, the reversed values of cholinergic activity show the balancing character of the autonomic nervous system.

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CHAPTER 6

Adventitial nerve fiber density in extracranial carotid and popliteal aneurysms

Submitted for publication

M.G.J. Nederhoff*²

V.E.C. Pourier* MD¹

M. de Bakker⁴

S.A.M.W. Verlinde²

A. Vink MD PhD³

G.J. de Borst MD PhD¹

R.L.A.W. Bleys MD PhD²

* authors equally contributed to this manuscript.

¹ Department of Vascular Surgery, University Medical Center Utrecht, Utrecht University, The Netherlands.

² Department of Anatomy, University Medical Center Utrecht, Utrecht University, The Netherlands.

³ Department of Pathology, University Medical Center Utrecht, Utrecht University, The Netherlands

⁴ Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht University, The Netherlands

ABSTRACT

Introduction

Extracranial carotid artery aneurysm (ECAA) and popliteal artery aneurysm (PAA) are of rare vascular pathology with a heterogeneous etiology and unknown natural course. Arterial wall innervation and especially sympathetic fibers may relate to aneurysmal growth patterns. The aim of this study was to quantitatively investigate the innervation of ECAAs and PAAs in comparison to non-diseased arteries.

Methods

Carotid (n=10) and popliteal (n=10) aneurysm wall samples of patients that underwent open surgical repair were compared with extracranial carotid (n=6) and popliteal (n=6) control arteries. All tissue samples were immunohistochemically stained for overall nerve fibers (oNF) with anti-protein gene product (PGP) 9.5 and for sympathetic nerve fibers (sNF) with tyrosine hydroxylase (TH). Total stained area was determined for both stainings. The nerve bundles were counted manually per field of view and recalculated to counts per mm². Also, scattered fibers and vasa vasorum associated branches were counted and analyzed as subgroups.

Results

ECAA revealed a non-significant higher number of scattered fibers in the aneurysmal wall as compared to controls. In popliteal arteries, no difference between aneurysms and controls was observed. Nerve density was higher in ECAA as compared to PAA for both oNF and sNF. In control samples, carotid arteries showed higher oNF and sNF density than popliteal arteries.

Conclusions

ECAA showed higher sympathetic nerve densities than popliteal aneurysms, which is in accordance with the topographical differences as found in control arteries. The non-significant increase in scattered nerve fiber density in ECAAs compared to carotid artery (CA) control arteries suggests that outgrowth of sNF may be related to aneurysm formation. These observations need to be validated in larger histological samples.

INTRODUCTION

Extracranial carotid artery aneurysm (ECAA) is a rare vascular pathology.^{1,2} Although there are no exact data available, the incidence of ECAAs is low. ECAA affects relatively young people around the age of 50 years. An aneurysm is generally defined as a dilation in diameter of 150% or more of the artery. It is known that most aneurysms will gradually grow over time and have an increased chance of rupture or in some cases, e.g. popliteal artery aneurysms (PAA) may thrombose and/or result in distal embolization.¹ However, for ECAA this growth pattern and related natural course is still unknown. Therefore, understanding the pathophysiology of aneurysms is important and may provide insights in the natural clinical course. It may also lead to a better treatment approach for these young patients affected.

Different etiologies for ECAA have been described including atherosclerosis, dissection, trauma and infection. Histological analysis revealed dissection and degeneration as the two distinctive underlying mechanisms in ECAA and different inflammatory cells have been observed in the walls of these aneurysms². In addition a decrease in extracellular matrix has been described which, like in abdominal aneurysms, is likely caused by inflammation in the arterial wall.^{2,3} Like ECAAs, PAAs are peripheral aneurysms associated with co-presence of other aneurysms (abdominal aorta), but they can also occur in isolation.^{4,5} Inflammation has been described in vessel walls of PAA, suggesting a link between arterial wall inflammation and aneurysm formation.⁵

The autonomic nervous system (ANS) has the potential to influence inflammatory responses. The main parasympathetic neurotransmitter acetylcholine has been described to effectively deactivate macrophages, thus inhibiting the inflammatory response.^{6,7} Sympathetic influence is found to operate pro- as well as anti-inflammatory.⁸ Stimulation of the vagus nerve in animal models and in clinical treatment of rheumatoid arthritis resulted in a reduction of inflammation.⁹⁻¹¹ Vascular innervation is heterogeneous with respect to location, artery type, nerve fiber density and transmitter content.¹⁴ Moreover local changes of nerve fiber density have been associated with various conditions and diseases including ageing, Alzheimer's disease, peripheral arterial disease, diabetes mellitus and with experimental studies of anosmia.^{12,16} The above-mentioned factors support the assumption that the ANS plays a role in the inflammatory process, which may be related to vessel wall changes including aneurysmal dilation.

To better understand the role of vessel wall innervation and its influence on ECAA formation, we examined the sympathetic and overall innervation of ECAA and PAA vessel walls. To our knowledge, the innervation of ECAA or any peripheral aneurysm has not been examined before. The aim of this study was to investigate whether there is a quantitative difference in innervation between ECAAs and PAAs as compared to healthy controls.

METHODS

Subjects and sample collection

Ten ECAA samples were available from patients that underwent surgical treatment in two participating hospitals (University Medical Center Utrecht, and St. Antonius hospital, Nieuwegein, the Netherlands) from March 2004 until June 2013. The operation indication for ECAs was made after multi-disciplinary conference and was based on symptoms, growth and size of the ECAA. All aneurysmal samples were stored in the hospital Biobank and the collection took place under approval of the local medical ethics committees of the two participating hospitals (Verenigde Commissies Mensen Gebonden Onderzoek, St. Antonius Hospital Nieuwegein and Medical Ethical Committee of the University Medical Center Utrecht). All patients provided written informed consent. To compare ECAs with other peripheral aneurysms, ten PAA samples, also collected in the hospital Biobank, were analyzed. As controls, six carotid artery (CA) and six popliteal artery (PA) non-aneurysmal samples were extracted from human bodies that entered the department of Anatomy (University Medical Center Utrecht) through a donation program. From these persons written informed consent was obtained during life that allowed the use of their entire bodies for educational and research purposes. Control samples were excised from human bodies preserved in 4% formaldehyde.

All samples were handled according to a standardized protocol¹⁷. After resection the specimens were fixed in 4% formaldehyde for seven days. Specimens were then decalcified for another seven days in ethylene diamine tetra-acetic acid (EDTA), dehydrated and embedded in paraffin. Subsequently, the samples were cut in 5µm sections and mounted with Entellan (Merck, Whitehouse, NJ, USA).

Immunohistochemical staining

As a standard procedure all sections were treated for antigen retrieval by heating them for 20 minutes in a 95°C, 0.01M citrate solution with pH=6. Sections were then immunohistochemically (IHC) labeled with polyclonal anti-PGP 9.5 antibodies for staining of any type of nerve fibers (anti Protein Gene Product 9.5, Dako, Glostrup, Denmark, cat.nr: Z5116), or labeled with polyclonal TH-antibodies for sympathetic nerve fibers (anti Tyrosine Hydroxylase, Pel-Freez, Rogers, USA, cat.nr: P40101) and stained with LPR (Liquid Permanent Red, Dako, Glostrup, Denmark, cat.nr: K5355). At 10x magnification on light microscopy, sections were digitized as field of view (0.57x0.87mm) images of the adventitial-medial border with a micro camera (Olympus DP71, associated with Olympus microscope BX53). The number of images depended on the size of the slice and every third picture was used for quantification. All results are recalculated to, and presented as counts per square millimeters (mm²).

Quantification and statistical analysis

PGP 9.5- and TH-immunoreactivity were quantified and expressed as an area percentage of the total standard picture size, using the image analysis software ImageJ¹³. Furthermore, nerve fibers, as clustered in bundles, those in close vicinity of vasa vasorum and scattered

nerve fibers were counted manually per field of view and recalculated to counts per mm² (Figure 2a-b). Any cluster of nerve fibers, separated from the surrounding tissue by an epineurium, was considered a nerve bundle. All nerve fibers surrounding one particular vessel in the vasa vasorum were counted together as one. Other stained nerve fibers, not in a bundle or near the vasa vasorum, were considered scattered nerve fibers and received one count per ten individual fibers.

Statistical analysis

A nonparametric One-way Anova with an uncorrected Dunn's multiple comparisons test was performed with Prism7 (GraphPad Software, La Jolla California USA, www.graphpad.com) to compare total and sympathetic innervation in healthy and aneurysmal carotid and popliteal arteries. Data are presented as mean and a p-value of <0.05 was considered statistically significant.

Quantification method, by ImageJ as well as manually, was validated by comparing in duplo quantification by two different observers to provide inter- and intra-observer correlations. With power=1.00000 and $\alpha=0.05$, together with a large number of subjects, an intraclass correlation was achieved of 0.80 resulting in an acceptable bias of maximum 5 for area (M=72.8), and max 0.13 for counted fibers (M=1.09) quantification (see appendix 1.)

RESULTS

Patient characteristics

The median age of the ECAA patients was 58 years (range 25-76), five were male. Of the ten included ECAA patients, nine were symptomatic. Ten PAA samples were included of nine patients, with a median age of 74 years (range 49-82). Seven patients had a symptomatic PAA. Patient characteristics are presented in table 1.

Aneurysms

Most of the ECAs were located in the internal CA (n=8, 80%). Of these eight carotid arteries, six were in the distal carotid artery and the other three in the proximal internal CA. An example of an ECAA in the internal CA is shown in figure 1. The majority of the aneurysms was located on the left side (n=6, 60%). The mean diameter was 12.5mm and 27mm for saccular (n=4) and fusiform aneurysms (n=6) respectively. The presumed etiology of the ECAA was degeneration (n=9) and dissection (n=1). Of the PAAs six out of ten (60%) were located on the right side. The mean diameter of popliteal aneurysm was 32mm. Specimens' characteristics are presented in Table 2.

Control samples

Post-mortem non-aneurysmal carotid specimens from six cadaveric donors with a mean age 89 (range 75-98) and PA samples from six cadaveric donors with a mean age of 73 (range 57-89) were used as controls.

Table 1. Patient characteristics.

	ECAA patients (n= 10)	PAA patients (n=9)
Gender male (%)	5 (50)	8 (89)
Age (mean), years (range)	58 (25-76)	74 (49-82)
Hypertension (%)	5 (50)	6 (67)
MI (%)	1 (10)	1 (11)
COPD (%)	0 (0)	3 (33)
Statin use (%)	4 (40)	4 (44)
Connective Tissue disease (%)	0 (0)	0 (0)
Smoking (currently/past) (%)	3 (30)	2 (22)
DM (%)	1 (10)	2 (22)

Symptoms			
ECAA		PAA	
TIA	3 (30)	Distal embolus	4 (44)
Stroke	1 (10)	Rupture	1 (11)
Local compression ^a	1 (10)	Pain	1 (11)
Contralateral symp.	4 (40)	Edema	1 (11)

Abbreviations: ECAA: extracranial carotid artery aneurysms, PAA: popliteal artery aneurysms, MI: Myocardial infarction, COPD: chronic obstructive pulmonary disease, DM: diabetes mellitus. ^aincludes local pain, mass or hoarseness. Symp= symptoms.

Figure 1. A 3-D image of an extracranial carotid aneurysm located in the right internal carotid artery.

Innervation density

In the IHC staining method of overall and sympathetic nerve tissue, different antibodies were used. Anti-PGP 9.5 and anti-TH bind to different sites in the tissue and TH stains obviously more intense than PGP 9.5. Consequently, in quantification, TH can present more counts per square millimeter than PGP, which may cause some confusion since the sympathetic nerves are by definition a part of the total innervation. The comparisons in this study were therefore made only between tissues stained in the same batch. Presence of sympathetic fibers was not quantitatively compared with overall nerve counts.

Figure 2a. Histology of a PGP 9.5 stained (oNF) carotid artery aneurysm. Yellow arrows: nerve bundle, white: vasa vasorum associated fibers, black: scattered fibers.

Figure 2b. Histology of a TH stained (sNF) carotid artery aneurysm. Yellow arrows: nerve bundle, white: vasa vasorum associated fibers, black: scattered fibers.

Immunohistochemistry yielded satisfying specific results from PGP 9.5 as well as TH staining procedures. Images were clear and well suited for quantification (Figure 2a-b). Validation of the quantification method resulted in satisfying values (appendix 1).

