Bernburg
Dessau
Köthen

Hochschule Anhalt
Anhalt University of Applied Sciences

FB Angewandte Biowissenschaften und Prozesstechnik (BWP)

Development of an *in vitro* model for studying aneurysms

Master Thesis

Submitted in accordance with the requirements for the degree of Master of Science (M.Sc.)

Jenny Richter

Matr. Nr.:4047944

Study course: Biotechnologie

1st supervisor: Prof. Dr. Mägert

2nd supervisor: Dr. Korossis

Date of delivery: 27.11.2014

Abstract

The aims of this project was to develop an arterial aneurysm using either enzymatic or laser degradation of the arterial wall without affecting the viability of the tissue and to cultivate the arteries under pulsatile flow conditions in a vascular bioreactor with a view to investigate the progress of the disease. Characteristics of aneurysms are the degradation of smooth muscle cells, collagen and elastin. Detached smooth muscle cells and degradation of the collagen matrix and elastin fibres were observed in arteries degraded with enzymes elastase and collagenase. Only remnants of the arterial wall were detected after cultivation. This might be a suitable model for late stage aneurysms. Arteries treated with the laser system showed no charring or heat damage of the not dissected area. Collagen matrix, smooth muscle cells and elastin fibres were intact. A clear defined cut was made in a depth of 200 µm and tissue was removed. Following cultivation of these arteries a dilation of the laser-eroded area was observed. This model can mimic atherosclerotic aneurysms, when plaques weaken the *tunica media* of the blood vessel wall and rupture. Limitations of this study were contamination of the bioreactor system and a low number of cultivations. The aim to generate a living arterial aneurysm *in vitro* was not achieved. Tissue viability decreased to the level of negative controls after cultivation.

Acknowledgements

My thanks go to my supervisors Prof. Dr. Mägert and Dr. Korossis for their help. Thank you for making this project possible. I want to thank Dr. Korossis for the possibility to work at CrossBIT and for his guidance through my project. I want to express my thanks to my co-supervisor Dr. Morticelli. Thank you for answering all my questions, thank you for your great work as a lab manager and thank you for your help with the bioreactor handling. My thank goes to the all the other members of the Mitral Valve group for their help every time I needed a third hand and for answering my questions. I also want to thank Dr. Will and Dr. Richter from ROWIAK for their help using the Tissue Surgeon. Thank you Dr. Böer for the possibility to use your lab and thank you for your advices. I also want to express my thanks to Mrs. Klingenberg for her instructions in immunohistochemistry work and her advises in the laboratory-everyday. I want to thank Miss Lau for her help in the lab. My thanks go to Dr. Theodoridis and Dr. Hinz for helping me to get porcine arteries.

My sincere thanks go to my family for supporting me during my academic studies. I want to thank my parents for the great job they do as parents. Thank you for giving me the opportunity to find my own way and for your guidance. Thank you Undine and Brigitte for helping me with this thesis. My deepest thank goes to my amazing boyfriend Igor, for his moral support, for calming me down when I needed it and always making me smile.

Table of Content

List of Abbreviations

ATP	Adenosin Triphosphat
BSA	Bovine Serum Albumin
CASO	Casein Soy
CDU	Collagen digesting unit
DMEM	Dolbeccos modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
ECM	Extracellular matrix
FCS	Fetal Calf Serum
HBSS	Hanks buffered salt soluion
LDL	Low density proteins
MMP	Matrix metalloproteinase
mo-LDL	Minimal oxidized LDL
MTT	3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
PBS	Phosphat buffered saline
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SMC	Smooth muscle cells
TIMP	Tissue inhibitor of metalloproteinases TIMP
U	Units

List of Figures and Tables

1. Introduction

1.1. Introduction

Blood vessels provide a complex transport system for the blood ensuring the oxygen and nutrition supply of every cell in the body and also the excretion of CO_2 and metabolic products (1).By transporting messengers, antibodies and cells from one part of the body to another, the blood is important for various cellular responses including the immune response (1) and the interaction of organs via hormones. In addition, the blood flow is also important to keep the pH and the temperature in the body constant (1). Oxygen-rich blood from the blood vessels in the lungs flow into the heart. This muscle pumps the blood through the arteries in the body where it passes the arterioles and reaches the capillaries. The capillaries are thin-walled blood vessels. Here the of oxygen and nutrition are exchanged between the blood and the cells. The blood, now poor in oxygen, flows from the capillaries into the venoles and re-enters the heart via the veins. From there the blood flows back into the blood vessels of the lounge and will be again enriched with oxygen (2). To provide an adequate distribution system for the blood, blood vessels near the heart need to resist the pressure of the fluid pumped out of the heart. These vessels have to be able to enlarge if more blood is needed in a special region of the body or to narrow, if less blood is needed. The luminal site has to present messengers that prevent clotting of the blood inside the vessels and the vessels have to be embedded inside the surrounding tissue. To achieve those tasks blood vessels have three layers: the *tunica intima*, the inner layer; *tunica media*, the middle layer and the *tunica adventitia* as the outer layer. The shape of the layers is adjusted to the function and needs of the different vessels in the body (2).

An aneurysm is a local expansion of the lumen of the artery which is caused by congenital or acquired changes of the artery wall. Based on their pathology three different types of aneurisms can be distinguished : the *aneurysma verum* , the *aneurysma spurim* and the

aneuryma dissecans. The *aneurysma verum* is considered a true aneurysm where all three layers form the blood vessel are affected. The *aneurysma verum* can be sub-divided in fusiform and saccular forms (3). The *aneurysma spurim* or false aneurysm, is a hematoma around a defect artery wall with compression by the surrounding tissue. The appearance of false aneurysms is correlated to the use of cardiac catheterization (4). In *aneurysma dissecans* the artery is dissected and the wall is formed by the inner or outer media (5).

75- 80 % of all aneurysms are true aneurysms, 15-20 % are *aneurysma dissecans* (6). The most common aneurysms are the abdominal aortic aneurysm (> 50% of all aneuryms) , aneurysm of the thoracic aorta (26 %) and aneurysms of the popliteal artery (12 %) (3). One possibility to treat an aneurysm is to insert implants into the vessel, which will close the blood flow through the aneurysm. Small aneurisms can be closed by injection of thrombin (3). For cerebral aneurysms clipping (“craniotomy with clip ligation”) and coiling (“endovascular occlusion with the use of detachable coils”) are established methods (7).

1.2. Aims and Objectives

The aims of this study are to develop an arterial aneurysm using either enzymatic or laser degradation of the arterial wall, without affecting the viability of the graft and to cultivate the treated artery under pulsatile flow conditions in a vascular bioreactor with a view to investigating the progress of the disease. This project addresses the hypothesis that it is possible to generate a living arterial aneurysm *in vitro*, with a view to investigating the onset and progress of the disease

Benefits of an *in vitro* model are lower contamination risks, higher reproducibility, the supply of a closed system, the existence of standardized techniques and almost operator independent working. Thus, an *in vitro* model of an aneurysm provides a more cost effective system for testing drugs.

To achieve this aim a common carotid artery from a pig was cultivated in a vascular bioreactor. A laser was used to remove parts of the artery to create an aneurysm. Alternatively, an elastase/collagenase assay was used to create an aneurysm model.

2. State of knowledge and technique

2.1. Arteries

Arteries transport the oxygen rich blood from the lungs through the body. Depending on the structure of their wall characteristics, arteries are subdivided into elastic and muscular type of arteries. Arteries of the elastic type are blood vessels close to the heart, like the aorta or the pulmonary trunk. Muscular type arteries are the most common arteries in the body. They differ mainly in the structure of the media (2).

2.1.1. Histoarchitecture

Tunica intima

In muscular arteries, parts of the *tunica intima* are the endothelium, a basal lamina and a sub-endothelia layer (2); (1). The endothelium is one close layer of flat, polygonal cells, connected via tight junctions, gap junctions and adherens junctions. The cells are based on the basal lamina and covered with a thick glycocalyx providing a negative charge of the endothelium. The longitudinal axe of the cells is in parallel to the blood flow. The plasma membrane shows a lot of caveolae. The endothelial cells form adhesion contacts with the extracellular matrix (ECM). At the inner site of the junction bunches of actin-filaments are inserted. They are orientated parallel to the blood flow. These stress fibres support the stability of the cells against the shear force of the blood flow (2).

The composition of the sub-endothelia layer depends on the age of the person. Children show a very thin sub-endothelia layer with less cells and ECM. In young adults smooth muscle which originate from the media cells can appear. The ECM of this layer consists of hyaluronan, different proteoglycans, collagenic and elastic fibres and micro fibrils (collagen

type VI and fibrillin) (2).In elastic arteries, the *tunica intima* shows clearly visible sub endothelial layer consisting of ECM and longitudinal orientated muscle cells (2).

Tunica media

The *tunica media* is the thickest layer of the artery wall. This layer of muscular arteries consists of smooth muscle cells and ECM (elastic and collagenic fibres, proteoglycans) produced by the smooth muscle cells. The muscle cells are arranged arranged circular ore spiral and connected by gap junctions (2). The media of elastic arteries consists of concentric elastic lamella with smooth muscle cells lying in between them. The muscle cells make contact via fibrilli –microfibrills and connect the lamella. Collagen fibres stabilize the construct which are embedded in a proteoglycan rich matrix (2).

Tunica adventitia

The *tunica adventitia* of muscular arteries is a connective tissue that fixes the vessel in the surrounding tissue. This layer contains fibroblasts, proteoglycans, elastic fibres and collagen fibres. Vasa vasarum (the blood vessels of the vessels) nourish the outer media. Nerve fibres surround the arteries but do not penetrate the media (2). In elastic arteries this layer is rich in vasa vasorum, penetrating even the outer layer of the media (2).

Membrana elastic interna* and *Membrana elastic externa

Synthesized by the smooth muscles of the *tunica media*, this layer consists of elastic fibres in vessels of the muscular typ. The fibres are arranged in two dimensions and orifices

enforce the membrane. Extensions of endothelial cells penetrate this orifices and form myoendothelial contacts with the smooth muscle cells of the media (2). In elastic arteries these layers cannot be easily distinguished from the elastic lamellas of the media. The membrana elastic externa can only be seen in big arteries (2)

2.1.2. Function

The main function of arteries is a suitable nutrition and oxygen supply of organs and extremities (3). Another function of arteries is the regulation of the blood flow into special parts of the body (1). The elastic arteries have a windkessel function which means that they transform the pulsatile blood flow from heart into a continuous blood flow (1).

The *tunica intima* of the arteries interacts with blood and performs the mass transfer between blood and tissue (1). Tasks of the endothelium are:

-Acting as a diffusion barrier: Tight junctions circumvent passive transport of macromolecular plasma content in the sub endothelial layer (2)

-Control adhesion: The endothelium presents a layer that prevents adhesion of platelet and leukocytes, but can express selectin and other adhesion molecules for emigration of leucocytes (2)

-Hemostasis: Secretion and surface expression of proteins, proteoglycans and coagulation factors prevent hemostasis, but stimulate blood clotting after injury of the blood vessel. Prostacyclin prevents aggregation of platelets; the von Willebrand-Faktor promotes adhesion of platelets and is stored in Weibel-Palade-Granula an endothelium- specific organelle. Weibel-Palade-Granula has P-selection molecules at the surface of their membrane, the P-selectin molecules are exposed during the exocytose of the granulum content (2).

-Regulation of the size of the vessel: the endothelium secrets vessel expanding (NO, prostacyclin) and vessel narrowing factors (endothelin) and has contact with the smooth muscle cells of the media via gap junctions (2).

-Synthesis of ECM components: the endothelium secretes the von-Willebrand-Factor which mediates adhesion between the endothelium and the subendotheliale layer (2).The *tunica media* ensure the maintenance of the blood pressure by regulating it (1). In elastic blood vessels this layer supports the windkessel function of the arteries. The *tunica adventitia* fixes the vessel in the surrounding tissue and ensures the stability of vessel wall (1). Membrana elastic externa and *membrana elastic interna* support the windkessel function of the vessels (1).

2.2. Veins

Veins transport the oxygen poor blood from the body into the heart. After passing the capillary system the blood pressure drops from 100mmHg to 5 mmHg (2). These vessels are thinner than the arteries with a thinner layer of smooth muscle cells.

2.2.1. Histoarchitecture

The structure of the intima is quite similar to the one of arteries. Parts of the intima form the venous valves (2). The media of veins are very different, depending on the part of the body where they are embedded. It can be thick or very thin. The layer of smooth muscle cells can range from a few layers to many layers. Some veins have more longitudinal than circular muscle cells. Elastic and collagen fibres enforce the muscles (2). The media is similar to a connective tissue with interwoven muscles with no coherent media (1). The adventitia can contain longitudinal smooth muscles, especially those of the *cavitas*

abdominalis. It has more vasa vasarum than arteries, penetrating the media and some nerve fibres (2). The membrane elastic interna can also be present in veins where it is mostly discontinuous (2). It is not cohesive (1).

2.2.2. Function

Tasks of the veins are to collect and to store blood. They ensure a suitable transport of blood back to the right half of the heart (3).The venous valves are Intima duplicates. They facilitate the transport of the blood from the body to the heart (1). In middle and small veins, the *tunica media* shows spiral bunches of smooth muscles, interwoven with an elastic net of collagenic fibres, which regulates the muscle tone (3).The *tunica adventitia* absorbs the forces from the outside of the vein and guarantee that the vessel does not collapse (3). Membrana elastic externa and *membrana* elastic interna provides the elasticity of the vein. The elasticity of veins is 200 times higher than the arteries (1).

2.3. Comparison of veins and arteries

Arteries and veins show the same general structure, consisting of three layers. The inner layer, named *tunica intima* is in contact with the blood and can be subdivided into endothelium and subendothelial layer (2). This layer “communicates” with the blood and the middle layer of the vessel by secreting messengers. The *tunica media*, the middle layer contains smooth muscle cells (2). This layer can control the blood flow by contraction of the smooth muscle cells. In addition it provides the elasticity of the vessel. The external layer, named *tunica adventitia*, is a connective tissue that embeds the vessel into the surrounding tissue (2).