In both CA and PA, no significant difference was observed between aneurysms and control samples for overall type nerve fibers (oNF) and sympathetic nerve fibers (sNF) stained area (Figure 3), total number of bundles (Figure 4), vasa vasorum associated fibers (Figure 5) and scattered fibers (Figure 6). In CA, however not significant, loose scattered oNF as well as sNF showed an increased density in the aneurysms as compared to controls (oNF scattered fibers: Mean control CA 0.93 vs. ECAA 9.19 counts/mm², $p=0.133$ and sNF scattered fibers: Mean control CA 0.65 vs. ECAA 5.76 counts/mm², $p=0.100$; Figure 6). Further analysis showed a strong correlation between values in oNF and sNF, equally in measured areas and nerve counts

High scores of overall innervation correspond with sympathetic stained samples.

In control samples, oNF, as well as sNF, was found to be higher in carotid than in popliteal

Table 2. Sample characteristics. Data presented as numbers unless otherwise indicated. Abbreviations: RCC: right common carotid artery, LICA: left internal carotid artery, RICA: right internal carotid artery.

	ECAA (n=10)	PAA (n=10)
Location	1 Left bifurcation 5 LICA, 3 RICA 1 RCC	6 Right
Shape	4 saccular 6 fusiform	
Size, mean in mm (range)	Saccular: 12.5 (11-15mm) Fusiform: 27 (13-33mm)	32 (8-67mm)
Symptomatic (%)	9 (90)	8 (80) 2 (20) unknown

samples. This was significant for oNF area and vasa vasorum associated fibers (oNF area control CA 165.00 vs. control PA 10.70 counts/mm², $p=0.006$ and vasa vasorum associated fibers control CA 0.95 vs. control PA 0.04 counts/mm², $p=0.013$; Figure 3 and 5) and showed a trend for number of sNF bundles (sNF bundles control CA 0.59 vs. control PA 0.03 counts/mm², $p=0.059$; Figure 4). This higher nerve density in the carotid compared to the PA was even more pronounced in the aneurysmal groups. In aneurysms, oNF and sNF area (Figure 3), numbers of bundles (Figure 4), vasa vasorum associated fibers (Figure 5) and scattered fibers (Figure 6) were all significantly higher in ECAAs than in PAAs (Table 3).

Figure 3. Comparison of (3a) overall nerve fibers (oNF) and (3b) sympathetic nerve fibers (sNF) as total stained area per mm², in arteries with and without aneurysm. Groups: Control carotid artery (N=6), ECAA (N=10), control popliteal artery (N=6) and popliteal artery aneurysm (N: oNF=7, sNF=10).

Figure 4. Comparison of (4a) overall nerve fibers (oNF) and (4b) sympathetic nerve fibers (sNF) in number of bundles in arteries with and without aneurysm. Groups: Control carotid artery (N=6), ECAA (N=10), control popliteal artery (N=6) and popliteal artery aneurysm (N: oNF=7, sNF=10).

Figure 5. Comparison of counts of (5a) overall vasa vasorum associated nerve fibers (oNF) and (5b) sympathetic vasa vasorum associated nerve fibers (sNF) in arteries with and without aneurysm. Groups: Control carotid artery (N=6), ECAA (N=10), control popliteal artery (N=6) and popliteal artery aneurysm (N: oNF=7, sNF=10).

Figure 6. Comparison of counts of (6a) overall scattered nerve fibers (oNF) and (6b) scattered sympathetic nerve fibers (sNF) in arteries with and without aneurysm. Groups: Control carotid artery (N=6), ECAA (N=10), control popliteal artery (N=6) and popliteal artery aneurysm (N: oNF=7, sNF=10).

Table 3. Means and significance of nerve fiber counts in carotid (ECAA) and popliteal (PAA) aneurysms vs. control arteries.

A. oNF, Overall nerve fibers stained with PGP 9.5				B. sNF, Sympathetic nerve fibers stained with TH.			
Overall nerve fibers				Sympathetic nerve fibers			
PGP 9.5	Contr CA vs ECAA			TH	Contr CA vs ECAA		
	mean	mean	p		mean	mean	p
Area	165	362	0,743	Area	37,3	110	0,364
Bundles	0,40	1,44	0,300	Bundles	0,59	0,79	0,667
VV associated	0,95	1,67	0,997	VV associated	0,46	1,57	0,213
Scattered	0,93	9,19	0,133	Scattered	0,65	5,76	0,100
	Contr PA vs PAA		p		Contr PA vs PAA		p
Area	10,7	38,9	0,510	Area	29,0	20,3	0,510
Bundles	0,03	0,06	0,596	Bundles	0,03	0,15	0,404
VV associated	0,04	0,08	0,721	VV associated	0,27	0,25	0,765
Scattered	0,44	0,16	0,633	Scattered	0,52	1,15	0,833
	Contr CA vs Contr PA		p		Contr CA vs Contr PA		p
Area	165	10,7	0,006	Area	37,3	29,0	0,721
Bundles	0,40	0,03	0,189	Bundles	0,59	0,03	0,059
VV associated	0,95	0,04	0,013	VV associated	0,46	0,27	0,401
Scattered	0,93	0,44	0,197	Scattered	0,65	0,52	0,706
	ECAA vs PAA		p		ECAA vs PAA		p
Area	362	38,9	0,030	Area	110	20,3	0,023
Bundles	1,44	0,06	0,043	Bundles	0,79	0,15	0,048
VV associated	1,67	0,08	0,012	VV associated	1,57	0,25	0,004
Scattered	9,19	0,16	0,000	Scattered	5,76	1,15	0,032

Abbr: Contr CA= control carotid artery, ECAA = extracranial carotid artery aneurysm, Contr PA = control popliteal artery, PAA= popliteal artery aneurysm, VV = vasa vasorum.

DISCUSSION

The main results of this explorative study are a non-significant higher rate of scattered sympathetic nerve fibers in ECAAs as compared to control CAs. This was observed for both oNF and sNF scattered fibers. Second, a higher nerve density was found in ECAA as compared to PAA samples, but also in carotid controls as compared to popliteal controls. The difference in nerve density was more obvious in the aneurysm groups than between the controls and was found for both overall and sympathetic innervation.

More scattered fibers in ECAAs than in controls could mean that growth of these fibers is drawn to the aneurysmal site. They could fulfill a function in the development of the aneurysm. In other words, that they actively take part in processes, whether or not inflammatory, that weaken the arterial wall or make an attempt to minimize a primary

damage by mechanical or infectious causes. The relation between innervation and inflammation has formerly been described¹⁴⁻¹⁶ and, because of the inflammatory nature of atherosclerotic aneurysms, might provide an explanation for the increased presence of nerve fibers in the aneurysmal arterial wall. The specific innervation in ECAA resulting from carotid dissection could not be studied due to the limited number of ECAA samples available in our biobank.

Preservation

Experience has shown that negative effects of long term formaldehyde preservation on the quality of nerve staining could be expected. However, all samples were preserved following the same protocol in duration of 7 days and concentration of 4%. Furthermore, as standard treatment antigen retrieval was executed on all sections to restore disturbing tissue changes before IHC was performed. All samples were thus equally treated in preservation as well as staining method.

Location

While ECAs tend to develop close to the carotid bifurcation, popliteal aneurysm formation is expected to be initiated by vascular injury, most probably caused by mechanical forces following strong bending of the knee. As such, location will be a factor of importance in the initiation of aneurysm formation.

The CA is a resistance artery and especially the internal branch is closely involved in pressure regulation and perfusion of the brain. Neural pressure sensitive receptors in the carotid sinus keep blood pressure and flow to the brain within acceptable range²². Baroreceptor tissue has been described to be visible using PGP 9.5 staining and is reported to be mainly located in the medial portion of the proximal internal carotid artery (ICA)²³. The ECAA we included were mostly (60%) located in the distal CA, while three were located near the bifurcation/proximal CA and one was located in the common carotid artery. This could potentially have influenced the observed nerve density in our ECAA samples, with a relative higher expected nerve density in the samples derived from the more proximal CA.

Inflammation

Results suggest that the increased density of scattered fibers in the CA is in part caused by sympathetic fibers (Table 4). In rodent studies with experimental myocardial infarction (MI), inflammatory cell synthesized Nerve Growth Factor (NGF) leads to sympathetic nerve sprouting in the peri-infarcted area^{24,25}. In another study, NGF stimulates nerve growth as well as angiogenesis in and around the injured arterial wall in the mouse²⁶. This provides evidence of a simultaneous outgrowth of nerves and blood vessels upon inflammatory cell regulated NGF-release in order to protect damaged tissue²⁷. This principle reaction might explain the denser innervation found in diseased vessels and we therefore emphasize further research should be aimed at inflammatory processes in aneurysm development.

Table 4. Correlations between PGP 9.5 (oNF, overall innervation) and TH (sNF, sympathetic fibers) stained results.

Correlation	oNF : sNF
Area	$R^2=0,53$
Bundles	$R^2=0,48$
VV assoc.	$R^2=0,70$
Scattered	$R^2=0,79$

Limitations

Due to the rarity of ECAA samples available for histological staining and the limited number of ECAA patients undergoing surgery, the present analysis could only be performed on a small number of cases. In addition, the majority of the selected ECAA patients were symptomatic and had a surgical treatment, thus the results of this explorative analysis apply to symptomatic ECAA patients that underwent surgery.

The mean age of ECAA was higher in controls and in PAA patients. In cerebral arteries an association had been found between age and increased arterial wall innervation²⁸. However, there are distinct differences in arterial wall anatomy between intracranial versus extracranial carotid arteries; namely the external carotid artery is considered an elastic artery while the internal carotid artery is considered a muscular artery²⁹. An important difference between the intracranial and extracranial portion of the ICA is the tunica media. The intracranial portion lacks a layer of the elastic lamina and this change occurs in the cavernous portion of the carotid artery³⁰. Aneurysm nerve density could also depend on stage of the aneurysm development, age and physical condition of the donor. Furthermore, innervation patterns can only be studied ex vivo and noninvasive imaging techniques will be useful to serve as a diagnostic approach in vessel wall characteristics assessment.

CONCLUSIONS

In this explorative study on a small but unique set of ECAA and PAA arterial wall samples, ECAAs showed a higher overall nerve density as compared to PAAs in overall and in sympathetic staining. Overall nerve densities measured as stained area as well as vasa vasorum associated nerve fibers in control CAs showed higher values as compared to those in control PAs.

Increased density of scattered nerve fibers in ECAAs compared to control CAs, not significant in this study, could suggest that outgrowth of small adventitial nerve fibers from pre-existing bundles may be related to aneurysm formation. The role of innervation in aneurysm growth patterns needs to be validated in larger histological sample sets.

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CHAPTER 7

Implantable vagus nerve stimulator for small laboratory animals

Under submission

M.G.J. Nederhoff^{1*}

E. Boskovic³

B.J. van Dongen³

R.L.A.W. Bleys¹

G. Pasterkamp²

¹ Department of Anatomy, ² Laboratory for Experimental Cardiology,

³ Department of Medical Technology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands

ABSTRACT

Introduction

Immune processes in inflammatory diseases are highly regulated by the autonomic nervous system. The vagus nerve serves as the major route of action of the parasympathetic branch of the autonomic nervous system. Stimulation of the vagus nerve (VS) causes attenuation of inflammatory responses that follows a stimulus. Animal models that facilitate research in neuro-inflammatory regulation are limited to single stimulation sessions. Repeated surgical intervention to administer vagus nerve stimulation causes distress for the animal and has unwanted neuro-hormonal consequences. To enable stress-less repeated long term vagus nerve stimulation in small laboratory animals there is a need for a small implantable device.

Method

Materials to use, dimensions and electronical settings were determined. 16 male C57Bl/6j mice were administered a single 30sec vagus nerve stimulation as a control for inflammatory responsiveness. 3 mice had dummy stimulators implanted to study acceptance by the body and subsequently 11 mice underwent implantation of functioning vagus stimulators. A decrease of heart rate was used as proof for effective vagus stimulation. Parameters like toleration by the body, function and work span of the stimulator were examined.

Results

We have developed an implantable and programmable vagus nerve stimulator for small laboratory animals that enables repeated vagal stimulation sessions. Animals did not show any sign of distress caused by the implant or by the vagus stimulation. After 3 days, stimulators in half of the implanted animals were functioning properly while the other half ceased working due to a broken electrode. The longest effective working period was 29 days.

Conclusions

VS for up to three days without stress for the animal was established with this device. Minimal manipulation in the cervical area can activate the vagus nerve of high responders but not in low responding subjects. Further experimentation is needed to develop VS for longer periods.

INTRODUCTION

The immune system plays an important role in the maintenance of daily health. Immune cells respond to environmental threats and endogenous danger signals in order to protect and support tissue growth and repair. The innate immune system functions in a delicate balance since hyper responsiveness, just as underperformance, may have life threatening effects. In the last decade it has become increasingly apparent that the autonomic nervous system (ANS) is of major importance for the maintenance of the inflammatory balance¹⁻⁴. Activity of the parasympathetic nervous system attenuates inflammatory responsiveness while sympathetic activity is associated with increased as well as decreased inflammation^{5,6}. In laboratory rodents, the release of pro inflammatory cytokines like tumor necrosis factor alpha (TNF α) and interleukin 1 beta (IL-1 β) is inhibited upon electrical stimulation of the vagus nerve (VS)^{7,8}. This effect is measurable in blood for approximately 72 hours in rats⁹. Simultaneously, vagotomy enhances the release of these inflammatory cytokines^{7,9,10} and therefore promotes inflammatory injury.

These findings led to the definition of “the Cholinergic Anti-Inflammatory Pathway” (CAIP)¹¹. The CAIP explains how an inflammatory trigger like a pathogen or trauma initiates immune cells to release cytokines like TNF α , IL-1 β , IL-6 and TGF- β , leading to an inflammatory response. The afferent vagal terminals sense these pro-inflammatory cytokines whereupon the signal is processed in specific vagal nuclei in the brain stem like the dorsal motor nucleus and the nucleus of the solitary tract. As a response, an inhibitory signal is then returned to the body via vagal or sympathetic efferent fibers. The release of acetylcholine blocks the $\alpha 7$ -subunits of nicotinic acetylcholine receptors, thus inhibiting further release of pro-inflammatory cytokines¹²⁻¹⁴. Although literature shows abundant proof for systemically measurable effects like a decrease in inflammatory responsiveness in the blood, there is also evidence for local inhibition of inflammatory activity by autonomic innervation, relaying the anti-inflammatory stimulus to neurotransmitter producing immune cells^{6,15}. As a consequence, the CAIP can protect the threatened body from serious tissue damage and reduce mortality.

Implantable electrical vagus stimulating devices have been used in human patients for approximately 15 years,^{16,17} to treat epilepsy, heart failure¹⁸ and experimentally patients with rheumatoid arthritis¹⁹ or inflammatory bowel disease²⁰.

In human patients, the electrode is connected to the left vagus nerve to avoid cardiac complications since the right vagus nerve mainly supplies the sinus (atrial) node. The left vagus nerve is thought to innervate the atrioventricular node²¹ although extensive research showed no differences in cardiac response between left or right stimulation of the vagus nerve^{22,23}. Minor adverse side effects like hoarseness, tinnitus, nausea and vertigo have been reported during VS but none that persist after stimulation has stopped^{24,25}.

Research is required to understand the deeper mechanisms and the potential clinical value of VS. Patients suffering from chronic or acute inflammatory diseases, such as atherosclerosis

and post infarct remodelling of the heart, may benefit from new insights in the neuro-immune axis. Atherosclerosis for example, might be sensitive to attenuation by vagal activity since the before mentioned inflammatory cytokines are also involved in this disease²⁶ but people suffering from other inflammatory ailments may equally benefit from vagus stimulation. Better understanding of these processes may lead to a more precise pharmacological or behavioral therapeutic approach.

The most frequently used animal model in inflammatory disease research is the mouse. Many genetically modified mouse models have been developed that mimic chronic inflammatory diseases. Many studies are published on attenuating effects of single VS sessions on inflammatory processes but research into the effects of repeated or long-term VS is, so far, not possible in these small animal models.

Therefore, the development of a small implantable vagus-stimulating device would be of great importance for the promotion of future VS research in the mouse.

We report the first experience with an in house developed implantable vagus nerve stimulator for mice that allows acute and short term repetitive activation of the vagus nerve.

METHODS

Animal handling

Animal procedures were all performed in accordance with the Guide for the Care and Use of Laboratory Animals, with prior approval by the Animal Ethical Experimentation Committee, Utrecht University, the Netherlands²⁷. A number of 16 mice was used for the inflammatory responsiveness control, 3 mice were implanted with dummy stimulators to examine the acceptance by the body and 11 mice were used for experimental implantations, in total 30 mice for this study. All animals were male C57Bl/6j mice of ca. 12 weeks of age and derived from an official supplier (Charles River Labs, 's Hertogenbosch, the Netherlands). Surgical procedures in detail are described later in this article.

Euthanasia

At the end of each individual experiment 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 for further examination. When, during the time span of the experiment, animals would have reached a humane endpoint as described by the Guide for the Care and Use of Laboratory Animals²⁷, they were euthanized similarly.

Requirements

A useful implantable vagus stimulator has to meet some basic required properties. It has to function properly and has to cause only a minimum of inconvenience. That means that it has to have appropriate dimensions and should consist of materials that are well accepted

by the animal body and can easily be sterilized. Thereby, it has to elicit no more than the desired effects and have a long term working capacity.

Technical description

The body of the stimulator contains all electronic parts and was constructed in the technical department of our institute (MTKF, University Medical Center, Utrecht, NL). It was embedded in a two-component acrylate resin cast (Poly-pox THV 500 with Poly-pox 255, Polyservice, the Netherlands). This material proved to provide the required protection and insulation of all electronic parts, including the battery. It is also well accepted by the mammal body. Embedding was executed in two steps in a vacuum stove to remove the surplus of dissolved gases from the viscous substance. The leads were welded onto the print board before the first layer of resin cast was applied. After this first resin layer had cured, the leads were placed over the stimulator body to the opposite side and the device was moved into a mould. A round stainless steel plate Ø10mm was connected to the negative lead and placed on the side of the infrared (IR) sensor, the “skin-side” of the stimulator body. This plate functions as the negative electrode. Then, the second layer of compound for the full cast was applied. In this way bodily fluids cannot follow its way along the leads to the electronic parts. After 24 hours, when the resin had fully cured, the device was trimmed off to get rounded edges and a smooth surface. Finally, a very thin layer of medical grade silicone compound was applied to cover the cast (Elastosil a07 RTV-2, Wacker Chemie, Munchen, Germany). The latter was done to promote a rapid encapsulation of the device in the subcutaneous tissue. Final measures of the stimulator body are 23x13x3.5mm with a total weight of 3.3±0,1 grams (Figure 1).

Figure 1. Dimensions of stimulator body

Figure 2. A: Stimulator body with lead, B: carotid cuff and C: electrode

The monopolar positive lead consists of Teflon coated stainless steel, braided from 7 strands of each 7 wires. This lead is 50mm long, it has a diameter of 0,15mm and ends up in a coiled PtIr-electrode with 5 windings and an inner diameter of 0.15mm (with thanks to Medtronic, Bakken Research Center, Maastricht, the Netherlands) for connection to the vagus nerve (Figure 2). With a length of 50mm the lead is long enough not to be pulled tight when the animal moves. Compared to a single wire, a braided lead is flexible and more resistant to metal fatigue such that a mouse, frequently turning its head and bending its neck in all directions, will not easily break the wire. The coiled electrode allows a continuous contact to the nerve which is not rigid or fixed because the nerve is able to freely move through the coil when the mouse is moving. The electrode itself is fixed and isolated in a silicon flap attached to a 3mm silicon cuff, $\varnothing$ 0.8x1.4mm, that is positioned around the right carotid artery. (Figure 3)

The choice for the materials did depend on strength, conduction and the possibility for easy insulation to prevent short-circuiting. The outer materials had to be bio-acceptable and inert to bodily fluids. At the same time, materials had to be easy to sterilize.

Electronic qualities

A button cell battery (type CR1220-1FCE) supplying the necessary energy was chosen with the aim to achieve a functioning period of more than 2 months. A small chip makes the stimulator programmable for power, frequency and for shape and width of the pulse wave. An IR sensor is built in to percutaneously switch the device on or off by the use of an IR-flashlight. At start-up, the stimulator starts the programmed stimulation session. Between the stimulation sessions, the stimulator switches to a “deep sleep mode”, using minimal energy for just a clock function and thus enhancing the battery’s longevity.

Figure 3. Silicon Cuff (1) around carotid artery (CA). A: open with vagus nerve (VN) in coil electrode (4) on the silicon flap (2). B: Flap closed with silk 2-0 ligatures (5). (3) lead approaching from caudal direction.

Electronical settings

VS settings were defined according to experiences in our laboratory and information in recent literature^{9,28}. Parameters as power and frequency for provable effective VS at minimal energy consumption were set at 1mA, 1k Ω and 10Hz for 30 seconds. A square pulse with a width of 0.5msec and a repolarization phase was found to be most appropriate (Figure 4). The repolarization phase prevents accumulation of electrical charge and therefore unnecessary tissue damage.

The VS effect of inflammatory inhibition is found to slowly drain away to meet baseline values in approximately 72 hours⁹. Therefore a repetition time of 48 hours was accepted as sufficient to keep the animal in a permanent state of reduced inflammatory responsiveness.

The anatomy of the vagus nerve can be differentiated in A, B and C fibers. B-fibers that conduct the pulses causing a heart rate (HR) decrease require a stronger current than A-fibers to be recruited²⁹. C fibers are mainly afferent which means that a visual HR decrease indicates a nearly complete stimulation of the efferent vagus nerve, including the part that affects the inflammatory responsiveness. As a proof of proper stimulation a minimal decrease in HR of 25% was accepted (Figure 5).

Figure 4. Square pulse of 1mA, 1k Ω and a pulse width of 0.5msec. A: 10 pulses/sec. B: enlarged pulse with clearly visible repolarization phase (rp).

Physical acceptability test

Dummy stimulators (100% Teflon, silicon coated) with the same size and weight as the functional ones and equal in outer material, were implanted in three mice to study the acceptance by the body and the level of inconvenience for the animal. Also locations for implantation on the animals' back or in the flanks were evaluated to determine the least inconvenient position for the animal.

Inflammatory responsiveness control

Tumor necrosis factor alpha (TNF α) is one of the first pro-inflammatory cytokines being released upon an inflammatory trigger and therefore blood levels of this cytokine are a surrogate measure for inflammatory responsiveness upon a stimulus.

As a control for effective vagal stimulation, sixteen male C57Bl/6j mice of 27.3 ± 1.2 g were anesthetized with FDD-mix. Eight animals received a 30 seconds stimulation session performed with an external pulse generator but with an identical square pulse train as from the implantable stimulators (30s, 1mA, 1k Ω , 10Hz, 0.5msec pulse width). Another number of eight mice underwent a sham procedure in which no electrical stimulation took place. In this sham procedure the sensitive vagus nerve was exposed but not touched to prevent any unwanted vagal response. Fifteen minutes after VS but still anesthetized, the abdomen of the animals was opened and whole blood samples were drawn through the diaphragm from the cardiac apex and kept on ice in heparinized tubes. Within <1 hour, 100 μ l of each of the samples was stimulated at 37 $^{\circ}$ C with 100 μ l lipopolysaccharide (LPS) solution (100 ng/ml LPS, Sigma-Aldrich: cat.nr. L2880) to stimulate the immune response. After 6 hours of incubation, levels of TNF α were determined with ELISA (mouse TNF-alpha Quantikine Kit, R&D systems, cat.nr: MTA00B) ^{30,31}.

Figure 5. Heart Rate decrease of 26% during vagus nerve stimulation (1mA, 1kΩ and 10Hz).

Sterilization

To sterilize the stimulators after production, they were kept for two hours in a 1% Virkon-S solution (Dupont Company, Netherlands) and were then flushed with sterile saline. Subsequently they were dried and stored in a dry and sterile plastic sealable bag. Shortly before implantation, the devices were put in 70% alcohol for ca. 60 minutes where after they were flushed and kept in sterile saline for at least one hour before implantation.

Surgical implantation

Eleven Male C57Bl/6j mice of 26.0 ± 1.4 grams were anesthetized with FDD-mix. A bit of ointment was applied to protect the eyes against dehydration (Terra Cortril, Pfizer, Capelle a/d IJssel, the Netherlands) and body temperature was monitored and kept at 37 ± 0.5 °C. All four limbs were attached to the leads of an ECG recorder. Right cervical area and 4 sq. cm in the right flank were depilated and cleaned with 70% ethanol.