But based on different functions, arteries and veins differ in their structure. Veins store and collect the blood. 85 % of the circulating blood is stored in the veins (1), to keep all the blood, veins are 200 times more dilatable than arteries. Venous valves prevent a blood flow back into the capillary system and away from the heart. Arteries have to withstand a much higher blood pressure than veins. The average blood pressure in the arteries is approximately 100 mmHg, compared to 5 mmHg in the veins (1). Thus the smooth muscle cell layer in the media is thicker in arteries than in veins. Arteries near the heart, like the aorta and the pulmunary trunk (2) transform the pulsatile blood flow of the heart into a continuous flow (windkessel effect), thus they have a dilative and thick wall (2) to persist the pressure and transform the discontinuous flow.

2.4. Pathology

Altered blood vessel function can contribute to pathological conditions including arteriosclerosis, the hardening of a vessel; vasculitis, an inflammatory disease, vessel tumours and aneurysms, a widening of the vessels diameter. Aneurysms can also be a result of other diseases of the vessels.

2.4.1. Arteriosclerosis /Atherosclerosis

Arteriosclerosis is the generic term for diseases including the thickening of the arteries, causing wall rigidification and consecutive loss of elasticity. Arteriosclerotic diseases are atherosclerosis, indicated by encapsulation of lipids in the intima and formation of fibrous plaques; calcification of the media, mostly occurring in older patient (above 50 years); and arteriolosclerosis/- hyalinosis, a disease of the small arteries and arterioles (5).

Atherosclerosis

The development of an atherosclerotic thrombus can be graduated in five stages: the initial phase, the inflammatory phase, the formation of foam cells and fatty streaks, formation of fibrous plaques, and formation of complex lesions and thrombosis (5).

The initial phase starts with a dysfunction of the endothelium. As a result low density proteins (LDL) and other apoprotein B containing lipoproteins of the blood can pass the junctions between the endothelial cells passively. High density proteins may take the cholesterin back to the blood. If the LDLs are not removed, metabolic products of vessel cells, e. g. radicals, will oxidate them (5).

The inflammatory phase is characterized by accumulation of minimal oxidized LDL (mo-LDL). This can trigger the production of chemokines, promoting the adhesion and immigration of monocytes from the blood. Transformation of monocytes to macrophages may be promoted, too. In addition, mo-LDL induces the increased expression of adhesion molecules on the endothelial cells. These molecules can interact with the adhesion molecules of the monocytes, thus facilitating the migration of trans endothelial monocytes and t-lymphocytes into the proteoglycan layer of the intima (5).

Formation of foam cells can appear,when the macrophages absorb the LDLs very quickly. The mo-LDLs have to be modified extensively, meaning a “high oxidation”. Reactive oxygen species (ROS) secreted by endothelial cells and macrophages modify the LDLs. Macrophages in the intima bind to scavenger receptors, receptors with high affinity to LDL. This binding facilitates and accelerates the admission of high oxidized, aggregated LDL in comparison to LDL from the blood. Foam cells appear, accumulate and die. Dead foam cells release a great amount of free and partly high oxidized lipids (5).

Formation of fibrous plaques:

Changes in the intima which lead to formation of chemotactic matter, result in immigration of numerous lymphocytes. Interaction of CD40 and CD40-ligand on T-lymphocytes and

macrophages stimulate the production of INF-γ. This stimulates an inflammatory reaction in the developed lipid plaque. Cytokines and growth factors like interleukin-6 and basic-fibroblast-growth-factor are secreted during the inflammatory reaction and promote the immigration of smooth muscle cells and their proliferation. The fibrous plaque appears (5).

Development of a complex lesion and thrombosis:

INF-γ inhibits the production of ECM by the smooth muscle cells. This process and the proteinases (collagenase, gelatinase, and stromolysin) secreted by the macrophages lead to an instable matrix. The risk of atheromatious ulceration increases. Inflammation also contribute to thrombosis and plaque destabilization. Calcification seems to be an active, regulated process, where pericyte like cells play a role. Thrombus formation is a result of plaque rupture and of the release of tissue factors (5).

2.4.2. Aneurysm

An Aneurysm is a local expansion of the lumen of the artery which is caused by congenital or acquired changes of the artery wall (5). Causes of these changes might be infections for example with Chlamydia pneumonia; inflammations; infiltration of inflammatory cells; genetic disorders; connective tissue disease; oxidative stress; necrosis and apoptosis of smooth muscle cells and a remodelling of the extracellular matrix of the vessel wall (8). Positive effects of antibiotic treatment and a correlation between antibodies against C. pneumonia in men and the expansion of abdominal aortic aneurysm are evidences for the correlation between an infection with C. pneumonia and the occurrence of aneurysms (8).

Elastin and collagen are two major components of the vessel wall. Elastin is a key protein of the extracellular matrix (ECM) and provides the integrity of the vessel wall. A degradation of elastin in the *tunica media* is associated with the dilation of the vessel wall (8). Collagen is a major component of the adventitia (9), a degradation of this protein is correlated with the rupture of aneurysms (8). The extracellular matrix contains

concentrically arranged collagen and elastin fibrils. Derangement of the structure and ultra-structure such as the cross linking of elastin fibrils are observed in aneurysmatic tissue (10). Other features are an increase in the collagen content and a decrease in the elastin content. This is accompanied by an increase of the microfibrillar proteins and a decrease in the smooth muscle cells in the *tunica media* due to necrosis and apoptosis of the cells (9); (8). Matrix metalloproteinase (MMP) activity was detected in aneurysms (8); (9). These enzymes are collagenases and elastases which degrade the extracellular matrix of the blood vessel wall. Collagenase-1 (MMP-1), stromelysin-1 (MMP-3), the 72-kDa gelatinase (MMP-2) and the 92-kDa gelatinase (MMP-9), macrophage elastase (MMP- 12) show elevated activity in aneurysms (9); (8). MMP 9 is secreted by macrophages and MMP 12 was detected near elastin fiber fragments. These fragments could increase the MMP activity or attract macrophages and create a positive feedback loop (9); (8). Abdominal aortic aneurysms show "inflammatory infiltrate consisting of T cells, B cells, and macrophages" (9). Genetic disorders that may be a reason for aneurysm formation are alterations in the gene regulation of MMPs and tissue inhibitor of metalloproteinases (TIMPs), which regulate the activity of MMPs. False folding of the protein fibrillin like in Marfan syndrome leads to a weakening of the elastic tissue. The resulting inelastic media of blood vessels make them prone to aneurysm formation (11). In atherosclerosis the macrophages enter the media of the vessel wall and produce MMP, which degrade the vessel wall. The formation of an atherosclerotic plaque leads to an inadequate oxygen and nutrition diffusion and thus an ischemia of the smooth muscle cells in the media. Smooth muscle cells synthesize parts of the ECM in the blood vessel wall. Apoptosis and necrosis of smooth muscle cells result in less ECM production and lead to a weakening of the vessel wall (11).

Branching points of blood vessels are prone to aneurysm formation, because of structural irregularities in the collagen matrix, discontinuities in the smooth muscle cell layers in the *tunica media* and increased hemodynamic stress due to radius variations and curvature. A prolonged elevated wall shear stress induces the production of matrix metalloproteinase (12), (10).

Aneurysma verum or true aneurysm, where all three layers form the aneurysm are effected; aneurysma spurim or false aneurysm, a hematoma around a defect artery wall with compression by the surrounding tissue; and aneurysma dissecans, where the artery is dissected and the wall is formed by the inner or outer media (5) can be distinguished. Aneurysma verum can be sub-divided into fusiform and saccular forms (3). 75- 80 % of all aneurysms are true aneurysms, 15-20 % are aneurysma dissecans (6). The appearance of false aneurysms is correlated with the use of cardiac catheterization (4).

Differentiating between different causal pathogenic aneurysms, one can distinguish between atherosclerotic aneurysm, congenital aneurysm, dissection of the aorta, inflammatory aneurysm and arteriovenous aneurysm (5).

Atherosclerotic aneurysm

As a consequence of atherosclerosis, fatal changes in the media of the artery take place. These changes are: reduction of the mediamyocytes, loss of elastic fibres and scar formation at the media. The atheroma may cause an attendant inflammation of the media and adventitia, leading to weakening of the artery wall. The consequence is an expansion of the whole vessel wall. In few cases, the atheroma rupture and blood can penetrate the media. An atherosclerotic dissection of the artery develops (5).

Congenital aneurysm

These are localized aneurysma verum caused by congenital violations of the ECM of the media. During lifetime, the affected vessels expand and can rupture (5).

Dissection of the aorta

A tear of the intima and media allows the blood flow into the generated false lumen. The blood may re-enter the right lumen distal from the first tear. Potential factors are congenital defects of the vessel wall, inflammation, atherosclerosis, hypertonia or trauma. Damage of the collagenic-elastic fiber frame of the media leads to weakening of the wall, so tangential shear forces of the pulse wave can tear the media. Defects of the intima can lead to tears, so blood can flow into the predamaged media. Diseases of the connective tissue (e.g. in Marfan syndrome) can cause elastica defects, filled with mukoid material. Ischemia of the middle media may have the same effect. Reasons for such starvation of the media are for example inflammations of the vasa vasorum or a lacking nutrition of the intima caused by atheroma plaques, leading to a longer diffusion distance (5).

Inflammatory aneurysm

In this disease, an inflammation of the vessel wall causes the weakening of the wall. A mycotic aneurysm is caused by a bacterial infection. More examples are the syphilitic aneurysm and the aneurysm occurring in polyarteriitis nodosa (5).

Arteriovenous aneurysm

A part of a vein is connected with an artery and expanded like an aneurysm. Reasons are a congenital defect, trauma ore inflammation (5).

The most common aneurysms are the abdominal aortic aneurysm, the thoracic aneurysm and the popliteal aneurysm (3). The abdominal aortic aneurysms (AAA) is the most

frequent aneurysm with an incidence of > 50 % of all aneurysm, mostly infrarenal (95 %) (3). 26 % of all aneurysms effect the aorta thoracalis and 12 % the a. popliteal (3).

2.4.3. Therapy

Atherosclerosis:

Surgeons can perform a balloon angioplasty, insert a stent into the blood vessel; do a bypass surgery or mechanicaly disrupt or displace the thromubus. Another possiblity is local fibrinolysis, the lysis of the thrombus via medicaments (3).

Aneurysm:

One possibility to treat an aneurysm is to insert implants into the vessel, which will close the blood flow through the aneurysm. Small aneurisms can be closed by injection of thrombin (3). Kurosawa, et al. reported different methods for treating abdominal aortic aneurysm. These methods include surgical repair; endovascular repair, anti-hypertensives, statins and antibiotics (8); (13). The beta- blocker propranolo, is thought to lower the blood pressure and to have an effect on the matrix proteins. Statins stabilize the serum lipid level, have anti-inflammatory effects and thereby prevent the progression of atherosclerosis, one reason of aneurysm formation. Angiotensin-converting enzyme inhibitors can inhibit and stimulate MMPs, a case study showed a preventive effect against rupture of the aneurysm in patients. Angiotensin receptor blockers are reported to decrease the matrix degeneration in Marfan syndrome. Macrolides and Doxycycline are antibiotics that can be used to treat abdominal aortic aneurysm. Macrolides work against *Chlamydia pneumonia* and Doxycycline might work as an inhibitor of MMPs (8).

For cerebral aneurysms clipping, coiling and the use of flow diverters are established methods (7), (12). Clipping is an open surgical repair, performed by a craniotomy and clip ligation of the aneurysm. Coilling is the endovascular occlusion of the aneurysm with the use of detachable coils (7). Flow diverters are flexible constructs with a mesh that divert the blood flow away from the aneurysm and allow the formation of a thrombus inside the aneurysm (12).

2.5. Lasers in medicine

Different kinds of lasers can be used for different tasks. Carbon dioxide laser are used as a non-contact scalpel for superficial tissue ablation. The excimer laser is useful for reshaping corneas and the flash lamp pumped dye laser to close small blood vessels (14).

Thermal laser therapy

The CO_2- Laser (10600nm) is a useful tool as a non contact scalpel for inaccessible areas like the brain or the upper airways. It is used for the ablation of small lesions. Because the beam of the laser cannot be transmitted via flexible fibres, it can only produce heomostasis in vessels well below 1 mm diameter (14). Laser that work in the near infrared like the Nd:YAG laser (1064 nm) and the semiconductor diode laser (805 nm) can produce effects through up to 10 mm of tissue. Nd:YAG lasers can vaporize tissue when high power is applied; coagulation of the tissue with hemostasis can be achieved, and will heal with fibrosis or slough. Nd:YAG lasers can be transmitted via thin fibres (14).

Interstitial laser photocoagulation

The fibres which guide the laser light to lesions in solid organs go through percutaniously inserted needles (14). The diseased tissue can be gently coagulated (using a 3 W-laser), so dead tissue can be resorbed by healing mechanisms. Advantages of this method are no effects on the overlying normal tissue no cumulative toxicity, no surgical wound and no scar formation (Brown, 1998). This technique is used to treat heatic metastases, breast cancer, benign adomas for small isolated metastases or adenoma removement (14).