The right cervical skin was opened laterally, parallel to the trachea for approximately 12mm. Then the vagus nerve was exposed and gently prepared free from surrounding tissue over a length of approximately 6mm without squeezing or stretching the vagus nerve. A small pre-fabricated silicon cuff with flap (Figure 3) was placed around the carotid artery at the level of the exposed vagus nerve in a way that the vagus nerve could be positioned on the silicon flap.

For the stimulator body, an incision of approximately 18mm was made in the skin of the right flank. With blunt scissors, a subcutaneous pocket was created and the sterile stimulator was gently placed into this pocket with the battery and IR-sensor positioned on the skin side. The lead with electrode was tunneled subcutaneously, underneath the pectoralis major and the sternocleidomastoid muscle to arrive in a position in the neck area, parallel and adjacent to the vagus nerve. The cervical end of the lead was curved in a Z-shape to get the coil-electrode in line with the nerve.

With assistance of a small blunt steel hook, the vagus nerve was guided in the coil. When positioned, the coil which still can be moved freely over the nerve was placed on the flap close to the carotid cuff. Then, the flap was closed with the attached braided silk 2.0 sutures, thus electrically isolating the coil from the surrounding tissue. To keep the coil in position, the lead was fixed with two ligatures to the carotid cuff and ca. 1 cm distal into connective tissue. Then the cervical skin was loosely closed and the function of the stimulator was checked.

With an IR flashlight, the stimulator was activated and a first stimulation train of 30 seconds was executed to check the functionality of the device. If the ECG recording showed the desired minimal decrease of 25% in HR, the stimulator function was considered effective and both neck and flank were closed with vicryl 4.0 sutures (Vicryl plus, V392ZH, Ethicon, Johnson and Johnson inc.). Subsequently the mouse was disconnected from the ECG and anesthesia. After a dose 0,1mg/kg of pain killer (Temgesic®, Reckitt Benckiser, UK) the animal was placed back in its cage and kept on a warm pad to recover. A second dose of pain killer was administered the next morning during animal inspection.

Regular functioning inspections

After 24 hours of recovery the animals were anesthetized with 4% (induction) and 2% (maintenance) isoflurane in air:O₂ (2:1) via a nose cuff. Eye ointment (Terra Cortril) was applied to prevent ocular dehydration and body temperature was kept at 37 °C. All four limbs were attached to the leads of an ECG recorder.

The stimulator was stopped to reset and switched on again with an IR flashlight to start the 30 seconds stimulation train and again bradycardia was used as proof for an effective stimulation. Subsequently, the anesthesia was stopped to let the animal recover. This ca. 5 minutes taking control procedure was repeated on day 3, day 7 and twice weekly thereafter.

When a stimulating device did not show a proper stimulation anymore, i.e. a clear bradycardia, the animal was euthanized and the stimulator was excised. Blood was sampled and the area of implantation was studied for position of the electrode, tissue adhesion and inflammatory reactivity.

RESULTS

Inflammatory responsiveness control

Sixteen male C57Bl/6j mice were weighed and randomly split up in a sham group (mean BW 27.5 ± 1.4 g) and a group that received 30 seconds of VS (mean BW 27.2 ± 1.2 g). All operations proceeded according to plan and all but one VS-animal showed the required minimum of 25% decrease in HR. Blood was drawn 15 minutes after VS and stimulated with LPS. These samples were measured with ELISA for TNF α as a score for inflammatory responsiveness.

An average decrease of 45.5% (ns) of TNF α levels was shown (Figure 6) in the blood plasma of sham operated compared to vagally stimulated mice, resp. 157.0 ± 90.3 vs. 85.6 ± 34.9 pg/ml ($p=0.130$).

Figure 6. levels of TNF α in LPS stimulated blood in Sham(N=8) vs VS (N=8) treated mice. $p=0.130$ (Mann-Whitney)

Location and biological acceptability

Silicon coated Teflon dummy stimulators were implanted to define the biological acceptance by the body and to find the best location with the least inconvenience for the mouse. The first chosen location of implantation of the stimulator just behind the right scapula was not convenient. The stimulator tended to slowly move downwards, thereby putting tension on the lead. A more appropriate and stable position was tried and found in the right flank (Figure 7).

Dummy implants were generally encapsulated in connective tissue in ca. five days and stayed nicely in position for over three months without any visible inconvenience for the animal.

The same results were found when fully functioning stimulators were implanted. The measures of $23 \times 13 \times 3.5$ mm did not hamper the animals in their normal behavior. The rounded edges prevented irritation to the covering skin and the underlying tissue. Long-term implantations did not result in any obvious adverse effects. Post mortem verification showed a reliable encapsulation and stable position of the stimulator in the animal.

The electrodes were also tightly encapsulated in connective tissue in a way that it was difficult to post mortem examine the condition of the vagus nerve in the electrode coil.

Figure 7. location of stimulator body and position of the lead

Sterilization procedure

The sterilization procedure with 1% Virkon S and alcohol 70% works satisfactory. Respecting this protocol prevents infections and subsequent rejection responses while enabling rapid encapsulation in the surrounding subcutaneous tissue.

Maximum functional time

The functional life of the device depends on the used battery type and the stimulation program. The battery type (CR1220-1FCE) is chosen for the optimal combination of minimal size, low weight and maximal power. These batteries lose only negligible amounts of energy in time and the device is equipped with a system that switches into a phase of hibernation between the pulsing periods, a “deep sleep mode”. With the used stimulation protocol, the maximum functional time of the stimulator will be six or more months which is far beyond the desired duration time.

Implantation

The implantation procedure as previously described was satisfactory and fast. Drawbacks were found in a deviating anatomy of the vasculature in the surgical area when positioning the electrode. Small branches from the carotid artery did sometimes impede an easy access to the carotid artery itself and to the location where the electrode has to be connected to the vagus nerve. In two mice a serious bleeding of these collateral branches of the carotid artery occurred. In another animal a rupture of the jugular vein caused a strong bleeding when tunnelling the electrode from the flank to the cervical area. These bleedings were not easy to stop and although they were not life threatening, they caused a longer duration of the implantation procedure and weakening of the animals' condition. Therefore, those three mice were excluded.

Appropriate functioning of the stimulator

From initially 11 operated mice with three animals excluded, eight implantation procedures as described in the methods section were successfully executed. Effective stimulation by >25% heart rate reduction was verified before the anaesthesia was ended. The first three days after implantation, the animals were twice daily inspected and body weights were registered. Except a minor loss of 0.4 g (=1.5%) in body weight in the first two days, no serious health conditions were observed. During stimulation, the animals did not behave differently compared with pre-implantation.

In the following period, proper functioning of the stimulator was checked in isoflurane anesthetized animals. Again, ECG was recorded and bradycardia was used as evidence for an effective vagal stimulation. At day 1 a greater than 25% reduction in HR was found in two of the eight animals. Four others showed a visible decrease but less than 25% and stimulation in two animals did not generate any reduction in HR anymore. The one experiment with the longest operating time generated >25% HR reduction at day 29 after implantation but failed to show any effect two days later (table 1).

Table 1. Three excluded animals and 8 implantations on day 0.

HR decrease	day:	0	1	3	7	12	20	29	31
	<25%	8	2	2	2	1	1	1	0
	>25%	0	4	2	0	1	0	0	0
	nd	0	2	4	6	6	7	7	8
	excluded	3							

Functioning of implanted vagus stimulators in days. HR decrease larger than 25%, smaller than 25% and not detectable (nd). Maximum period of effective stimulation is 29 days.

Examination of the stimulator after termination of the animals showed that all stimulators were still working properly but the contact of the electrode to the cervical vagus nerve bundle was lost. In 2 experiments the electrode had broken from the lead. In the others, the electrode was still in the right place, kept in position by newly formed connective tissue.

Stimulating the nerve caudally from the electrode with an external stimulation device evoked a HR response. Stimulating the cranial part however, did not show any effect indicating that the vagus nerve in the electrode coil did not conduct the stimulator pulses anymore.

Adverse effects

During the period between implantation of the devices and termination, the animals were checked for health and general wellbeing. After an initial minor loss of body weight animals were again back at start weight at day two. Within a week after surgery the mice ate, played and fought as they did before the implantation. The size and position of the device did not impede the animal in its normal behavior, nor did they cause bald spots or wounds in the fur.

DISCUSSION

The autonomic nervous system appears to be the great regulator of immune function. In this respect the parasympathetic branch plays an important role as a suppressing force in the inflammatory response. Since the introduction of the Cholinergic Anti-Inflammatory Pathway¹³ biomedical researchers are increasingly interested to unravel the complex interplay between the immune system and the autonomic nervous system. A better understanding of these principles may help in the development of therapies or ways to prevent inflammatory disease. Many studies have been performed in which laboratory animals were vagally stimulated to investigate the attenuating effects of a single vagus stimulation session on inflammatory processes³²⁻³⁶. Effects of stress free vagus nerve stimulation over an extended period have so far not been examined.

The developed stimulator provides an effective means for short term repetitive vagus stimulation in a rodent model.

The reduced size, rounded shape and small weight of 3.3g of the developed device do not hamper the animal in its normal behavior and do not lead to any obvious stress to the animal. Furthermore, the used materials do not cause adverse effects like irritation or rejection by the bodily system.

The construction of the electrode proved to be a technical challenge. The vulnerability of the murine vagus nerve of only 80 μm in diameter requires an electrode that does not put any pressure on the nerve. Simultaneously, it has to provide a good electrical contact to the nerve and a thorough isolation from the surrounding tissues. Since the cervical area is populated with significant numbers of various nervous tissue and musculature, care should be taken that stimulation only effects the vagus nerve and no other tissues. With the choice for a unipolar electrode we were able to reduce the size and rigidity but at the same time, an adequate isolation became more important because of a greater chance for dispersion of the current over surrounding tissue. With the coil-shaped electrode we think we have found a suitable design in which the nerve is able to freely glide through when the animal

moves. Thereby, we form the lead close to the electrode in a Z-shape to bring the lead in line with the muscular tissue and the electrode in an optimal position parallel to the vagus nerve by which unnecessary tension on the lead by movement of the animal is prevented. Still, some improvement of the electrode is desired to prolong the work span of the device. The connection of the electrode to the lead is of mechanical nature and in future development could this be dealt with by trying alternative solders or new fixing methods. The position of the electrodes from stimulators that stopped operating was checked in terminated animals. The electrodes showed often an abundant encapsulation. Despite our careful attention there may still have been enough mechanical stress on the nerve to cause tiny movements in the wrapped up coil which stimulates outgrowth of connective tissue around the electrode. This could have been exacerbated by the enveloping flap which is made from silicon, a material that stimulates encapsulation.

This outgrowth may have caused consequential pressure on the nerve and that in turn could have forced the nerve to cease functioning.

To check the situation, the cranial end of the vagus nerve was stimulated with an external pulse generator with the previously used stimulation settings but this did not cause any effect on the heart rate. Stimulating the caudal vagus nerve however, did cause the expected HR decrease, indicating that the vagus nerve in the electrode was interrupted.

Further examination of the electrode showed that connective tissue had also grown into the electrode coil in a way that it was too difficult to exactly determine the presence or position of the vagus nerve in the coil. This ingrowth of connective tissue could have caused the pressure on the nerve that led to the interruption. In future experiments this problem may be solved by the use of implantable silicone compound to isolate the coil from the environment and therefore from collagen ingrowth.

Generally, a complete implantation with operational check takes ca. 60 minutes. Unexpected complications like different vascular anatomy elicit extra risk for bleeding and therefore require extra caution and time.

Immediately after implantation while the animals are still anesthetized, the stimulators are checked for proper functioning by switching the device on which starts the first programmed stimulation session. In former studies the hearts of some animals stopped contracting during stimulation to start again immediately after 30 seconds when the stimulation stopped (Figure 5). In conscious animals however, the heart normally continued working and no behavioral changes in the animals were observed during vagal stimulation.

A test stimulator was programmed to stimulate 30 seconds every 12 hours with the same settings as used in the implanted devices (10Hz, 1mA, 1k Ω , pulse width of 0.5msec). Submerged in saline, this test stimulator has been functioning for more than eighteen months. Changing the program to continuous stimulation with the same stimulation settings will reduce the working capacity to approximately one month.