Photodynamic therapy

This method involves the administration of photosensitising drug and followed by treatment with low power red light usually from a laser. Both collagen, and the elastin of connective tissues remain largely unaffected. So there is less danger to the mechanical integrity of the hollow organs. Healing takes place with more regeneration and less scarring occurs. Because one has to deliver both, the drug and the light, and a close collaboration between scientists and clinicians is needed, the therapy is more complicated (14). Photodynamic therapy is most useful for the treatment of early invasive cancers like localised cancers of the oral cavity, small cancers of the major airways, oesophagus, stomach and colon and cancer of the prostata and pancreas. It is also used to treat dysplasia in the mouth, oesophagus , major bronchi, bladder and vulva and skin conditions like basal cell carcinoma, actinic keratoses and psorieasis (14). With this technique, it is possible to achieve necrosis of mucosa without damaging the underlying muscle. The risk of incontinence and impotence when cancers of the postata are treated can be reduced. Treatment of localized infections may be possible, because many bacteria take up photosensitiser and can be killed with red light (14).Possible photosensitisers are porfirmer sodium (Photofrin), meso-tetra hydroxyphenyl chlorin (mTHPC, Foscan) and 5-amino

leavulinic acid (ALA, Levulan) (14).Drawbacks of the method are, that it cannot be used for tumors that spread beyond the wall ot the organ of origin; treating of sensitive areas (mouth, skin) may be painful, healing take several weeks and photosensitiesers can cause some skin photosensitivity to sunlight (from 2 days to 3 month) (14).

Low-level laser therapy

Low-level laser therapy can induce cellular proliferation, stimulate mitochondrial activity, increase ATP production, synthesis of DNA and RNA. It can also activate signalling cascades including the production of ROS and NO release, activate caytochrome c oxidase and modify intracellular organelle membrane activity, calcium flux and expression of stress proteins. Other effect can be reduction of pain, anti-inflammatory effects and support of wound healing (15). “Low intensity laser irradiation has been shown to induce stem cell activity by increasing migration, proliferation and viability, activating protien expression and inducing differentiation in progenitor cells” (15).

Lasers and the treatment of vascular disease

After ballon angioplasty or insertion of a stent, restenosis of the treated blood vessel is a problem. Stenosis is the narrowing of the the lumen of blood vessels (11) , restenois is the narrowing of the lumen after treatment of narrowed blood vessels. Restenosis is related to proliferation of smooth muscle cells from the media. Experiments have shown that the use of 5-amino leavulinic acid in pthotdynamic therapy can suppress the cell proliferation without increasing the risk of thrombosis or weakening the mechanical strength of the arterial wall (14).

2.3. Bioreactors

Bioreactors are dynamic culture devices that provide a native like culture environment and allow to monitor and control the physicochemical environment. This devices can provide a wide range of physical stimuli and enables the adaption of culture conditions (16). Bioreactors used for Tissue engineering are dishes and flasks, Spinner flasks and tissue specific Bioreactor systems for tissues like cartilage, ligament, cardiovascular tissue (17). A commercial bioreactor for human skin exists (14), (17). Advantages of bioreactors are a lower contamination risk, higher reproducibility, the supply of a closed system, the existence of standardized techniques, almost operator independent working, a greater traceability and a greater scalability. The systems have to mimic mechanical load, perfusion pressure and hormonal stimulation of the native tissue (16).

2.3.1. Vascular Bioreactors

Bioreactors can provide appropriate biochemical and mechanical stimuli in a controllable environment (18). For arteries, pulsatile perfusion bioreactors are used. Devices for the cultivation of cardiomyocytes can apply unidirectional stretch to the material. Autologous vascular cells can grow on tubular, biodegradable scaffolds, while the bioreactors apply pulsatile radial distension in a controlled manner (17).

The first engineered vascualar graft model was cultured in a fixed wall reactor in 1986 by Weinberg and Bell. In 2003, Nasseri et al. used a bioreactor with continuous flow and perfusion of the vessel lumen. Mironov combined perfusion with continuous ore pulsatile flow and functional capacity for longitudinal strain in 2003. They used periodic variations of longitudinal strain. A combination of pulsatile flow with perfusion inside and outside the lumen was created by Bilodeau in 2005. It was used for 3D regeneration of arterial tissue on a cylindrical scaffold (16).

2.3.2. Bioreactors and drug testing

The use of bioreactor allows make automated, repeatable, scalable and clinically sustainable biological processes possible (16). Therefore they can be used as a model system for the investigation of cell functions in specific environmental conditions like concentration of oxygen, CO_2 and nutrition , biomechanical factors, hydrodynamic conditions, physical stimuli (16). The liver is a central organ for detoxification, metabolism, excretion of drug and for this reason it is often used for drug testing (19) (20). A hollow fiber perfusion reactor with a volume of 0.5 ml for drug testing was created, using human liver cells. The bioreactor is made of multiple interwoven capillary layers for gas and medium exchange . (19) (20). Darnell and colleagues used it for identification of enzymes involved in drug metabolism (20). Riches et al developed a bioreactor for the abdominal aortic aneurysm using the carotid artery of pigs. They focus on smooth muscle cells (21).

2.3.3. *In vivo* and vitro models for aneurysms

Investigation of aneurysm can be performed with animal models and *in vitro* models. Animal models are more cost and time consuming, but provide a complete biological environment and native hemodynamic conditions (22). Animal models used for *in vivo* studies of blood vessels are pig, rabbit, mouse, rat, turkey and dog (23). Each species has advantages and disadvantages when used as a model for human aneurysms. For example arteries of pigs show similarities to human tissue regarding the number of elastic lamellae and the relative proportions of elastin, collagen and SMC (9) but the high costs for animal husbandry and the large size of the animal make it difficult to perform studies with a high number of individuals (23). In Table 1 advantages and disadvantages of different animal models are listed.

Table 1: Advantages and disadvantages of animal models (23).

Table 1
Advantages and disadvantages of different animal species for vascular research

Animal	Advantages of species	Disadvantages of species
Mouse	Relatively low costs and small size of species permit maintenance of large animal stocks	Physical size of the vasculature very small, hindering surgical manipulation
	Relatively easy to handle	Genetically manipulated models can be expensive to purchase
	Well-characterized genome facilitates genetic manipulation	Unlike humans, mice most frequently develop AAAs in the suprarenal aorta
	Males exposed to angiotensin II are more susceptible to AAA than females [25]	Small quantities of tissue to analyze
Rat	Relatively low costs and small size of species permit maintenance of large animal stocks	Genetic manipulation is difficult compared to mice [26]
	Larger size of vessels compared to mice facilitates surgical manipulation and generates large amounts of experimental tissue	
	Relatively easy to handle	
Canine	Large peripheral arteries	Poor public perception of studies based in canine species
	Able to survive prolonged anesthesia	High cost and large size may prohibit investigations using large numbers
	Show a lack of spontaneous endothelization of prosthetic surfaces in a similar manner to humans [27]	Variable aneurysm formation following elastase perfusion [28]
Swine	Similar arterial morphology to humans	Variable aneurysm formation following elastase perfusion [28]
		Peripheral arteries are smaller than those of canine species
		Relatively difficult to handle
		High cost and large size may prohibit investigations using large numbers
Rabbit	Relatively low costs	Poor public perception of studies based in rabbit species
	Relatively easy to handle	
Sheep	Large arteries	High cost and large size may prohibit investigations using large numbers
	Coagulation system is closer to humans than that of the swine or canine [27]	
Turkey	Form spontaneous aneurysms [30]	
	Male birds are predisposed to lesions [29]	

Several methods for the induction of aneurysm including chemical induction, surgery or genetic modifications of the animal have been described (23). Chemicals which are used to create an *in vivo* model are elastase, calcium chloride and angiotensin II. When using elastase, the blood vessel is perfused with an elastase solution for a certain time to induce the aneurysm. A method using a calcium chloride treated gauze to induce blood vessel dilations has been described. The gauze is exposed directly to the *tunica adventitia* of the blood vessel for 10 min and removed afterwards. Angiotensin II infusions are performed via subcutaneous micro pumps. Surgical methods can be used to alter the blood flow, replace a part of an artery with a vein patch or replace a part of the blood vessel with an equivalent from another species. Blood flow alterations can be achieved by artificial reduction of the blood vessel diameter, increasing the velocity of the blood flow and the turbulence, or ligation of arteries, thus increasing the blood volume passing the blood vessel. The

increased wall shear stress can induce an aneurysm. Genetically modified animal models described in the literature are deficient in the apolipoprotein E, thus creating a hyperlipidemic mouse model. In addition these mice are deficient in TIMP or Blt1 genes. TIMPs are inhibitors of MMPs, which play a key role in the formation of aneurysms. Blt1 is a receptor with a high affinity to leukotriene B4 and induce a signalling pathway which results in the production of MMPs. Another described method is the angiotensin II infusion of blood vessels in apolipoprotein deficient mice (23).

In vitro models are less cost and time intensive, but have limited abilities to mimic the *in vivo* situation in humans. These models are used to investigate the blood flow in aneurysmatic blood vessels (24) and test medical devices like stents (25) and surgical techniques like coiling (26). Commonly used models range from bare polymer tubes (26) , polymer tubes sodded with endothelial cells, so called blood vessel mimics (25) to organ cultures of blood vessels from animals (9). Deplano used a fluid, consisting of 350 ppm Xanthan gum, 0,9g/ L sodium salt and 20 % glycerin (w/w) to mimic the flow dynamics of blood and polymer tubes made of estane. The blood analogous show similar behaviour to blood under high shear rates, but flow dynamics of blood differs under low shear rates due to the higher viscosity of blood under low shear stress (24). Baráth et al used a blood analogous consisting of 33 % glycerol and 66 % water, a Newtonian fluid. Blood behaves as a Newtonian fluid in blood vessels larger than 0.5 mm in diameter (27).

3. Materials and Methods

3.1. Materials

3.1.1. Bioreactor system

The bioreactor system consist of two parts, a vessel unit and a continuously stirred tank reactor (UniVessel). A detailed description of the system is given in figure 1. The UniVessel is used as a medium reservoir. oxygen, pH and temperature probes are placed in the UniVessel and connected to a supply tower and control panel. A six blade flat radial turbine inside the UniVessel ensure adequate mixing of the culture medium. The air inlet of the UniVessel is connected to the CO_2 and O_2 supply for aeration. Incoming air enters the bioreactor via a sparger ring. Air filters ensure the sterility of the incoming and out-going air. A condenser is placed before the air outlet of the stirring bioreactor. The cooling loop of the condenser consists of a bottle which is placed on ice and a peristaltic pump. With this system the water inside the bottle is cooled down to the required temperature and and then pumped from the bottle to the condenser and backwards. Silicon and marprene tubes are used to connect condenser and bottle. Marprene tubes are used because they are more resistant to the forces appearing during the pump process. Temperature, pH, agitator speed, CO_2 and air inlet can be monitored via a control panel. Gas inlet, agitator speed and a temperature plate under the UniVessel can be controlled via the control panel.

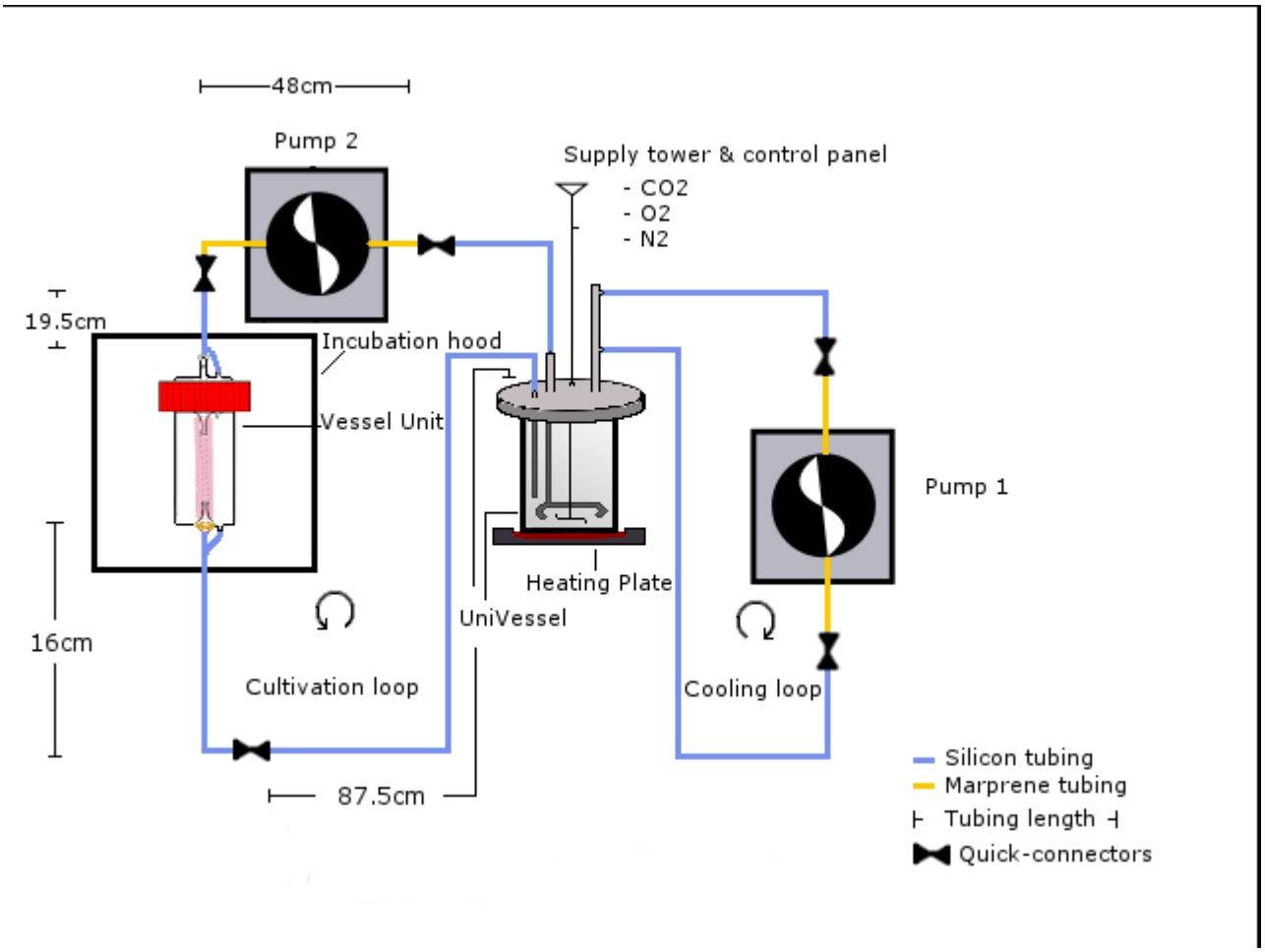

Figure 1: Setting of the bioreactor system (28). The UniVessel is used as a medium reservoir. Medium is pumped from the UniVessel to the vessel Unit and backwards. Aeration, agitation of the medium and temperature control take place in the UniVessel via a gas supply, a stirrer and a heating plate under the Univessel. The cooling loop ensures an adequate temperature of the condenser. The vessel unit is placed in an incubation hood. The artery is placed in the vessel unit (pink tube).

The vessel unit is placed in an incubation hood for temperature control. As shown in figure 2 the vessel unit is a glass pipe with two inlets and two outlets. One outlet and one inlet are realized as glass tubes inside the glass pipe. These glass tubes are used to fix the blood vessel in between (pink tube in figure 1). A Y connection is used to divert the incoming medium flow to a flow inside the lumen of the blood vessel (Inlet inner part (Ii); see figure 2) and a medium flow at the adventitia side of the artery (Inlet outer part (Io) see figure 2). A Y connection is used to reunion the flow after the vessel unit.

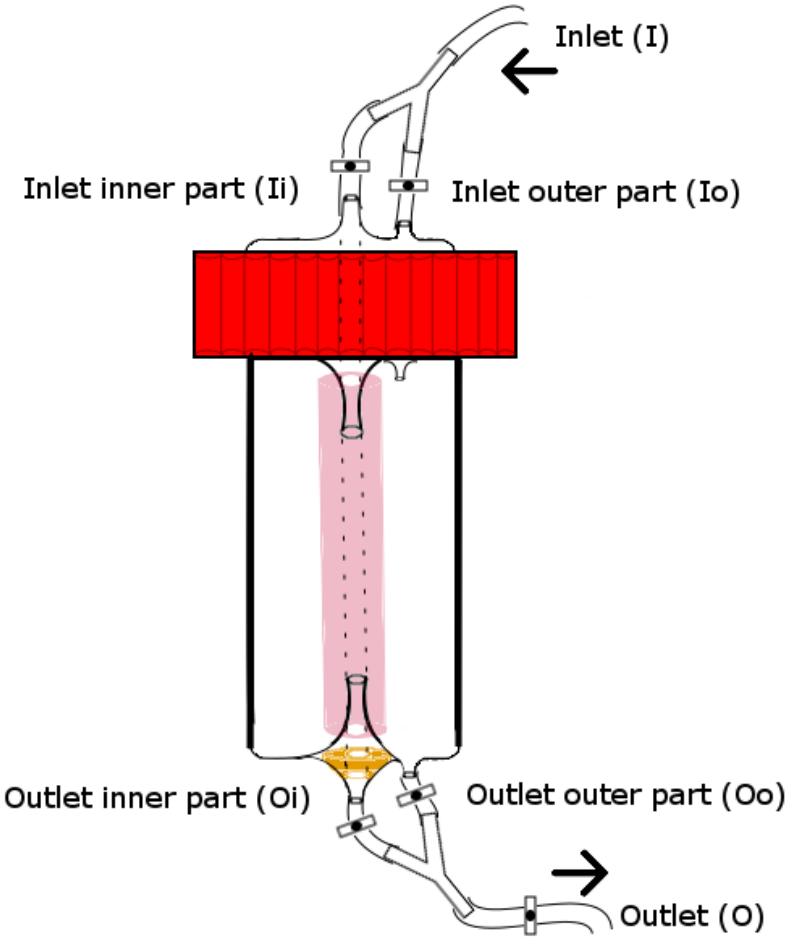

Figure 2: Structure of the vascular bioreactor (28). The artery (pink) is placed between the inlet (Ii) and the outlet (Oi) of the inner part. Incoming medium flow is diverted into a flow inside the artery (inner part) and a flow outside the artery (outer part). At the outlet of the vessel unit the two flows are reunited.

Vessel unit and UniVessel are connected via quick connectors to marprene and silicon tubes. The medium flow is regulated via a peristaltic pump.

3.1.2. Laser System

Laser sections are performed with the Tissue Surgeon from ROWIAK (figure 3). This systems works with an Amplitude T-Pulse 500 laser at a wavelength of 1030 nm and a pulse of 300 fs.

Figure 3: Laser system provided by ROWIAK

3.1.3. Solutions

Solutions used for this project are listed in Table 2.

Table 2: Media used for this study

Name	Ingredients
Culture medium	10 % (v/v) FCS, 1 % (v/v) Glutamax;1 % (v/v) Penicillin/ Streptomycin in DMEM
Stock solution MTT	5 mg/ml MTT
5x CASO Bouillon	0,15 g/ ml dissolved in distilled water
Blocking solution for immunohistochemistry	1 g/L BSA, 0.05 % (v/v) Tween 20 and 10 % (v/v) FCS in PBS
PBS-1 % BSA	1 g/L BSA in PBS
PBS-0.05 % Tween	0.05 % (v/v) Tween 20 in PBS
Weigert's iron hematoxylin	50 % (v/v) Weigert's solution A and 50 % (v/v) Weigerts solution B
Phosphotungstic/Phosphomolybdic acid	25 % (v/v) Phosphotungstic acid; 25 % (v/v) Phosphomolybdic acid and 50 % (v/v) distilled water
1 % acetic acid	1 % (v/v) Acetic acid in distilled water.

3.2. Methods

3.2.1. Isolation and preparation of arteries

Carotid arteries were taken from 6 month old pigs from the local abattoir. The arteries were removed using sterile gloves and a sterile scalpel and transferred to a 50 ml tube containing culture medium (DMEM with 10 % FCS, 1 % Glutamax, 1 % Penicillin-Streptomycin) at room temparature. After 20 min of transport, the tissue was dissected under sterile conditions. Fat and surrounding tissue were removed. For treatment with the laser system or enzymes the tissue was turned inside out using autoclaved pasteur pipettes and cable strep. The cable strep was inserted in the artery and two sterile forceps were used to turn half of the artery inside out. The sterile pasteur pipette and a sterile forceps were used to push the other half of the tissue inside out. This procedure was performed in a petri dish containing culture medium (DMEM with 10 % FCS, 1 % Glutamax, 1 % Penicillin-Streptomycin). All procedures were performed at room temperature.

3.2.2. Sterility test

To ensure the sterility of the tissue a piece of the artery was minced using forceps and a scalpel in a Petri dish under sterile conditions. The minced tissue was transferred to sterile 50 ml tube filled with 30 ml of 5 x CASO bouillon. The incubation was performed in an incubator at 37 °C. After 14 days the turbidity of the CASO bouillon was assessed. Medium with turbidity indicated a contamination.

3.2.3. Viability of tissue

The MTT tetrazolium salt colorimetric assay was used to measure the viability of the isolated tissue. This assay is based on the cleavage of the yellow tetrazolium salt to an insoluble purple formazan dye by living, metabolically active cells.

To determine the viability of the isolated tissue, a piece of tissue was cut under sterile conditions and minced in a petri dish. 50 ± 10 mg of tissue were transferred to an autoclaved and weighed i 1.5 ml eppendorf tube. The weight was determined with a fine balance. The tissue was covered with 400 ml of PBS with calcium and magnesium. To start the MTT assay 200 ml of a MTT solution (5 mg/ml MTT dissolved in PBS with calcium and magnesium) was added and the tissue was incubated for 4 h at 37 °C and 300 rpm in a thermomixer. Afterwards the tubes were centrifuged at 14.000 g for 5 min and the supernatant was removed. 1ml of DMSO was added under sterile conditions and the tissue was incubated for 17 h (300 rpm, 37 °C). The samples were centrifuged at 14.000 g for 5 min and absorption of 100 µl of supernatant mixed with 100 ml of DMSO measured at and 570 nm and 620 nm.

3.2.4. Laser treatment

Arteries were turned inside out and put in a cell culture flask with 90 ml of culture medium. After transport (45 min at room temparature), the samples were put in a homemade glass chamber and cut with the laser system. An area of 3,5 x 5 mm was cut up to a depth of 150 µm. The cut was performed twice with an energy of 300 nJ and a power of 2546 mW. The tissue was removed with an eye scissor and forceps. To visualize the cut pieces of tissue were left around the cut.

3.2.5. Enzyme treatment

Arteries were treated locally with 1 ml of a low melting point agarose, containing Collagenase (380 CDU /ml) and Elastase (25 U/ ml or 75 U/ml), using a paint brush. After four hours of incubation in 37 °C the enzyme gel was removed and samples were washed in HBSS with 10 % FCS for 45 min (100 rpm on a shaker). The gel was applied at the adventitia or the intima of the blood vessel. Incubation was done with medium and in the absence of culture medium.

3.2.6. Cultivation

Dynamic cultivation was performed with the bioreactor. The bioreactor system was cleaned before use as follows: Korsolex (30 min), distilled water (10 min), mucasol (30 min), distilled water (10 min), 70 % Ethanol and distilled water (10 min). After cleaning the bioreactor system was separated in two parts using quick connectors. The quick connectors and air filters were covered with aluminium foil and fixed with autoclave tape. After autoclavation (121 °C for 45 min) the system was left to dry at 60 °C for 3 days. Afterwards, the UniVessel was filled with 400 ml of culture medium with 0.0005 % PPG. The arteries were fixed on barbed fittings inside the vessel unit using a surgical suture. Vessel unit and UniVessel were connected to the BIOSTAT II system and pumps. The cultivation has been performed for 5 days. Chosen parameters were a flow rate of 262 ml/min, an air inlet of 95 ccm oxygen and 5 ccm carbon dioxide, a temperature of 37 °C and an agitator speed of 30 rpm. Static cultivation was performed in a cell culture flask. The artery was cultivated with 90 ml of culture medium in the cell culture flask in an incubator.

3.2.7. Immunohistochemistry

Samples were covered with the cyro-embedding media OCT on a cold plate and stored in at -18 °C wrapped in aluminium foil until sectioning. The OCT blocks were cut in a cryo-microtome into 7 µm thick sections which adhere to the slide. After each cut, the slides were put in solution of 99.5 % Ethanol for 5 sec. Up to three sections were put on a slide. The slides were air-dried for 30 min. After fixation using -20 °C cold Acetone for 8 min samples were air-dried for 10 min. Sections were rehydrated for 5 min in PBS with 1 % BSA followed by an incubation with 100 µl of blocking solution (1 % BSA, 0.05 % Tween 20 and 10 % FCS in PBS) per slide for 30 min to block unspecific binding. Three washing steps with PBS-1 % BSA followed. For incubation with antibodies the slides were put in a chamber with a lid with moist tissue at the bottom of the chamber. The tissue slides were incubated with the respective primary antibody diluted in PBS-1 % BSA. For the CD31 antibody a dilution of 1:500 was used, for the myosin heavy chain antibody a dilution of 1:100 was used. 100 µl of antibody solution was used per slide. After incubation for 60 min at room temperature samples were washed three times with PBS with 0.05 % Tween 20 for 5 min at room temperature. Incubation with the secondary antibody Alexa Flour 555 goat antibody IgG followed. The second antibody was diluted 1:250 in PBS and incubation was performed at room temperature for 45 min in a chamber with a lid to ensure light protection. Slides were washed 3 times in PBS-0.05 % Tween for 5 min and mounted. After 3 days in the fridge the samples were investigated under the fluorescence microscope.

3.2.8. Histology

Samples were fixed in 10 % neutral buffered Formalin for 3 h at room temperature. After fixation the tissue was embedded in paraffin blocks. The blocks were cooled down for 30 min in at -18 °C and than sections of 7 µm were cut and put in a 37 °C water bath.

Afterwards 2 sections were put on a slide. The slides were dried on a heat plate for 24 h, deparaffinated, stained and rehydrated.

For deparaffination, staining and rehydration of the samples 200 ml of solution in a glass cuvette was used for up to 10 slides. To deparaffinate the samples Xylene and a row of decreasing Ethanol concentrations were used as follows: 2 times in Xylene for 10 min, 2 times in 99.5 %, Ethanol for 5 minutes, 2 minutes in 95 % Ethanol, 2 min in 70 % Ethanol and 1 min in distilled water.

A Masson Trichrome Staining and Elastica van Gieson Staining were used to analyse the histoarchitecture of the samples. For the Masson Trichrome staining following procedure was used after they were deparaffinised : 5 min Weigert's iron hematoxylin (50 % Weigert's solution A and 50 % Weigerts solution B), 5 min running tap water, 1 min distilled water, 6 min Biebrich scarlet-acid fuchsin, 1 min distilled water, 5 min Phosphotungstic/Phosphomolybdic acid, 5 min Aniline blue, 2 min 1 % Acetic acid, 1 min distilled water and rehydration. The steps in the protocol used for the Elastica van Gieson staining after deparaffination were 11 min Weigert’s resorcinfuchsin, 1 min running tap water, 5 min Weigert’s iron hematoxylin (50 % Weigert's solution A and 50 % Weigerts solution B), 1 min running tap water, 2 min Picrofuchsin, 1 min 70 % Ethanol and rehydration.

For rehydration samples were washed with solutions of increasing Ethanol concentration and Xylene: 5 min 95 % Ethanol, 2x 5 min 100 % Ethanol and 2x 5 min Xylene. After rehydration the slides were mounted with Corbit Balsam and examined under a light microscope.

3.2.9. Statistical analysis

Statistical analysis was performed with Word Excell 2010. A one factor ANOVA was perfomed following the T-Method.

4. Results

4.1. Disinfection of arteries

After dissection the arteries need to be disinfected to ensure sterile conditions but treatment with antibiotic can also affect the viability of the tissue. To find the optimal disinfection method three different antibiotic solutions were tested. 1) A mixture of 250 µg/ml Amphotericin B, 0.5 mg/ml Gentamycin, 10 % FCS and 1 % Glutamax in DMEM; 2) a solution containing 0.5 mg/ml Gentamycin,0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in PBS and 3) a solution containing 0.5 mg/ml Gentamycin,0.2 mg/ml Polymixin B , 0.05 mg/ml Vancomycin , 10 % FBS and 1 % Glutamax in DMEM. The tissue was incubated with the respective antibiotic solution for 30 min at 37 °C in an incubator. Prior and following antibiotic treatment the viability of the tissue was determined using the MTT assay. Tissue treated with Formalin at 37 °C for 30 min was used as a negative control.