In the existing version of the stimulator, the stimulation program is entered before the cast is applied and the program cannot be amended once the stimulator is casted. With an IR

flashlight the device can be switched on or off and if desired, start a new stimulation session. In next versions however, it will also be possible to adjust the pulse program and electrical settings in an implanted stimulator from a PC by Bluetooth connection.

The implanted devices yielded effective vagus stimulation for an average of 10 days with a maximum of 29 days. That means that the intended goal of a 2 months working period is at the moment of completion of this study not yet achieved. Following experiments will be aimed at extension of the work span of the device.

The chosen pulse program yields a non-significant ($p=0.130$) decrease from 157.0 to 85.6pg/ml TNF α in LPS challenged blood (Figure 6). That means a 45.5% reduction of the inflammatory response which is comparable to findings in literature^{7,12}. Changing the electric current or the frequency can increase or decrease the cholinergic effect²⁸ and therefore the inflammatory response. In this study the used stimulation settings were very convenient. As part of an earlier study in our laboratory, 14 mice underwent VS and were compared with 9 sham-operated animals. (Figure 8). The result was a significant decrease from 121.6 to 45.2pg TNF α per ml blood, i.e. a reduction of 62.9% ($p=0.013$). Looking more closely at the Sham TNF α values in the figures 6 and 8 a division can be detected between one cluster that is equal to the VS values and another that remains at higher levels (red encircled). Reconsidering the surgical process, small trauma was inflicted when the cervical area was opened to expose the carotid artery and the vagus nerve, in particular where small arterial branches had to be moved to access the site of (sham-) implantation. Literature shows that even minor vascular injury is able to cause attenuating effects on inflammatory responsiveness^{37,38}. Tissue injury following surgery and trauma already leads to a depressed

Figure 8. levels of TNF α in LPS stimulated blood in current study A, (Sham: N=8, VS N=8) and in earlier study B, (Sham: N=9, VS N=14). While VS treated animals show all decreased values, the sham-operated appear to be divided in animals that do and those that do not respond to the sham procedure. High and low responding subjects are clustered.

cell mediated immunity³⁹, an effect that is similar to VS effects. Moreover, in this sham-procedure the vagus nerve could have easily been touched or moved with evoked unwanted parasympathetic triggers as a result. The found division could even be more pronounced when among mice, there are low- and high responders to VS. In conclusion, in surgical operations like these, care should be taken to prevent undesired manipulations to the vagus nerve and surrounding tissue because the threshold for a parasympathetic trigger is easily crossed.

CONCLUSIONS

With the device described in this study, short term repetitive vagus nerve stimulation without stress for the animal is feasible. The availability of the device allows research of autonomic influences on inflammatory processes over a period up to three days in the free moving animal. Further experimentation with technical improvements will bring VS up to a month within reach. Extra care should be taken to avoid any unnecessary manipulation to the vagus nerve and trauma to the surrounding area. High as well as low responders should be taken into account.

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CHAPTER 8

General discussion

INTRODUCTION

Some two decades ago, the first scientific reports on anti-inflammatory effects of the parasympathetic nervous system were published ¹⁻⁴. Later, these effects were described as the cholinergic anti-inflammatory pathway (CAP) ⁵ and nowadays, the relation between the autonomic nervous system and the immune system is supported by a vast amount of scientific evidence and has become generally accepted. Despite the popularity of the topic and the increasing list of publications the implications for biomedical science are not fully known. The chapters in this thesis describe studies that approach the topic from two distinct sides. In mouse studies the effect of vagus nerve stimulation (VS) on tissue damage and development of function loss after myocardial infarction (MI) or ischemia-reperfusion (IR) was examined. In observational studies in human aneurysm and atherosclerotic specimen studies we tried to shed a light on the relation of innervation and pathologic processes in the diseased arterial wall. With the development of the implantable vagus nerve stimulator we think we can give a stimulating impulse to research on this topic.

ANTI-INFLAMMATORY EFFECT OF VASCULAR INJURY

Blood sampling and arterial injury lead to an identical parasympathetic effect and cause a serious decrease of inflammatory responsiveness (**Chapter 2**). Anatomically, caudal branches of the vagus nerve do not innervate the femoral artery. Therefore anti-inflammatory effects of femoral injury must function by other than vagal routes. Irrespective of the used anesthetic and the time under anesthesia arterial injury decreases inflammatory responsiveness with 64%. The arterial wall is quite sensitive. Figure 1A in Chapter 2 shows that even gentle manipulation to expose the femoral artery (Sham group) generates some inhibitory effect on inflammatory cytokine production.

Blood sample volumes of 350µl or 750µl generated similar reduction of inflammatory responsiveness, indicating that the inhibiting effect of blood sampling on inflammation is caused by the sample procedure rather than by the sample volume (Chapter 2, Figure 2 and 3).

This blood sampling procedure consists of smoothly cutting a small piece of less than a millimeter from the animal's tail by which tiny arteries and veins are cut. This may be enough arterial injury to trigger inhibition of the inflammatory response. In follow-up studies we can include an extra group in which inflammatory responsiveness will be examined after a tail tip cut without blood sampling.

However, the decrease is evident and we would recommend to limit experimental blood sampling to only one time per experiment, preferably during termination. Furthermore, it is important to take due account of the effects of blood loss and vascular injury in surgical and experimental procedures. They may be of influence on experimental outcome specifically when inflammatory parameters are main determinants of an output parameter. In addition, in clinical studies the health and wellbeing of patients after intervention may be affected, a complication that has been discussed previously⁶.

A factor of importance is the choice of the artery involved since location and innervation of the artery, and composition of its wall may be of influence ^{7,8}. Ascending or descending aorta, femoral or cerebral arteries do all differ in size, wall thickness, elasticity, tissue composition and type and density of innervation. Therefore different effects from vascular injury on inflammatory parameters can be expected. It is not clear if the degree of arterial damage has to reach a threshold or is in a continuous association with the change in inflammatory responsiveness. Regarding the techniques that were compared in our study, crush and intimal scratching, results were similar and the type of injury did not appear to be a critical determinant. Of course, in the surgical approach of the vessel, harm to other tissues and bleeding could create a bias and should be avoided.

As we know from other conditions, organismal systems generally strive for homeostasis and it is therefore expected that an inflammatory imbalance will soon be abolished. As the effect of VS on inflammatory parameters is detectable for almost 72 hours ⁹, so may the immune effect of vascular injury be of temporary nature. This has to be a topic of future research.

MYOCARDIAL INFARCTION AND ISCHEMIA-REPERFUSION WITH OR WITHOUT VAGUS NERVE STIMULATION

In the studies on VS in the mouse the surgical methods were executed by experienced technicians and the very small number of unreliable dates were excluded. Nevertheless, partly due to the high standard deviation, the results did not show clear evidence for reduction of final cardiac damage and function loss at the moment of termination 48 hours after experimental MI (**Chapter 3**) or cardiac IR (**Chapter 4**).

Ejection fraction in both studies, MI±VS and IR±VS, did significantly decrease after MI and after IR. The groups that also received VS showed less decrease which may be an indication of the protective effect of VS. However, this difference was not significant ($p=0.075$) (Chapter 3, fig3+4 and Chapter 4, fig2A+B).

In the analysis of the cardiac echoes it was often difficult to outline the 48h scans. Although some publications may show clear results with little variation two days after MI induction, our experiences are less positive. In the obtained scans the outlines of the left ventricle were often blurry because of disturbing movements of the heart. To execute an LAD-occlusion the pericardial cavity has to be opened and this impairs the stability of the beating heart. After MI-induction the heart restores its stable position after some days but in our study 48 hours proved to be too short.

TNF α levels were determined in LPS-stimulated blood at $t=48h$ as a measure for inflammatory responsiveness. The animals that received VS prior to MI did not show different levels TNF α than those with only MI (Chapter 3, fig6). However, the vagus nerve is very sensitive to tactile

stimulation. The MI group was also subject to a Sham-VS procedure which means an incision in the neck and exposure of the vagus nerve where after the neck was closed again. Although effort was made to cause as little as possible movement to the vagus nerve, it could have been unintentionally manipulated causing manual vagal stimulation. The incision itself and rupture of fasciae may also have initiated some inflammatory inhibition. The possibility for this undesired blunting of the inflammatory responsiveness also applies for the (Sham VS +) IR group (Chapter 4, fig3). Studies on side effects of physical trauma and a threshold experiment in which per group the vagus nerve is gradually manipulated stronger could provide a better understanding of the sensitivity of these tissues and possible impact on immune parameters.

As literature shows, the VS effect on TNF α release fades out in approximately 3 days⁹. The longer the post intervention period, the less demonstrable the effect will be and this may also be the case with the mentioned side effects.

The infarct size (IS) in the MI $\pm$ VS study was smaller (not significant) in the VS+MI than in the MI group.

IS was also a little smaller in the IR+VS compared to the IR alone while the area at risk (AAR) was somewhat bigger. Both were not significantly different. Nevertheless, the ratio IS/AAR is seriously smaller for the IR+VS group ($p=0.003$) which does support the protective effect of VS in cardiac IR in mice.

Neutrophils are attracted to the infarcted site to clear it from necrotic cells and cellular debris. Numbers of neutrophils in the mouse were reported to reach a peak at day 2 to three after MI¹⁰⁻¹². As expected more neutrophils were found in the infarcted area than in the remote area of the MI group but this was not observed in the MI+VS group. In the IR $\pm$ VS study more neutrophils were found in the infarcted than in the remote area as well but most neutrophils were observed in the border areas. While in the MI $\pm$ VS study many neutrophils were also counted in the remote areas, in the IR $\pm$ VS study the remote areas were almost complete deserted (Chapter 3, Figure 8, Chapter 4, Figure 4).

CD3⁺ lymphocytes were found all through the heart, in the remote and border areas as well as in the infarcted areas, in both mouse studies. The same was observed for numbers of macrophages (Chapter 3, Figure 10). No significant differences were detected between the MI or IR groups with or without VS. The chosen termination time at 48 hours may have been too early to find activity of macrophages or lymphocytes yet. At the time of termination pro-inflammatory macrophage (M1) numbers are expected to be increasing. It would be interesting to study a possible change from M1 to the anti-inflammatory type M2 cells to find out whether or not VS has influence on the moment of transition from the inflammatory to the reparative phase. For a future study a longer recovery period of at least 7 days would therefore be recommended. Those transitions could also be influenced by VS. The ANS may be the driving force in the first response upon small injury but also initiate anti-inflammatory and more reparative activity in a later stage.

Staining for VEGF was performed to possibly find an indication for early neovascularization, as an effect of VS. Without VS, VEGF is expected, just like CD3⁺ lymphocytes, to act in larger amounts in the reparative phase that starts at ca. 7 days after MI. We observed random presence of VEGF-activity indeed but no significant group differences were found.

DEVELOPMENT OF A VAGUS NERVE STIMULATOR FOR LAB ANIMALS

With the development of an implantable device that enables intermittent stressless vagus stimulation we can now study inflammatory related processes and diseases in small laboratory animals over an extended period. Long term studies on VS effects on the development of auto-immune or other inflammatory disease like atherosclerosis in genetically manipulated mice are within reach.

In the mouse, the neck area is the best location to approach the vagus nerve since more distal parts and its branches are too small and fragile for a proper positioning of stimulation electrodes. The vagus nerve is positioned in the carotid sheath and follows the carotid artery in the neck between strong muscles. The constant motion in the neck makes this location also sensitive for break. It has been a long search and many attempts were necessary before the actual coil-electrode was found to be satisfactory. The nerve is not fixed in the coil but can freely move through the coil on the cranial-caudal axis without losing contact. This is a great advance compared to previous fixed designs which caused the nerve to break within 24 hours. Still we think there are possibilities to improve the electrode. By using a smaller coil and thinner and more flexible leads the work span may improve.

In the animals that were administered a sham-VS procedure we discovered a difference in the degree of responsiveness (**Chapter 7**). In this procedure the neck area of these animals was opened and the carotid artery and vagus nerve were exposed. Here again, in the surgical procedure some minor arterial injury may have had an attenuating effect on inflammatory responsiveness. Some more sensitive animals responded as if they were administered a vagus nerve stimulation (Chapter 7, Figure 8) while others did not show any response. High and low responding to minor manipulations of the vagus nerve can thus provoke great undesired responses. In immunologic research and in surgical procedures anti-inflammatory side effects like these should be taken into account. And moreover, it would be of interest to explore possible similarities between the pathways that lead to the anti-inflammatory effects of both VS and arterial injury.