A solution containing 250 µg/ml Amphotericin B, 0.5 mg/ml Gentamycin in DMEM with 1 % Glutamax and 10 % FBS did not show an effective disinfection. Arteries treated with this solution were contaminated. The contamination is relfected by the "increase" in the viability of the tissue which can be seen in the MTT assay (see figure 4).

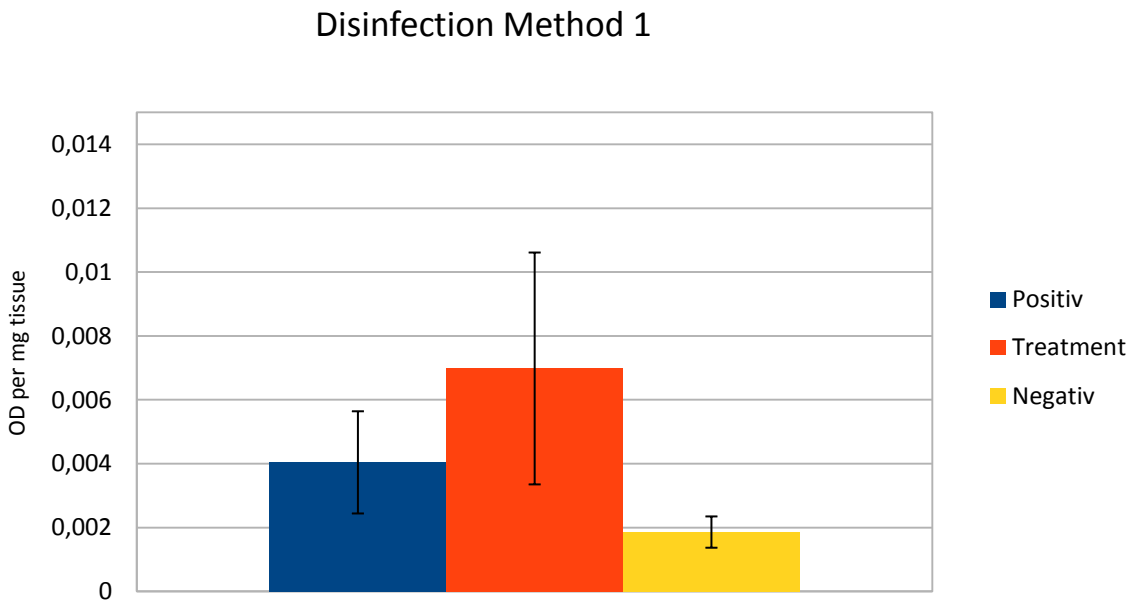

Figure 4: Viability after disinfection for 30 min with 250 µg/ml Amphotericin B and 0.5 mg/ml Gentamycin in DMEM with 1 % Glutamax and 10 % FBS. Arteries showed an increase of the tissue viability after disinfection. This indicates a contamination of the tissue and an inadequate disinfection. Positiv= native tissue, Treatment= Viability of tissue after disinfection; Negativ= Tissue incubated in Formalin for 30 min at 37 °C

Antibiotic treatment with a solution of 0.5 mg/ml Gentamycin, 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in PBS showed an effective disinfection. No contamination was observed after two weeks of sterility test. The MTT assay showed a statistically significant decrease of viability (see figure 5). This decrease might be due to a lack of nutrition. Using a solution with DMEM, FBS and Glutamax can improve the results.

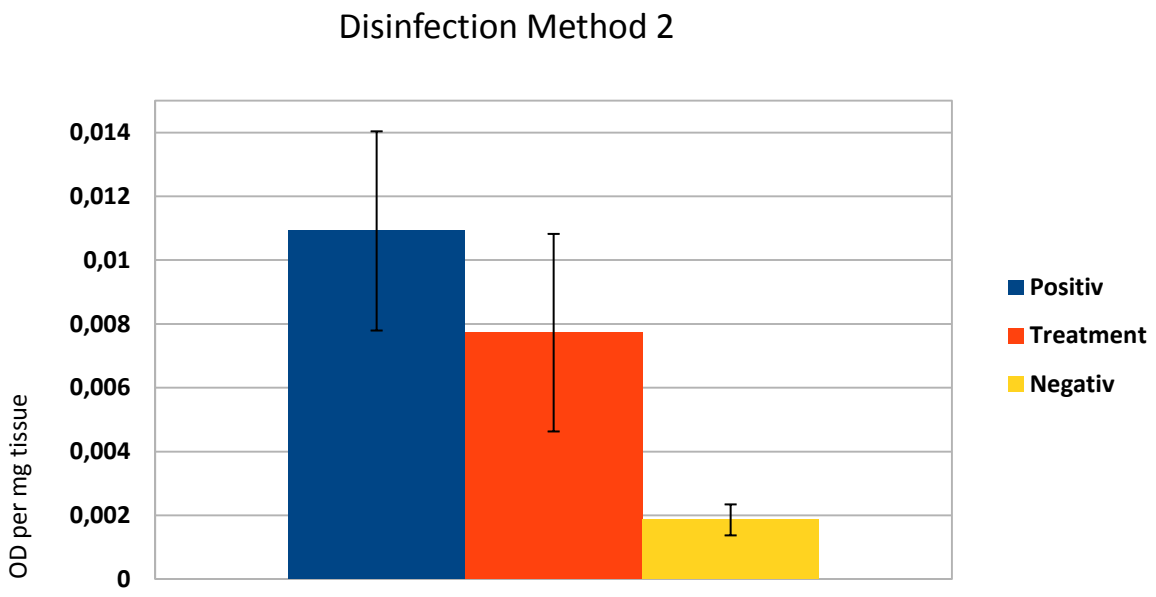

Figure 5: Viability after disninfection for 30min in 0.5 mg/ml Gentamycin; 0,2 mg/ml Polymixin B and 0,05 mg/ml Vancomycin in PBS. The tissue viability after disinfection was significantly decreased. A significant difference between the tissue viability after disinfection and the negative control was observed. Positiv: viability of native tissue; Treatment: viability of tissue after disinfection. Negativ: Viability of tissue incubated at 37 °C for 30min in formalin.

To test whether the decrease of viability using 0.5 mg/ml Gentamycin,0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in PBS was be due to a lack of nutritions, a solution with DMEM, FBS and Glutamax was tested. Viability tests of arteries treated with an antibiotic solution with 0.5 mg/ml Gentamycin,0.2 mg/ml Polymixin B, 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax showed no statistically different decrease of viability of the tissue after disinfection (see figure 6). The sterility test showed no contamination of the blood vessels. Thus this method was used as disinfection method for further cultivation experiments.

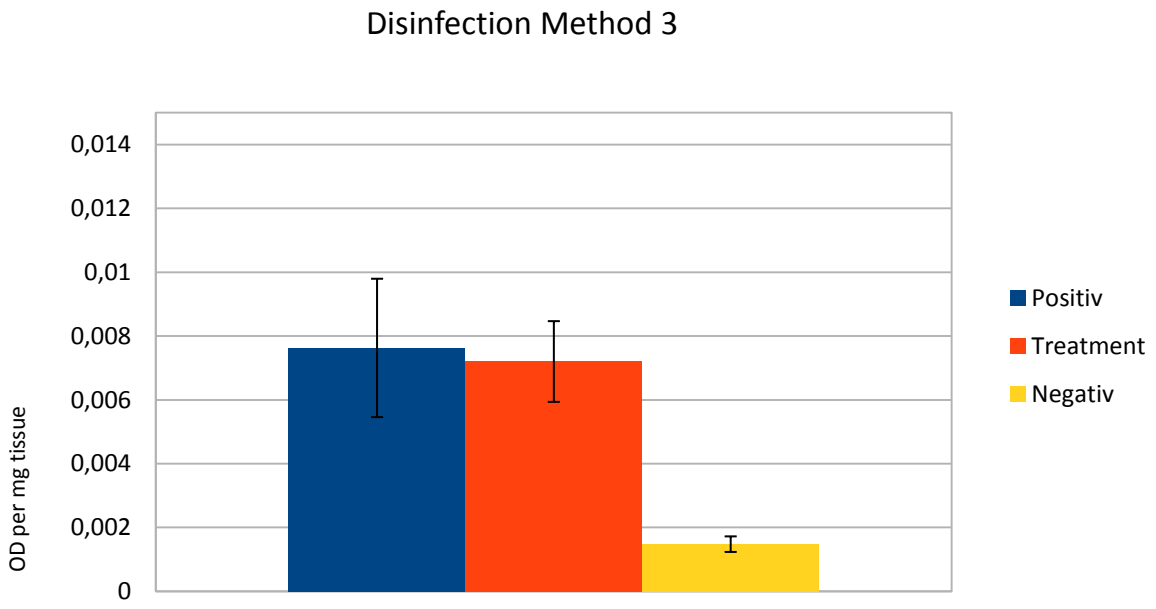

Figure 6: Viability after disninfection for 30min with 0.5 mg/ml Gentamycin; 0,2 mg/ml Polymixin B and 0,05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax. Tissue viability was not significantly decreased after disinfection. Positive: Viability of native tissue. Treatment: Viability of tissue after disinfection. Negative: Viability of tissue incubated in formalin (30 min; 36 °C)

4.2. Enzyme Test

4.2.1. Collagenase

The effect of the treatment of arteries with an enzyme gel containing 380 CDU of collagenase was investigated. Therefore, arteries were covered with the gel using a brush and incubated for four hours at 37 °C. The gel was applied at the adventitia or the intima of the arteries. The incubation was done with or without culture medium. Using the medium can dilute the enzymes and decrease the effect but the absence of the medium can affect the viability of the tissue.

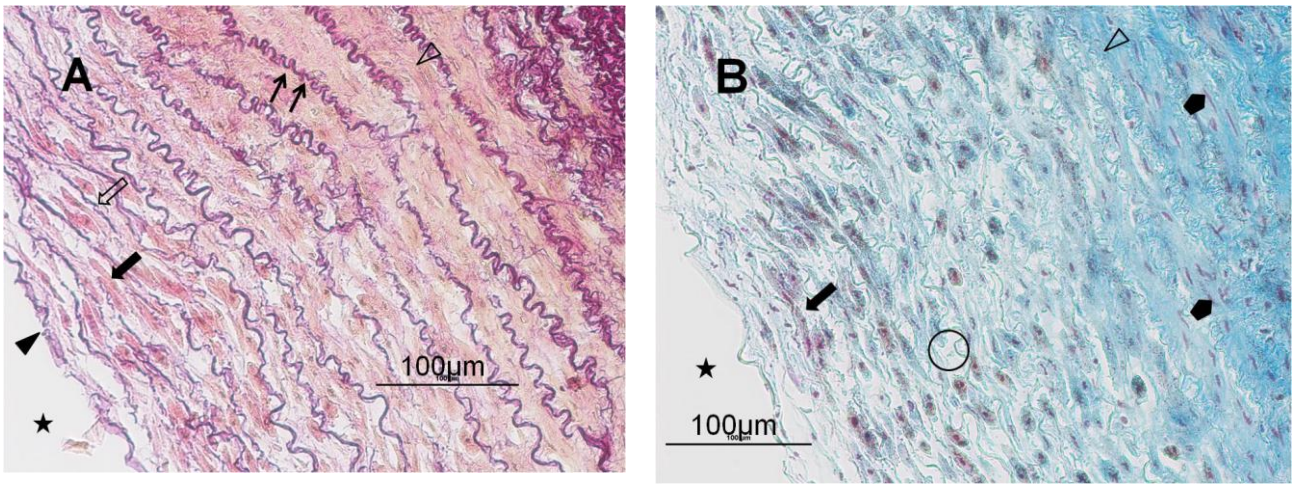

Figure 7: Elastica van Gieson(A) and Masson Trichrome staining (B) of a porcine common carotid artery treated with collagenase at the intima in the absence of medium. Stars indicate the lumen of the blood vessel. A: The tissue showed degradation of the membrane elastic interna (filled triangle) and disrupted elastic fibres (empty arrow). The smooth muscle cells detached at the intimal side of the media (filled arrow). The tissue near the adventitia showed intact elastic fibres (two arrows) and collagen (empty triangle). B: Smooth muscle cells near the intima were detached (filled arrow) but intact near the lumen (pentagons). Collagen is intact near the adventitia (empty triangle) but areas without collagen were observed near the intima (circle). The tissue was treated with a LMP agarose with 380 CDU/ml of collagenase at the intima. The tissue was incubated for 4 h at 37 °C, gel was removed afterwards and the sample was washed with HBSS with 10 % FCS for 45 min. the enzyme and decrease the effect but in the absence of medium the viability of the tissue can be effected.

Masson Trichrome and the Elastica van Gieson staining were used to examine histological changes of the tissue. Applying the gel at the intima of the blood vessel in the absence of culture medium showed a significant destruction of the tissue as shown in figure 7. The collagen showed empty areas. Cells of the *tunica intima* detached from the collagen matrix of the tissue. The destruction showed a gradient from the endothelium to the adventitia. Elastic fibres presented fragmentation at the intimal side of the media and normal shape at the adventitial side of the media. The *membrana elastic interna* was fragmented. Evenly distributed cells were observed in the media.

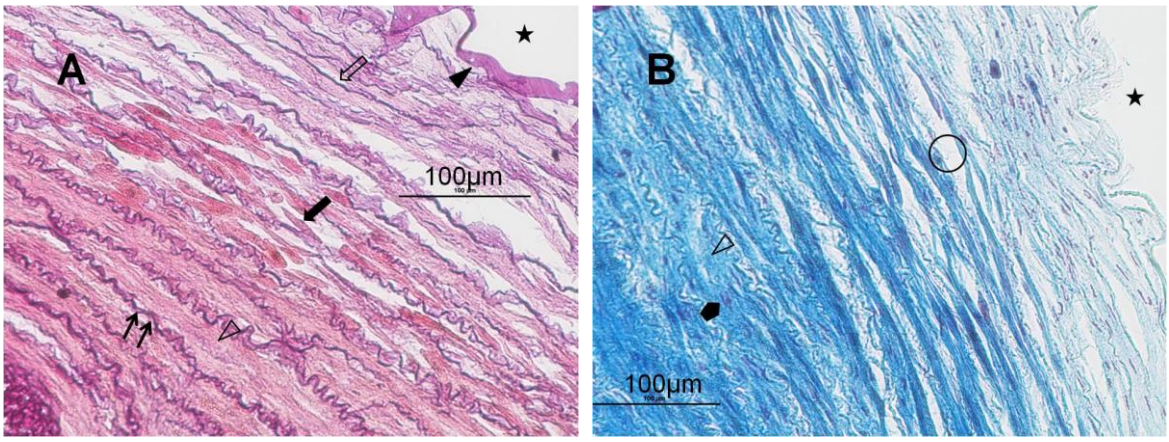

Figure 8: Elastica van Gieson (A) and Masson Trichrome staining (B) of a porcine common carotid artery treated with collagenase at the intimal side using medium. Stars indicate the lumen. A: Elastic fibres near the intima showed straightening (empty arrow), the membrana interna elastic detached (filled triangle), smooth muscle cells near the intima detached (filled arrow). Near the adventitia elastic fibres (two arrows) and collagen (empty trianlge) stayed intact. B: Near the intima areas without collagen were seen (circle), collagen matrix (empty triangle) and smooth muscle cells (nuclei shown by the pentagon) were intact. The tissue was treated with a LMP agarose with 380 CDU/ml of collagenase at the intima. The tissue was incubated for 4 h at 37 °C in culture medium, gel was removed afterwards and the sample was washed with HBSS with 10 % FCS for 45 min.

A decrease of the collagen matrix and a detachment of cells in the media and intima were observed when the gel was applied to the intima of the artery and the tissue was incubated in culture medium(see figure 8). The destruction of the collagen matrix was not as deep as in the experiment where the tissue was treated in the absence of culture medium. Areas free of matrix appear in the blood vessel wall. At the side of the adventitia the collagen matrix was intact. Elastic fibres showed straightening at the intimal side of the artery but were intact at the adventitial side of the media. *Membrana elastic interna* was detached and showed no fragmentation. Cells were evenly distributed in the media as seen in the native tissue.

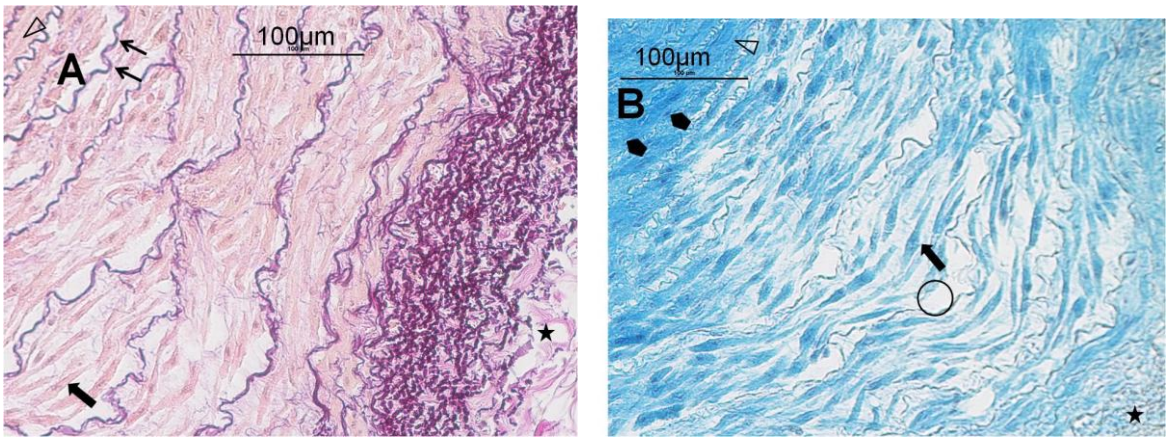

Figure 9: Elastica van Gieson (A) and Masson Trichrome staining (B) of a porcine common carotid artery treated with collagenase at the adventitia in the absence of medium. Stars indicate the adventitia. A: At the side of the intima collagen was intact (empty triangle). Elastic fibres (two arrows) and smooth muscle cells (filled arrow) detached. B: Collagen (empty arrow) and smooth muscle cells (nuclei shown by pentagons) stayed intact at the side of the intima. Near the adventitia smooth muscle cells detached (filled arrow) and areas without collagen appear (circle). The tissue was incubated with a LMP agarose containing 380 CDU/ml of collagenase at the adventitia. The tissue was incubated for 4 h at 37 °C, gel was removed afterwards and the sample was washed with HBSS with 10 % FCS for 45 min.

Destruction of the collagen matrix of the media was examined when the adventitia of the artery was covered with the gel. The sample was incubated in the absence of culture medium. Cells of the *tunica media* detached from the collagen matrix of the tissue. The greatest decrease in collagen was at the adventitial side of the media with less decrease at the intimal side. Elastic fibres showed straightening and were detached. Evenly distributed cells were observed in the media.

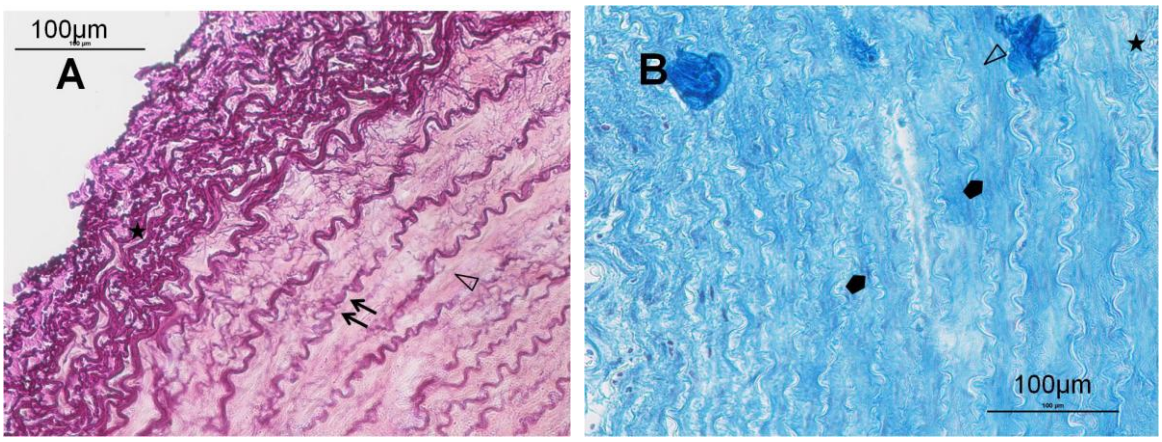

Figure 10: Elastica van Gieson (A) and Masson Trichrome staining (B) of a porcine common carotid artery treated with collagenase at the adventitia in the presence of medium. Stars indicate the side of the adventitia. A: Elastic fibres (two arrows) and collagen (empty triangle) were intact. B: Collagen (empty triangle) and smooth muscle cells(nuclei shown with pentagons) did not show differences to native tissue. The arteries were incubated with a LMP agarose containing 380 CDU/ml of collagenase at the adventitia. The tissue was incubated for 4 h at 37 °C in culture medium, gel was removed afterwards and the sample was washed with HBSS with 10 % FCS for 45 min.

No changes of the histioarchitecture of the blood vessel were detected when the enzyme gel was applied at the adventitia of the artery and the incubate was done in culture medium as can be seen in figure 10.These results suggest that the adventitia might act as a barrier for the enzyme.

4.2.2. Elastase

The effect of the elastase treatment was tested with a gel containing 25 U/ ml elastase. The gel was applied to the intimal and adventitial surface of the tissue and in the absence and presence of culture medium. Tissue treated in the absence of culture medium at the intima side of the artery showed a partial fragmentation of elastic fibres up to a depth of about 100 µm from the intima in the medial layer (see figure 11). Areas devoid of matrix were observed in the media. The *membrana elastic interna* was degraded. Tissue of the media near the adventitia showed intact elastic fibres and an intact collagen matrix. The number of cells in the media near the intima was decreased.

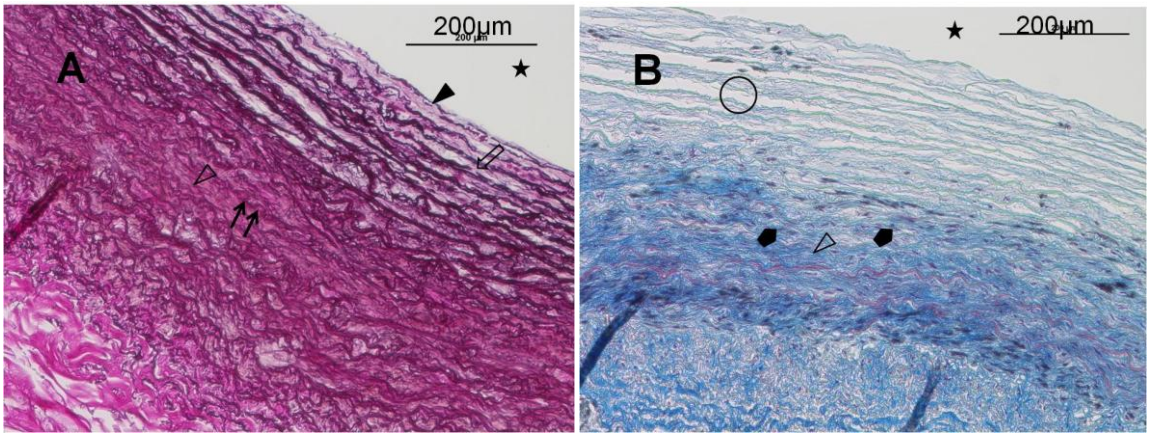

Figure 11: Elastica van Gieson (A) and Masson Trichrome staining (B) of a porcine common carotid artery treated with elastase at the intima in the absence of medium. Stars indicate the lumen. A: *Membrana elastic interna* (filled triangle) and elastic fibres (empty arrow) were degraded near the intima. Near the adventitia elastic fibres (two arrows) and collagen (empty triangle) were intact. B: The collagen matrix near the lumen showed empty areas (circle), smooth muscle cells were absent. At the adventitial side of the media, smooth muscle cells (nuclei indicated by pentagons) and collagen (empty triangle) were intact. LMP agarose with 25 U/ml of elastase was applied at the intima. The tissue was incubated for 4 h at 37 °C, gel was removed afterwards and the sample was washed with HBSS with 10 % FCS for 45 min.

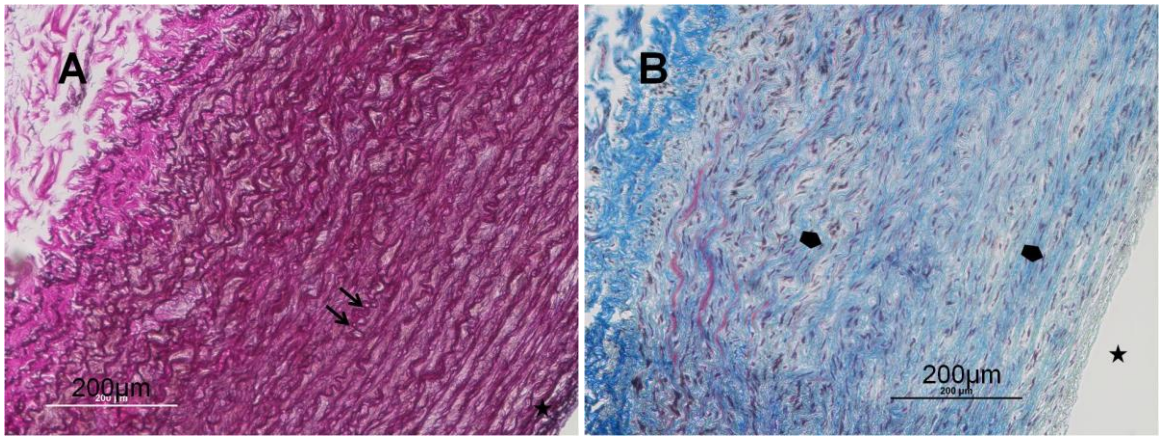

Figure 12: Elastica van Gieson (A) and Masson Trichrome staining (B) of a porcine common carotid artery. No changes to native arteries were seen. Stars indicate the side of the intima. A: Elastic fibres (two arrows) and collagen matrix were not affected. B: Smooth muscle cells (nuclei indicated by pentagons) were continuously distributed in the media. The tissue was treated with a LMP agarose containing 25 U/ml of elastase at the intima. The tissue was incubated for 4 h at 37 °C in culture medium, gel was removed afterwards and the sample was washed with HBSS with 10 % FCS for 45 min.