INNERVATION AND INFLAMMATION IN ATHEROSCLEROSIS

Atherosclerosis, is an inflammatory disease and is therefore sensitive to the activity and interaction of the sympathetic and parasympathetic nervous systems. Sympathetic nerve fibers appear to sprout from adjacent bundles and grow towards inflammatory activity^{13,14} and this is triggered by an up-regulation of neurotrophic factors like nerve growth factor (NGF) and transforming growth factor beta (TGF β). Moreover, some inflammatory cells are able to release tyrosine hydroxylase or acetylcholine and so support sympathetic or parasympathetic activity^{15,16}. In **Chapter 5** we discuss a study on a possible association between innervation and inflammatory activity related to atherosclerotic stages in human abdominal aortas.

Increasing numbers of T-cells indicate stronger inflammatory activity in further developing stages of atherosclerosis culminating in a peak in the stage of vulnerable atherosclerotic lesions. Total and sympathetic innervation also increase in further stages but peak in the progressive lesions where after they decrease in the vulnerable and stabilized lesions. Analysis shows a positive correlation between innervation and inflammation in atherosclerotic developmental stages. Total and sympathetic innervation show lower values in vulnerable lesions while parasympathetic fibers show a little increase. Simultaneously, in the total B-cells and in all T-lymphocyte groups numbers still increase in this stage (Chapter 5, Figure 3 and 4). That suggests that a decrease in sympathetic innervation precedes a reduction of inflammatory activity. In the stage of stabilized lesions innervation and T-cells all strongly decrease.

The parasympathetic values show decreasing numbers of nerve fibers until the progressive lesions group followed by a small increase. This is a reversed pattern compared to the total and sympathetic fibers demonstrating the balancing character of the sympathetic and parasympathetic systems. When the autonomic nervous system is out of balance electrical, mechanical or pharmaceutical intervention may be a valuable option. People who are genetically prone to inflammatory diseases like atherosclerosis, for example, may benefit from short daily VS sessions.

TH⁺ and ChAT⁺ staining yields pictures in which not all stained cells are neural since TH and ChAT are also released by other cells like lymphocytes and macrophages¹⁷⁻²¹. Automatized quantification of innervation by these parameters, executed by measures of intensity, is therefore not possible. The manual quantification of bundles, scattered fibers and vasa vasorum associated fibers that we performed is a validated and reliable method to obtain the actual presence of nerve fibers. Still these values may be an overestimation because it is sometimes difficult to distinguish between cellular dots and solitary fibers. Non-neural cells that stain for TH and ChAT have sympathetic and parasympathetic features respectively. They are able to stimulate NA and ACh synthesis and thereby mimic neural pathways that influence inflammatory activation. When mistakenly scored as nerve fibers, they still reflect sympathetic and parasympathetic activity.

Sympathetic nerve sprouting is induced by specific neurotrophic factors like NGF which is released by smooth muscle cells and inflammatory cells^{22,23}. The amount of this insulin-like neuropeptide is increased in inflamed tissues and stimulates growth of sympathetic and sensory neurons. Because NGF can stimulate the release of TH as well as acetylcholine, it can act pro- as well as anti-inflammatory. Therefore, it may have an important function in the regulation of inflammation in the arterial wall as well as in the response to MI and as such it deserves serious attention in future research regarding inflammation related processes.

INNERVATION OF ANEURYSMS

The study on aneurysms as described in **Chapter 6** quantitatively investigated innervation in aneurysmal carotid and popliteal arteries and compared the results with non-diseased arteries. In the discussion section of the chapter it was already mentioned that the number of included arterial samples was small because of a low availability. The number of patients that undergo surgery is limited. Although aneurysms are in principle continuously developing, they may do so at varying speed. At the moment of excision, they may have been in an inflammatory stronger or lesser active period which could have obscured a clear result from the measured neural markers. Comparison of innervation with the presence of inflammatory parameters like macrophages, lymphocytes and cytokines may provide further insight in this matter and a better understanding of the role of innervation with inflammation in the development of aneurysms.

Aneurysm development is accompanied by loss of elastin, continuous degradation and formation of collagen and remodeling of the arterial wall. When the type I and type III collagens that normally generate stability in the arterial wall get degraded by proteinases they are rapidly replaced by newly formed collagen which is less in quality and therefore enhances the process of arterial wall weakening and aneurysm formation²⁴. In our lab we attempted to distinguish older from more recently produced aneurysmal development by quantification of polarized pictures of Picrosirius Red stained collagen in aneurysms. This might have resulted in a method to determine the phase of development or inflammatory activity of the aneurysms but unfortunately it did not produce useful results. Nevertheless, examination of aneurysm samples on proportions of different collagen types and collagen architecture may lead to the desired information which in relation to neural and inflammatory activity could help in better understanding of aneurysm formation.

FINAL CONCLUSION

Literature shows that the autonomic nervous system has a strong regulatory influence on the immune system. Stimulation of the vagus nerve has an attenuating effect on inflammatory responsiveness. This attenuating effect may serve to prevent the body from excessive inflammatory damage in a protective immune response to a serious threat. Understanding of these anti-inflammatory principles may eventually lead to new therapeutic approaches towards inflammation related conditions.

The studies described in this thesis were performed to elucidate some actual questions on this subject but many questions remain. Whilst the sensitivity of the vagus nerve and the arterial wall, just like the risks of manipulating these tissues are evident, it is not clear how these reflexes work. More mechanistic details of pro- and anti-inflammatory actions of the ANS need to be further investigated. Great advances have been made in the development of VS as a therapy for rheumatoid arthritis²⁵ and inflammatory bowel disease²⁶. For cardiovascular disorders with an inflammatory component like a myocardial ischemic episode, heart failure and arterial wall diseases, the possible benefits of therapeutic VS in human patients are unknown thus far. Future research should particularly aim at obtaining extensive background knowledge on neural-immune interactions and to find ways to restore inflammatory balance. The vagus nerve stimulator which we have developed for small laboratory animals may be of great value for this research.

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ADDENDA

Samenvatting in het Nederlands

Review committee

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List of publications

Curriculum Vitae

SAMENVATTING IN HET NEDERLANDS

Het immuunsysteem

Het immuunsysteem met zijn vele verschillende functies is essentieel voor het behoud van het lichaam. Het vormt de eerste bescherming tegen bedreigingen van buitenaf zoals micro-organismen en toxinen. Daarnaast heeft het een belangrijke functie in het herstel van weefsels na schade veroorzaakt door trauma, (bio-)chemische stoffen of een periode van ischemie. Ook speelt het immuunsysteem een cruciale rol bij het verwijderen van verouderde en beschadigde cellen en van afvalstoffen. Het is daarbij van groot belang dat er accuraat onderscheid gemaakt wordt tussen gezonde en beschadigde cellen. Zowel het abusievelijk afvoeren van gezonde cellen als het niet afvoeren van zieke cellen levert gevaar op voor de gezondheid. Bij aanwezigheid van een deeltje dat niet in het lichaam hoort, reageert het immuunsysteem op proteïnestructuren van de indringer die het herkent als “niet-eigen” (PAMPs) of op stoffen die worden afgescheiden door beschadigde en afstervende cellen (DAMPs). Het start vervolgens een proces om de exogene of defecte cellen af te voeren en herstel van weefsel en functie in gang te zetten.

Ontsteking is een belangrijk instrument van het immuunsysteem en kenmerken van ontstekingsactiviteit zijn o.a. temperatuurverhoging, roodheid en oedeemvorming. Overactieve ontstekingsactiviteit vergroot de kans op ontstekingszieken en een te zwakke ontstekingsrespons geeft “foute” cellen de kans te overleven. Hier is dus een strikte regulatie van groot belang. In de laatste 20 jaar is steeds duidelijker geworden dat het autonome zenuwstelsel een sterk regulerende rol speelt in het onderhouden van deze balans.

Het autonome zenuwstelsel en inflammatie

In het efferente deel van het autonome zenuwstelsel (AZS) zijn twee functionele onderdelen te onderscheiden, het sympathische en het parasympathische zenuwstelsel. Het sympathische zenuwstelsel wordt met name actief in situaties van acuut gevaar waarbij alertheid is geboden. De belangrijkste neurotransmitter in dit systeem is (nor-)adrenaline (NA) dat vooral gemaakt wordt in de bijnieren maar ook door zenuwuiteinden en door bepaalde afweercellen afgescheiden kan worden. Het parasympathische zenuwstelsel is actief in de meer vegetatieve functies als spijsvertering, slaap-waak ritme en voortplanting. De neurotransmitter die hier de belangrijkste taken vervult is acetylcholine (ACh) dat vooral door parasympathische zenuwuiteinden wordt afgescheiden maar ook afkomstig kan zijn van ontstekingscellen. Het sympathische en parasympathische zenuwstelsel worden vaak gezien als functioneel tegengestelde systemen en in een aantal gevallen is dat ook zo. Toch kunnen ze beter beschouwd worden als complementair omdat een gebalanceerde werking van beide delen noodzakelijk is voor een goed functionerend systeem.

Naast de genoemde functies bij alertheid en bij rust heeft het AZS een belangrijke invloed op ontstekingsprocessen waarbij activatie van het parasympathische deel met name een vermindering van inflammatie tot gevolg heeft en de sympathische tak zowel pro- als anti-inflammatoir kan werken. Veel immuun cellen zoals macrofagen, granulocyten en lymfocyten hebben receptoren voor NA of ACh in hun membraan of hebben de mogelijkheid om deze

stoffen te produceren. Andere immuuncellen produceren stoffen die de aanmaak van NA of ACh in gang kunnen zetten. Dat zijn stoffen zoals tyrosine hydroxylase (TH) dat tyrosine omzet naar L-DOPA, een belangrijke stap in de aanmaak van NA, of choline acetyltransferase (ChAT), een enzym dat van belang is voor de aanmaak van ACh.

Zenuwen groeien naar hun doelgebied op geleide van “chemoattractants”, en groeifactoren, vaak tegelijk met vaatgroei. Een van de belangrijkste groeifactoren is nerve growth factor (NGF), een stof die door veel verschillende cellen geproduceerd kan worden, zowel pro- als anti-inflammatoire effecten kan hebben en van belang is bij herstel van beschadigd weefsel.

Activatie van de nervus vagus

De nervus vagus is de tiende craniale zenuw en voorziet het craniale, thoracale en grotendeels het abdominale gebied van parasympatische innervatie. Activatie van deze zenuw heeft een demping van inflammatoire activiteit en daarnaast afname van de hartfrequentie en bloeddruk tot gevolg. In wetenschappelijk onderzoek is aangetoond dat elektrische stimulatie van deze vagale zenuw de ontstekingsactiviteit doet afnemen bij acute dreiging van septische shock. Door deze vagus stimulatie (VS) bindt de vrijgekomen ACh aan de $\alpha 7$ subtype nicotinerge Acetylcholine receptoren ($\alpha 7nAChR$) wat weer leidt tot een inhibitie van inflammatoire responsiviteit. In bloed van vagus gestimuleerde muizen dat behandeld wordt met lipopolysaccharide (LPS) is een duidelijke vermindering te zien in de productie van pro-inflammatoire cytokinen. Dit effect is nog meetbaar tot 72 uur na VS. Deze reflex is uitgewerkt in de “Cholinergic Anti-inflammatoire Pathway” (CAP). VS is sindsdien met succes ingezet bij de behandeling van ontstekingsziekten als reumatoïde artritis en chronische darmziekten. Al langere tijd wordt elektrische VS gebruikt bij mensen die lijden aan epilepsie en tot dusver blijkt er nauwelijks sprake te zijn van negatieve bijwerkingen die voortduren na beëindiging van de stimulatie.