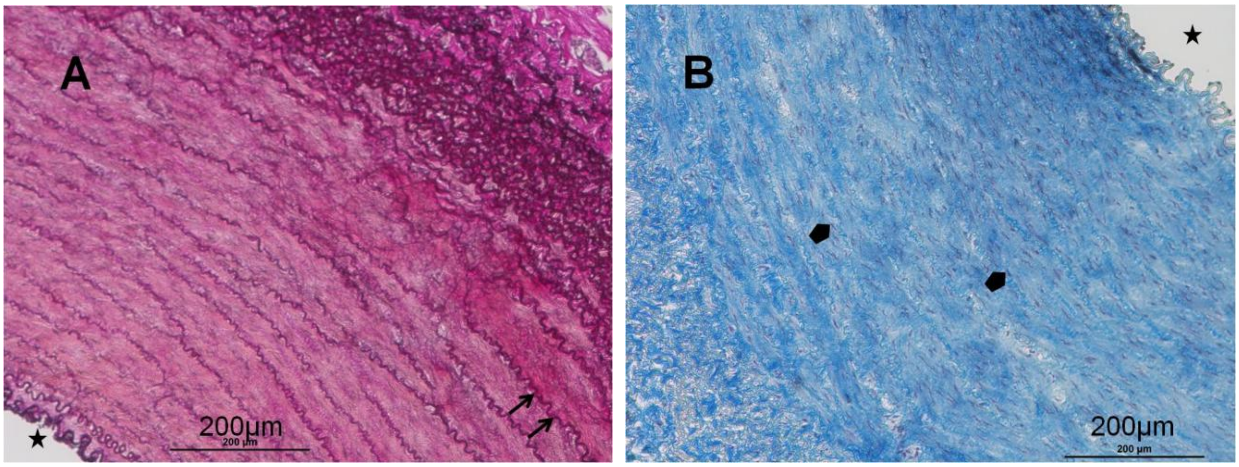

Figure 13: Elastica van Gieson (A) and Masson Trichrome staining (B) of a porcine common carotid artery treated with elastase at the adventitia in the absence of medium. No changes to native arteries were seen. Stars indicate the site of the lumen. A: Elastic fibres (two arrows), collagen matrix and intima showed no degradation. B: Smooth muscle cells (red nuclei indicated by pentagons) showed no difference to native tissue. The artery was treated with LMP agarose with 25 U/ml of elastase at the adventitia. The tissue was incubated for 4 h at 37 °C, gel was removed afterwards and the sample was washed with HBSS with 10 % FCS for 45 min.

Arteries covered locally with gel at the intima and incubated with culture medium showed no degradation of the elastin matrix as can be seen in figure 12. The *membrana elastic interna* and collagen matrix were intact. This might be due to the dilution of the gel by the culture medium. Cells were evenly distributed in the media.

Applying the gel at the *tunica adventitia* in the absence and presence of culture medium showed no effect on the elastic fibres and the collagen matrix of the tissue (see figure 13 and figure 14). *Membrana elastic interna* was intact and cells in the media were evenly distributed.

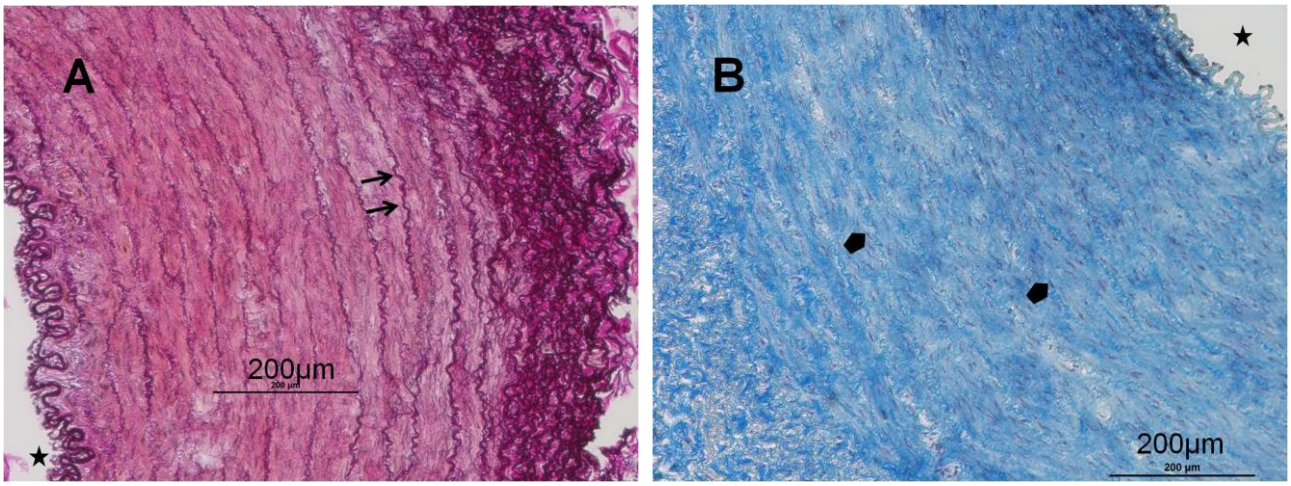

Figure 14: Elastica van Gieson (A) and Masson Trichrome staining (B) of a porcine common carotid artery treated with elastase at the adventitia in the presence of medium. No changes in the structure in comparison to native carotid arteries were observed. Stars indicate the lumen. A: Elastic fibres (two arrows) and collagen matrix were intact. B: Smooth muscle cells (red nuclei indicated by pentagons) showed no changes to native tissue. The tissue was treated with LMP agarose with 25 U/ml of elastase at the adventitia. The tissue was incubated for 4 h at 37 °C in culture medium, gel was removed afterwards and the sample was washed with HBSS with 10 % FCS for 45 min.

4.3. Laser Test

Arteries were cut with a laser system up to a depth of 150 µm and the tissue was removed with eye scissors and forceps under a microscope. To investigate the effect of the cut on the tissue architecture sections were stained utilizing both a Masson Trichrome and Elastica van Gieson staining. Both sections from arteries before and after removing the respective tissue by the laser were analysed. The figure 15 shows a cross section of an artery immediately after the laser-cut. A clear cut in the *tunica media* was observed with a distance of 200 µm to the intima. The difference between the depth of the 150µm cut and an observed cut depth of 200 µm might be due to the pressure caused by the weight applied during the cutting process to fix the tissue. The membrane elastic interna was intact. Collagen matrix and elastic fibres were destroyed at the side of the cut but stayed intact in the other parts of the tissue. Cells were evenly distributed in the media and the same shape compared to untreated tissue.

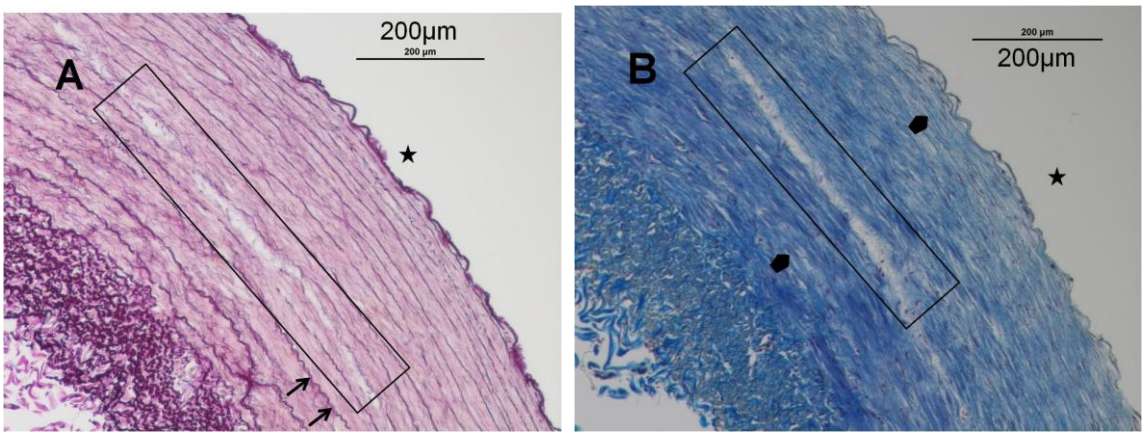

Figure 15: Elastica van Gieson (A) and Masson Trichrome staining (B) of cross sections of a porcine common carotid artery cut with the laser. Stars indicate the lumen. A: Elastic fibres (two arrows) were intact, the area of the cut (rectangle) showed no thermal injury. B: Smooth muscle cells were not affected outside the area of the cut (rectangle). The tissue was cut with the TissueSurgeon from ROWIAK with energy of 300 nJ and a power of 2546 mW two times in a depth of 150 µm.

Figure 16 shows that the removal of the tissue was successful. Some remnants of the tissue stayed at the borders of the cut. No fragmentation of the elastin fibres or loss of collagen in the tissue near the cut was observed. The membrane elastic interna stayed intact in the area of the artery where no tissue was removed.

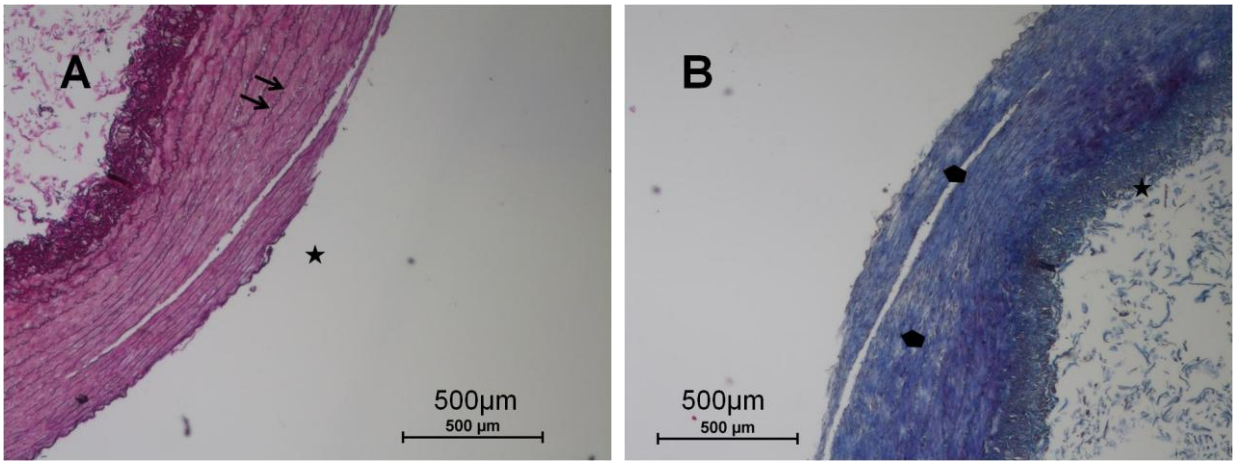

Figure 16: Elastica van Gieson (left) and Masson Trichrome staining (right) of cross sections of a porcine common carotid artery cut with the laser. Stars indicate the lumen. A: Elastic fibres outside of the cut were intact (two arrows). B: Smooth muscle cells (red nuclei indicated by pentagons) were not affected. The tissue was cut with the TissueSurgeon from ROWIAK with an energy of 300 nJ and a power of 2546 mW two times in a depth of 150 µm. Tissue was removed with an eye scissor and forceps.

4.4. Cultivation

4.4.1. Cultivation without treatment

To test the effect of dynamic and static conditions on the viability and architecture of arteries, the tissue was put in either a vascular bioreactor or a cell culture flask. Dynamic conditions to mimic the shear stress where achieved by an application of continues flow of the medium in the vascular bioreactor. To mimic static conditions the tissue was cultivated in a cell culture flask in an incubator. Arteries were cultivated for 5 days. Cell viability was investigated with a MTT assay. A staining against CD31 was used to visualize the endothelium and Masson Trichrome and Elastica van Gieson staining were used to investigate the architecture of the tissue.

Investigation of the tissue viability (see figure 17) showed a significant decrease of viability after antibiotic treatment. Samples cultivated under static or dynamic conditions showed no significant difference in tissue viability compared to the negative control. A high variation of viability values within one artery was observed. This might be due to a long waiting time at the abattoir. The medium got cloudy during the cultivation but a sterility test did not show a contamination. A formation of crystals in the bioreactor was seen under the microscope. Chosen cultivation parameters keep constant.