Cardiovasculaire ziekten

Cardiovasculaire aandoeningen vormen nog steeds de grootste doodsoorzaak wereldwijd. Ze zijn vaak gevolg van atherosclerose, in de volksmond “aderverkalking”, een alom voorkomende aandoening waarvan de eerste symptomen vaak al op jonge leeftijd aanwezig zijn. Atherosclerose begint met een kleine beschadiging in de arteriële vaatwand. Die kan bijvoorbeeld ontstaan door hypertensie, trauma (stoten) of door infectieuze activiteit. Zo’n kleine schade initieert een inflammatoire respons om het beschadigde weefsel af te voeren en het weefsel en de functie te herstellen. In dit proces kunnen lipoproteïnen (LDL) uit het bloed in de vaatwand worden opgenomen waar ze worden geoxideerd. In een poging dit geoxideerde LDL op te ruimen worden ze door macrofagen gefagocyteerd. Deze macrofagen gevuld met geoxideerd LDL vormen foam-cellen die de wand niet meer verlaten. Daardoor ontstaat accumulatie van vet waardoor de atherosclerotische plaque zich verder ontwikkelt. In de beginstadia kan het proces nog stoppen en kan de vaatwand nog volledig herstellen. In latere stadia echter kan de aangroei van plaque de doorstroming van bloed ernstig belemmeren. Ook kunnen er hematomen in de plaque ontstaan en kan de plaque scheuren waardoor er deeltjes van de plaque in de bloedstroom terechtkomen die verderop in de

stroom een afsluiting kunnen veroorzaken. In al deze fases spelen inflammatoire factoren een cruciale rol.

Ook bij herstel na een hartinfarct is het de ontstekingsactiviteit die zowel verantwoordelijk is voor het herstel als voor de uiteindelijke resterende schade.

Kortom, het immuunsysteem is essentieel voor het onderhouden van een goede gezondheid. De activiteit van inflammatie moet strak gereguleerd worden om weefsel en functieschade te voorkomen en het AZS lijkt die functie in belangrijke mate te vervullen. Cardiovasculaire aandoeningen behoren tot de meest voorkomende ziekten die vaak leiden tot vermindering van algemeen welzijn of de dood. Meer kennis van het immuunsysteem en ontstekingsactiviteit in het bijzonder, en van de regulerende invloeden daarop van het AZS kunnen een bijdrage leveren aan het verbeteren van de behandeling van hart en vaatziekten.

Indeling van dit proefschrift

In dit proefschrift wordt een aantal studies beschreven die tot doel hebben:

- 1: het vinden van een mogelijk gunstig effect van vagale activatie op cardiovasculaire aandoeningen.
- 2: het vinden van mogelijke associaties tussen innervatie en inflammatoire activiteit in aneurysmata en in de door atherosclerose aangedane arteriële wand.

Na een algemene inleiding in **hoofdstuk 1** worden in hoofdstuk 2 de effecten besproken van arteriële schade en herhaalde bloedafname op het aangeboren immuunsysteem in de rat. Katheterisatie is een veel voorkomende klinische interventie waarbij door perforatie noodzakelijkerwijs lichte schade in de vaatwand ontstaat. Een publicatie van Versteeg (2009) toonde aan dat patiënten die zo'n katheterisatie hadden ondergaan vervolgens een sterke afname in ontstekingsactiviteit vertoonden.

In een groep geanestheeerde dieren werd aan de femorale arterie van het dier experimentele schade toegebracht waarna de inflammatoire responsiviteit in het bloed werd gemeten door het te stimuleren met LPS. Het bleek dat toegebrachte arteriële vaatwandschade een sterk onderdrukkend effect heeft op de systemische ontstekingsactiviteit van het dier.

In een andere proefgroep werd bij de geanestheeerde dieren met tussenpozen van 25 minuten 3 maal bloed afgenomen en ook daar werd vervolgens de inflammatoire respons gemeten in het bloed. Opnieuw werd een afname van de inflammatoire respons vastgesteld die door de bloedafname was geïnitieerd. Deze afname was onafhankelijk van de hoeveelheid afgenomen bloed en van het soort anesthesie dat werd gebruikt. Net als katheterisatie is bloedafname een ingreep die in biomedisch onderzoek en in de medische praktijk veel plaatsvindt, soms in patiënten voor wie een afname van de immuun capaciteit risico's met zich meebrengt. Het is belangrijk om te weten dat inflammatoire parameters in het bloed worden beïnvloed door zelfs lichte arteriële schade en door herhaalde bloedafname.

Bij een hartinfarct wordt het dode en necrotische weefsel afgebroken door acute inflammatoire responsen en vervangen door collageen. Door deze ontstekingsactiviteit kunnen gezonde cellen aan de rand van het infarct ook beschadigd raken en verwijderd worden. Daarmee wordt het infarctgebied onnodig vergroot. Wanneer de perfusie van een ischemisch gebied na verloop van tijd wordt hersteld, bijvoorbeeld door percutane coronaire interventie ("dotteren") of door middel van een bypass operatie, ontstaat er een gebied met stervend weefsel waaromheen zich niet of slecht geperfundeed weefsel bevindt dat nog wel levensvatbaar is. Reperfusie veroorzaakt een heftige inflammatoire respons die een grote collaterale schade veroorzaakt met als gevolg een infarctgebied dat veel groter is dan het oorspronkelijke ischemische deel. Dit verschijnsel wordt reperfusieschade genoemd. Mogelijk kan periodieke vermindering van inflammatoire activiteit de totale schade beperken. **Hoofdstuk 3** behandelt een studie naar de effecten van VS op het herstel van weefsel en hartfunctie na een permanent myocardiaal infarct (MI) bij de muis. In **hoofdstuk 4** wordt hetzelfde gedaan met een studie naar effect van VS in een ischemie-reperfusie model (IR). De muizen werden onder volledige anesthesie gebracht en in de rechter zijde van de hals werd de nervus vagus 30 seconden lang elektrisch gestimuleerd. Omdat de vagale vezels die verantwoordelijk zijn voor de daling in hartfrequentie moeilijker te activeren zijn dat die verantwoordelijk zijn voor een afname in inflammatoire responsiviteit kunnen we frequentieafname beschouwen als indicatie voor een effectieve VS. In deze studies werd ECG registratie gebruikt en bij alle stimulaties werd een duidelijke afname van de hartfrequentie geconstateerd. Vervolgens werd de linker zijde van de thorax geopend, het hart werd geëxposeerd en de linker coronaire arterie (LAD) werd afgebonden. Bij de permanente infarct studie werd daarna de thorax gesloten om het dier bij te laten komen. Bij de reperfusie-studie werd na een periode van 30 minuten ischemie de perfusie weer hersteld waarna de thorax werd gesloten en de dieren bij mochten komen uit anesthesie. Ter vergelijking werden in zogenaamde Sham-groepen de dieren alleen geopend en weer gesloten waarbij er geen VS en/of geen ischemie werd geïnduceerd. 48 uur na chirurgie werden de dieren getermineerd en parameters voor inflammatoire activiteit en functie herstel werden gemeten. In alle dieren was zowel voorafgaand aan de behandeling als achteraf, vlak voor terminatie, de hartfunctie gemeten door middel van cardio-echografie. Infarctgrootte werd bepaald met behulp van kleuring met triphenyltetrazolium chloride (TTC). Hoewel er enig verschil was in ejectie fractie werden er bij het MI-model geen significante verschillen gezien in het herstel tussen de dieren die wel en geen VS hadden ondergaan. Ook waren er geen duidelijke verschillen te zien in de gemeten inflammatoire parameters in het hart en het bloed. Wel was de ratio *infarctgrootte:bedreigde zone* (IS:AAR) in de IR studie beduidend kleiner in de groep die VS had ondergaan. Enerzijds kan geconcludeerd worden dat de periode van 48 uur herstel na de MI of IR procedure te kort was om een duidelijk effect te sorteren. Anderzijds kunnen de resultaten betekenen dat VS een minder anti-inflammatoir effect heeft dan in de literatuur wordt gesuggereerd.

Het verband tussen innervatie en inflammatie in atherosclerose wordt in **hoofdstuk 5** verder onderzocht. In specimen van atherosclerotische abdominale aorta's wordt gekeken in hoeverre er een verband bestaat tussen aanwezige innervatie en ontstekingsactiviteit, en dat in relatie tot het stadium van atherosclerotische ontwikkeling. Er waren 5 achtereenvolgende stadia gedefinieerd, de eerste vier in oplopende fase van ontwikkeling; 1: normaal (N), 2: niet-progressieve intimale laesies (NIL), 3: progressieve atherosclerotische laesies (PAL), 4: zwakke (vulnerable) atherosclerotische laesies (VAL) en gestabiliseerde atherosclerotische laesies (SL), dus 5 groepen en 14 samples per groep. Met immunohistochemische kleuring werden de verschillende parameters in beeld gebracht en gefotografeerd bij een vergroting van 100x. Deze foto's zijn geanalyseerd m.b.v. ImageJ. Op de grens van de tunica media en de adventitia werden foto's gemaakt bij een vergroting van 100x zodanig dat de verhouding media:adventitia overeenkwam met ca. 20%:80%. De uitvoerende medewerkers waren geblindeerd voor de atherosclerotische fases van de specimen. De gebruikte antilichamen zijn weergegeven in hoofdstuk 5, tabel 1. Waarden werden bepaald voor totale aanwezigheid van zenuwweefsel, sympathische en parasympathische vezels, van totale aantallen T-lymfocyten, T-helper cellen, cytotoxische T-cellen, totaal B-lymfocyten en plasmacellen. Er bleek een positieve correlatie te bestaan tussen zowel totale als sympathische innervatie en inflammatoire activiteit van aanwezige T- en B-lymfocyten in de ontwikkeling van atherosclerotische laesies. Voor de parasympathische vezels bestond die correlatie niet. De aantallen van alle gemeten T-lymfocyten en die van totaal B-lymfocyten vertoonden een stijgende lijn door de achtereenvolgende atherosclerotische plaque ontwikkeling tot en met de VAL. Terwijl de totale B-lymfocyten daarna verder stegen bleken alle T-cel waarden in de SL te zijn gedaald. Zowel totale als sympathische innervatie stegen door tot in de PAL maar lieten in de VAL al een kleiner aantal aanwezige vezels zien en die waarden daalden verder in de SL. De parasympathische vezels laten tot en met de VAL een tegengesteld patroon zien en nemen net als de totale innervatie in de SL sterk af. Hoewel de waarden niet significant verschillen geeft dat omgekeerde patroon wel de sympathisch-parasympathische balans weer en komt het patroon overeen met stijgende en afnemende inflammatoire activiteit.

De daling van totale en sympathische innervatie in de VAL waar de lymfocyten nog stijgen, gevolgd door een daling van T-lymfocyten in de SL veronderstelt dat neuronale afname vooraf gaat aan afname van inflammatoire activiteit. Dat kan betekenen dat het ANS naast het initiëren van ontstekingsactiviteit ook de impuls kan geven voor de afname van inflammatie en daarmee voor stabilisatie van de laesie.

Het ontstaan van aneurysmata is niet helemaal duidelijk maar in de literatuur gaat men ervan uit dat er een kleine schade in de arteriële wand ontstaat door dezelfde oorzaak als bij atherosclerose. Dat wil zeggen door bijvoorbeeld trauma of infectieuze activiteit. Hoewel atherosclerose aanleiding kan zijn voor de vorming van een aneurysma komen aneurysmata ook voor zonder de aanwezigheid van atherosclerose. Het immuunsysteem reageert op zo'n schade met het inzetten van ontstekingsfactoren om de schade te herstellen en daarbij kan een zwakke plek in de wand ontstaan. De bloeddruk zorgt er vervolgens voor dat er

een uitstulping in de inmiddels instabiele vaatwand ontstaat. Inflammatoire processen regelen het opruimen van de beschadigde cellen die, indien reparatie niet mogelijk is, worden vervangen door collageen. De situatie blijft vaak instabiel waardoor een aneurysma vaak een permanent progressieve ontwikkeling doormaakt tot de vaatwand scheurt met een inwendige bloeding tot gevolg. Een dergelijke bloeding heeft, afhankelijk van de locatie, meestal ernstige gevolgen en is vaak dodelijk.

Evenals bij atherosclerose zijn inflammatoire processen betrokken bij alle fasen van de ontwikkeling van aneurysmata. In **hoofdstuk 6** wordt in twee grote arteriën bestudeerd in hoeverre de lokale innervatie verband houdt met de aanwezigheid van aneurysmata. In aneurysmata in de extracraniële arteria carotis (ECAA, N=10) en in de arteria poplitea (PAA, N=10) werd de aanwezigheid van totale innervatie en van sympathische vezels bepaald en vergeleken met die in de controle arteria carotis (Contr. CA, N=6) en de controle arteria poplitea (Contr. PA, N=6). Dezelfde antilichamen als in hoofdstuk 5 werden gebruikt voor de immunohistochemische kleuringen van totale innervatie (PGP-9.5) en voor sympathische vezels (TH). Bij de kwantificering van de innervatie werd onderscheid gemaakt tussen zenuwbundels omgeven door een epineurium ("bundles"), zenuwvezels die geassocieerd konden worden met de loop van een tak van het vasa vasorum ("vv-ass".) en losse vezels verspreid liggend in het weefsel ("scattered"). Daarnaast werd met ImageJ de totale hoeveelheid aangekleurde vezels bepaald en in een percentage van de totale oppervlakte weergegeven als "area".

In het algemeen was de innervatie zowel in controle carotiden als in ECAAs groter dan in de controle popliteas en de PAAs. Van totale innervatie en van sympathische vezels werden in ECAAs meer "scattered" vezels geteld dan in controle carotiden maar het verschil was statistisch niet significant. Een dergelijke verschil zou kunnen betekenen dat uitgroei van deze vezels verband houdt met de vorming van aneurysmata. Om onderbouwde uitspraken te doen is echter meer onderzoek nodig in een grotere groep samples. Helaas zijn die slechts beperkt beschikbaar.

Nadat anti-inflammatoire effecten van vagale activatie voor het eerst werden gepubliceerd heeft in de laatste 2 decennia onderzoek naar invloeden van het autonome zenuwstelsel op ontstekingsprocessen een hoge vlucht genomen. In-vivo onderzoek waarin gebruik wordt gemaakt van stimulatie van de nervus vagus (VS) maakt daar een belangrijk deel van uit en voor een aantal inflammatoire aandoeningen zijn inmiddels proefdiermodellen ontwikkeld, veelal in muizen en ratten. VS in kleine dieren is daarom beperkt tot een eenmalige sessie omdat de stress die het dier ondervindt bij herhaling van een dergelijke procedure de resultaten van het onderzoek zou kunnen beïnvloeden. Herhaalde stimulatie over een langere periode in een klein proefdier is tot dusver niet mogelijk gebleken waardoor onderzoek naar langdurige inhibitie van de inflammatoire respons door VS niet mogelijk is. Er is dus behoefte aan een vagus stimulator voor een klein proefdier waarbij verminderde inflammatoire responsiviteit verkregen kan worden over een langere periode zonder dat daarbij stress voor het dier ontstaat. In **hoofdstuk 7** wordt de ontwikkeling van een implanteerbare stimulator beschreven. Voorafgaand aan de bouw van de stimulator werd

eerst getest hoe en bij welke elektronische instellingen een afname in inflammatoire responsiviteit werd verkregen. Omdat de vagale vezels die zorg dragen voor een inflammatoire inhibitie sneller geactiveerd raken dan de vezels die afname van de hartfrequentie tot gevolg hebben, is afname van meer dan 25% van de hartfrequentie geaccepteerd als bewijs voor effectieve stimulatie. In die eerste groep werd bovendien de inflammatoire inhibitie getest door het bloed van gestimuleerde dieren met LPS te behandelen en daarin de hoeveelheid TNF α te meten.

Bij die ontwikkeling van de stimulator moest rekening gehouden worden met acceptatie van de gebruikte materialen door het lichaam van het dier. De vorm, de afmetingen en het gewicht werden zodanig bepaald dat het dier daar geen ongemak door zou ondervinden. Dummy stimulators van de gekozen materialen, met de bepaalde vorm en gewicht werden geïmplanterd en werden geschikt bevonden wanneer ze 3 maanden probleemloos in positie waren gebleven. Verder werden de juiste elektronische instellingen voor een effectieve VS uitgetest en bepaald. Daaruit ontstond een apparaat waarbij alle onderdelen inclusief de batterij (CR1220-1FCE) compact zijn gepositioneerd en zijn ingegoten in acrylaat. Daaroverheen is een coating van silicone aangebracht voor een betere inbedding en hechting aan het omliggende weefsel. De uiteindelijke afmetingen zijn 23x13x3.5mm met een gewicht van 3.3 gram en de hoeken en randen zijn afgerond. Het geheel wordt onderhuids in de rechter flank geïmplanterd (Hst.7, Figure 2 en 7). Vanaf de stimulator loopt een gevlochten kabeltje onder de huid en onder de musculus pectoralis major en de sternocleidomastoideus door en komt uit, parallel aan de carotis/nervus vagus. Aan het einde van dit 5cm lange kabeltje is de elektrode gesoldeerd.

De nervus vagus in de hals is het meest toegankelijk voor elektrische stimulatie maar in het gebied van deze zenuw ligt veel kwetsbaar zenuw- en vaatweefsel tussen sterke en veel gebruikte nekspieren. Door die positie is het weefsel zeer aan beweging onderhevig en de elektrode moet daar tegen bestand zijn. De ontwikkeling van een geschikte elektrode bleek daardoor het meest ingewikkelde deel van de stimulator te zijn. De eindversie van de elektrode bestaat uit een ca. 500 μ m lang spoelvormig geheel van platina-iridium (PtIr, 90/10%) en deze elektrode wordt om de vagale zenuw gepositioneerd. Ter plekke is om de arteria carotis een kleine silicone manchet aangebracht waaraan een flapje zit waarin de elektrode met daarin de zenuw wordt gefixeerd door het flapje dicht te binden zoals in figuur 2 en 3 van hoofdstuk 7 te zien is. Bij bewegingen van de nek kan de zenuw zelf nog vrij door de elektrode heen en weer bewegen.

Een aantal van 11 functionerende stimulators werd geïmplanterd en de werking werd gecontroleerd. Het stimulatieprogramma was bepaald en ingesteld op sessies van 30 seconden bij 1mA, 1k Ω en 10Hz elke 48 uur om een continu verlaagde inflammatoire respons te genereren. De implantatie duurde inclusief de controle op effectiviteit van de stimulatie maximaal 60 minuten maar sommige dieren vertoonden een afwijkende arteriële anatomie in de hals met veel collaterale takken die expositie van de vagus belemmerden en daarmee risico op bloeding vergrootten. Van de 11 geopereerde muizen werden er 3 geëxcludeerd door het ontstaan van een bloeding tijdens de operatie. De 8 overige implantaten functioneerden van 1 tot 29 dagen (Hst.7, table 1). Bij verwijdering van de

stimulator na terminatie van de dieren werd vastgesteld dat de meeste stimulators gestopt waren doordat ingroeiend collageen in de elektrode-spoel de druk op de zenuw zodanig vergroot had dat die niet meer reageerde op de stimulatie.

Conclusie: met de ontwikkelde nervus vagus stimulator is kortdurende repetitieve stimulatie mogelijk zonder ongerief voor het vrij bewegende dier. Effectieve stimulatie werd geverifieerd aan de hand van een afgenomen hartfrequentie op het ECG. Met een technische aanpassing aan de bevestiging van de elektrode op de zenuw kan de werkingsduur van de stimulator naar verwachting verlengd worden naar ca. 30 dagen. Ook het vinden van een alternatieve locatie voor de elektrode kan de functionaliteit van de stimulator nog verbeteren. Vanwege de gevoeligheid van de zenuw moet bij ingrepen in het cervicale gebied elke manipulatie van de nervus vagus vermeden worden om ongewenste triggering van inflammatoire inhibitie te voorkomen.

Algemene conclusies

Het autonome zenuwstelsel heeft een sterk regulerende werking op het immuunsysteem en met name ontstekingsactiviteit. Stimulatie van de nervus vagus heeft een reflexmatige afname van inflammatoire responsiviteit tot gevolg. Dit effect kan het lichaam beschermen tegen overmatige schade door ontstekingsresponsen die het gevolg zijn van weefselschade door bijvoorbeeld trauma of ischemie. Een beter begrip van deze principes kan uiteindelijk leiden tot een nieuwe therapeutische benadering van ontsteking gerelateerde aandoeningen.

De studies zoals in dit proefschrift beschreven proberen een antwoord te geven op actuele kwesties met betrekking tot neuronale invloeden op inflammatoire processen in het hart en de grote arteriën.

Hoewel het duidelijk is dat manipulatie van de vagale zenuw, evenals die van de arteriële vaatwand effect heeft op de systemische inflammatoire activiteit is nog niet helemaal bekend hoe deze reflexen werken. De meer mechanistische details van de pro- en anti-inflammatoire invloed van het autonome zenuwstelsel moet daarvoor verder onderzocht worden.

Er zijn al grote vorderingen gemaakt met het ontwikkelen van vagus stimulatie als therapie bij reumatoïde artritis en inflammatoire darmziekten zoals ziekte van Crohn en colitis ulcerosa. Naar effecten van vagus stimulatie bij humane patiënten met een cardiovasculaire aandoening is nog weinig bekend. Toekomstig onderzoek zou zich moeten richten op het vergroten van kennis rond interacties tussen het neurale en het immuunsysteem met het doel een verstoorde balans bij inflammatoire aandoeningen te herstellen. De ontwikkelde vagusstimulator voor laboratoriumdieren kan daarbij van grote waarde zijn.

REVIEW COMMITTEE / LEESCOMMISSIE

Prof. dr. G.J. de Borst

Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, the Netherlands

Prof. dr. D.P.V. de Kleijn

Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, the Netherlands

Prof. dr. M.C. de Ruiter

Department of Anatomy, Leiden University Medical Center, Leiden, the Netherlands

Prof. dr. J.P.G. Sluijter

Regenerative Medicine Center Utrecht, UMC Utrecht, Utrecht, the Netherlands

Prof. dr. J. van der Velde

Department of Physiology, VUmc, Amsterdam Cardiovascular Sciences, Amsterdam, the Netherlands

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1. **Nederhoff MGJ**, Verlinde SAMW, van Zuuren T, Pasterkamp G, Bleys RLAW. Effect of Vagus Nerve Stimulation on Tissue Damage and Function Loss in a Mouse Myocardial Infarction Model. *Int J Cardiovasc Res*. 2019;8(2). doi:10.4172/2324-8602.1000409
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6. **Nederhoff MGJ**, Boskovic E, van Dongen BJ, Bleys RLAW, Pasterkamp G. Implantable vagus nerve stimulator for small laboratory animals. *Submitted for publication*.

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CURRICULUM VITAE

Marcel Nederhoff, de auteur van dit proefschrift, werd geboren als zoon van een scheepstimmerman op 30 Augustus 1959 in Warmond (ZH) aan de rand van bos, weiden en bollenvelden. In die eerste jaren werd daar zijn interesse gewekt voor de natuur en alles wat daarin te vinden is. In zijn 10^e levensjaar verhuisde hij met zijn ouders naar Zeist. Na de middelbare school werd hij werkzaam bij het instituut voor Toegepast Natuurwetenschappelijk Onderzoek (TNO) waar hij zich door de jaren heen bekwaamde in experimentele microchirurgie ten behoeve van toxiciteitsonderzoek. In die periode volgde hij een grote variatie aan opleidingen en cursussen op MBO en HBO niveau. In 1991 hij werd aangenomen bij het Interuniversitair Cardiologisch Instituut Nederland (ICIN), onderdeel van de Koninklijke Nederlandse Akademie van Wetenschappen, op de locatie UMC Utrecht. Onder professoren Ruigrok en van Echteld ondersteunde hij daar projecten met magnetische resonantie (MRS en MRI) en breidde zijn chirurgische vaardigheden verder uit. In die periode behaalde hij een master in de Klinische en Gezondheidspsychologie aan de UU. Binnen zijn functie nam hij op den duur meer onderwijs gerelateerde taken op zich en droeg hij bij aan het opzetten van de cursus "Cardiac MRI in the Mouse". Toen deze muizen-MRI faciliteit naar Groningen werd verplaatst is de auteur onder professor Pasterkamp o.a. betrokken geweest bij het opzetten van een laboratorium voor cardiovasculair onderzoek bij muizen en verzorgde hij de onderwijscoördinatie voor de Experimentele Cardiologie. Hij raakte in die tijd geïnteresseerd in de inflammatoire processen in cardiovasculaire aandoeningen en in het bijzonder in de mogelijke invloed daarop van het autonome zenuwstelsel. In 2013 werd hij aangenomen bij de Afdeling Anatomie van het UMC Utrecht onder professor Bleys op het promotie-project dat heeft geresulteerd in dit proefschrift.