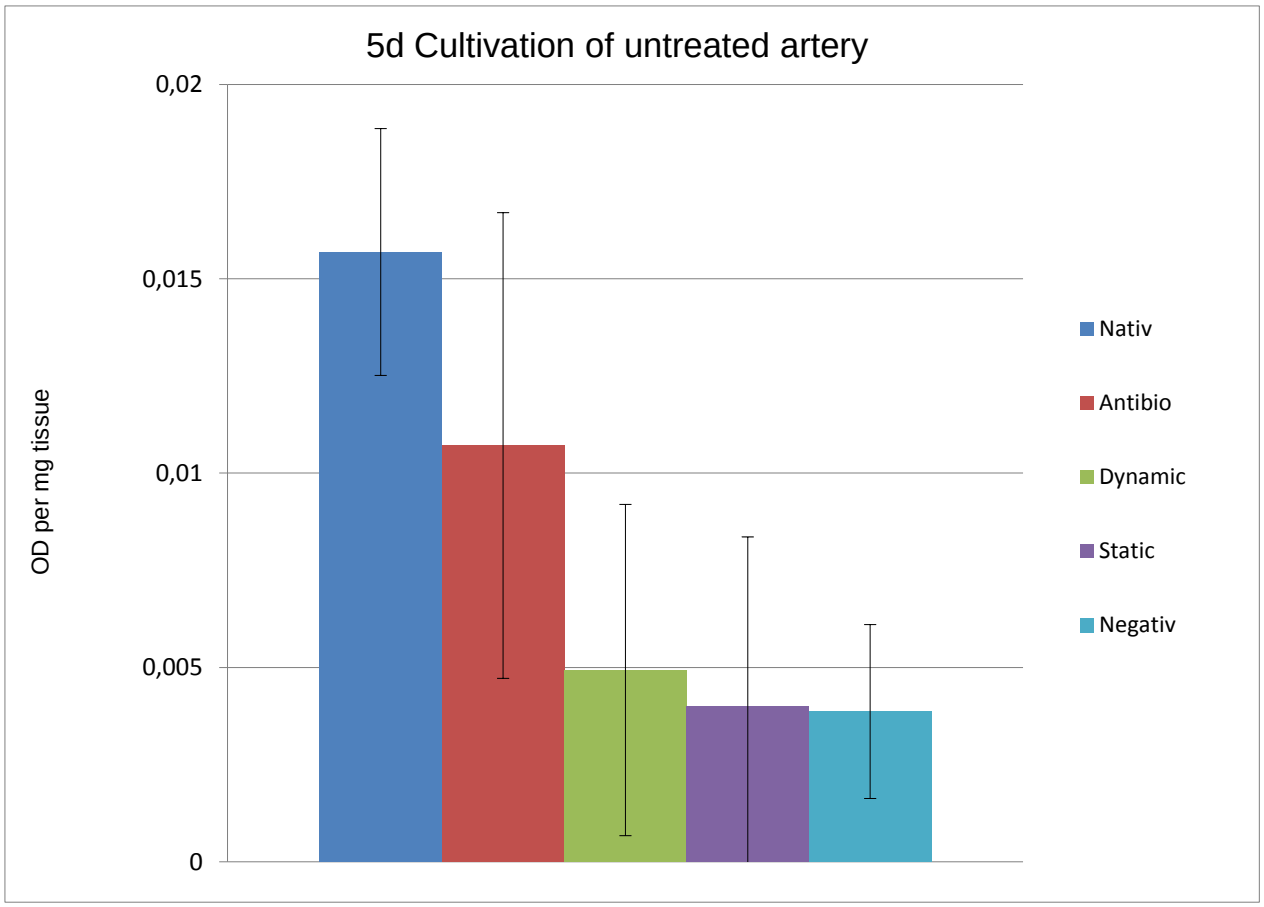

Figure 17: Viability after 5 days of cultivation. Tissue viability decreased significantly after disinfeciotn. Tissue cultivated for 5 days under dynamic or static conditions did not show a significant difference of viability compared to the negative control. A MTT assay was performed to determine the viability of 50 ± 10mg tissue. Porcine carotid arteries were taken from the local abattoir and disinfected with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax) for 30 min. Afterwards the samples were put in the bioreactor system for dynamic cultivation with a flow rate of 262 ml/min culture medium respectivly in a cell culture flask with 90 ml of culture medium for static cultivation at 37 °C. Tissue incubated for five days in 10 % NBF at 37 °C was used as a negative control. Native: tissue viability of native tissue; Antibio: tissue viability after disinfection. Dynamic: tissue viability after 5 days of cultivation under dynamic flow; Static: tissue viability after 5 days of cultivation in a cell culture flask. Negative: tissue viability after 5 days of incubation in formalin

To check the quality of the tissue a part of the native artery was taken for histological staining (Masson Trichrome and Elastica van Gieson) and immunohistochemical investigation (see figure 18 and figure 19). Masson Trichrome and the Elastica van Gieson staining showed an intact tissue. *Membrana elastic interna* , elastic fibres and the collagen matrix showed no abnormal structures.

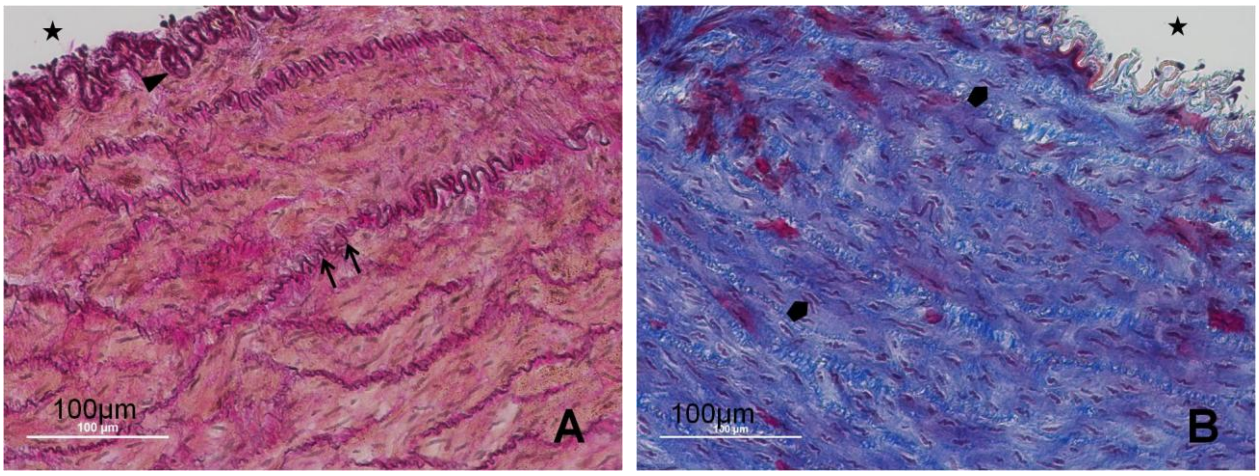

Figure 18: Elastica van Gieson (A) and Masson Trichrome staining (B) of a cross section of a porcine common carotid artery. Stars indicate the side of the lumen. A: Elastic fibres (two arrows) and *membrana elastic interna* (triangle) were intact. B: Smooth muscle cells (red nuclei indicated by pentagons) were continuously distributed in the media.

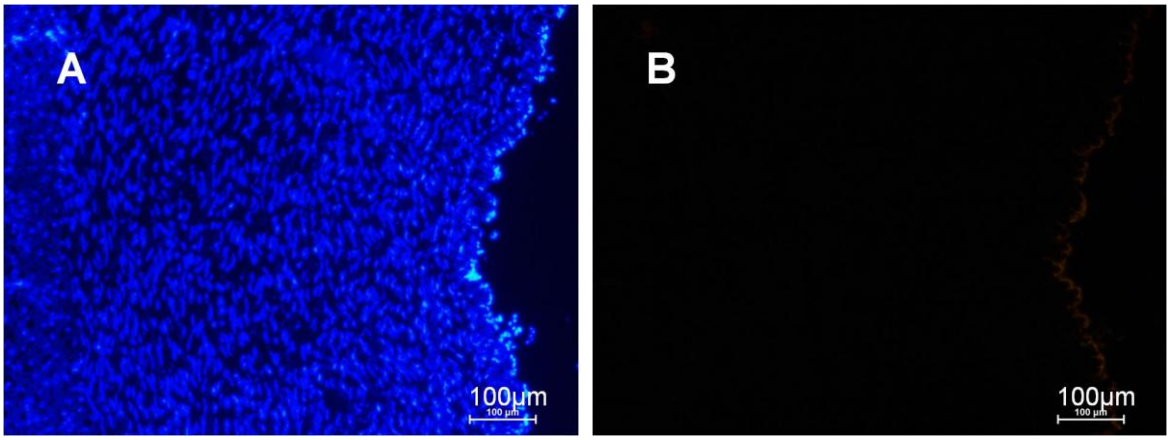

Figure 19: Cross section of a porcine carotid artery incubated with DAPI (A) respectively a CD31 antibody (B). The endothelium was intact.

In the media, evenly distributed cells were observed and in the media cell nuclei and red stained cells were seen in the Masson Trichrome staining. Incubation with a CD 31 antibody showed an intact endothelium of the artery.

The effect of antibiotic treatment on the histoarchitecture of the artery was investigated (see figure 20 and figure 21) to further clarify whether treatment would affect the architecture of the tissue. *membrana elastic interna*, elastic fibres and the collagen matrix did not show changes in comparison to the native tissue. The adventitia was intact. Cells

observed in the media and adventitia showed the same structure as in native tissue. The endothelium was intact (see figure 21).

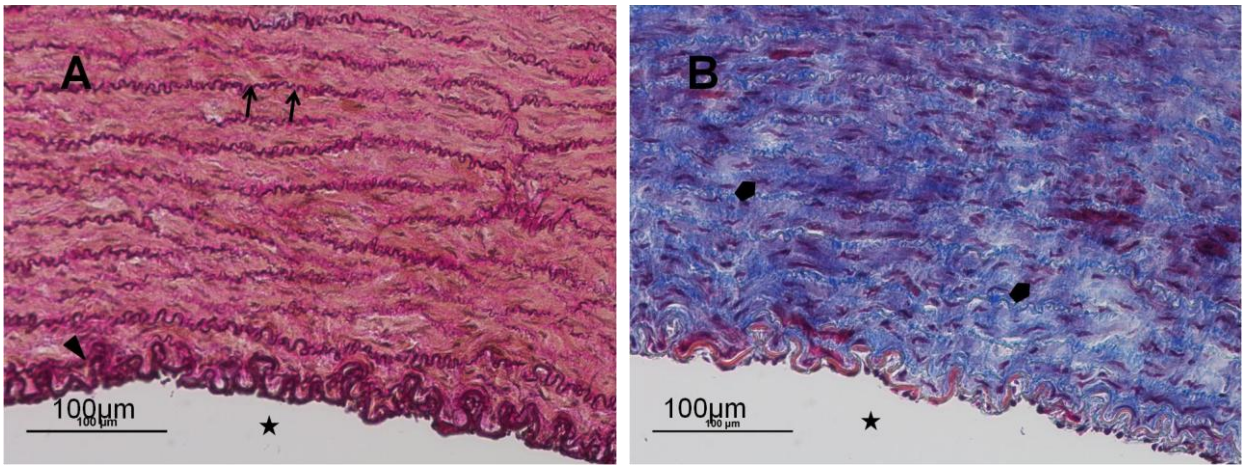

Figure 20: Elastica van Gieson (A) and Masson Trichrome staining (B) of a cross section of a porcine common carotid artery after 30 min incubation with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax). The antibiotic treatment had no effect on the architecture of the tissue. Stars indicate the lumen of the tissue. A: Elastic fibres (two arrows) and *membrana elastic interna* (triangle) showed no degradation or straightening. B: Smooth muscle cells (pentagons indicate the red nuclei of the cells) did not show morphological changes.

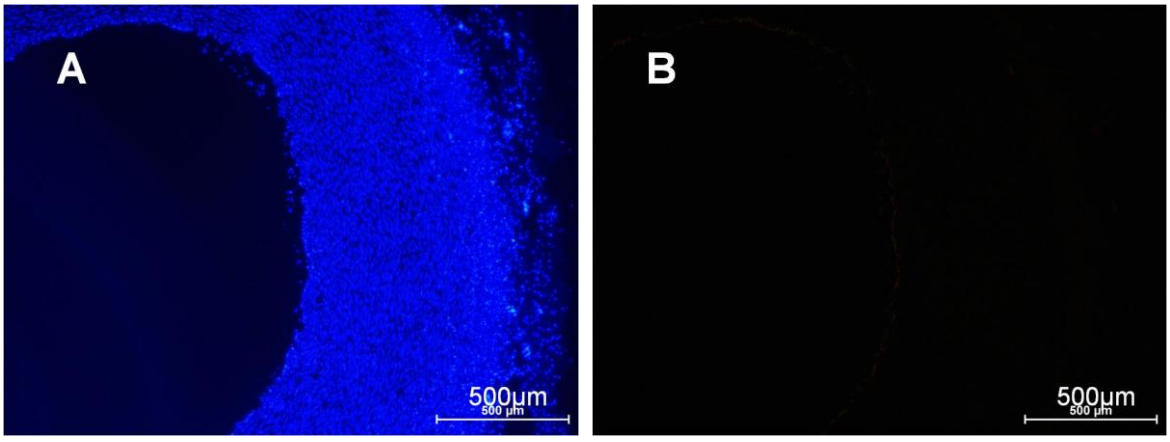

Figure 21: Cross section of a porcine carotid artery incubated with DAPI (A) respectively a CD31 antibody (B) after 30 min incubation with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax). The endothelium was intact.

After static cultivation, no changes in collagen and elastin content were observed. The *membrana elastic interna* and the elastic fibres in the media showed straightening. The

collagen matrix was intact. The endothelium showed no degradation as confirmed by staining against CD 31. Cells in the media were as evenly distributed as seen in native tissue. Cell nuclei were observed in the adventitia but less intense stained cell plasma was seen in the Masson Trichrome staining.

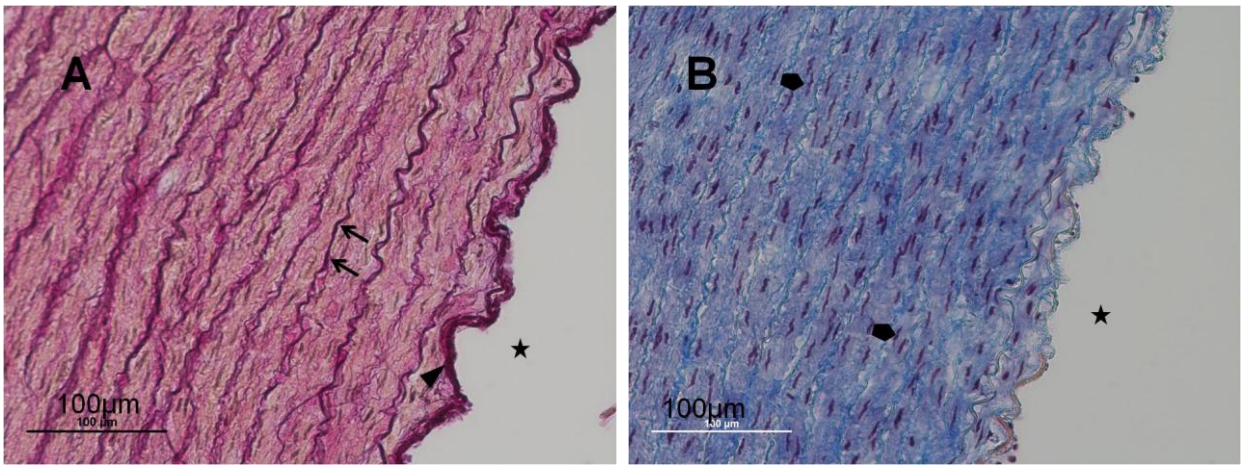

Figure 22: Elastica van Gieson (A) and Masson Trichrome staining (B) of a cross section of a porcine common carotid artery after static cultivation. Stars indicate the lumen of the tissue. A: Elastic fibres (two arrows) and *membrana elastic interna* (triangle) showed straightening. B: Number and distribution of smooth muscle cells (pentagons indicate red nuclei of cells) were not affected by the cultivation. The tissue was incubated with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax) for 30 min and static cultivation at 37 °C in 90ml of culture medium in a cell culture flask was performed.

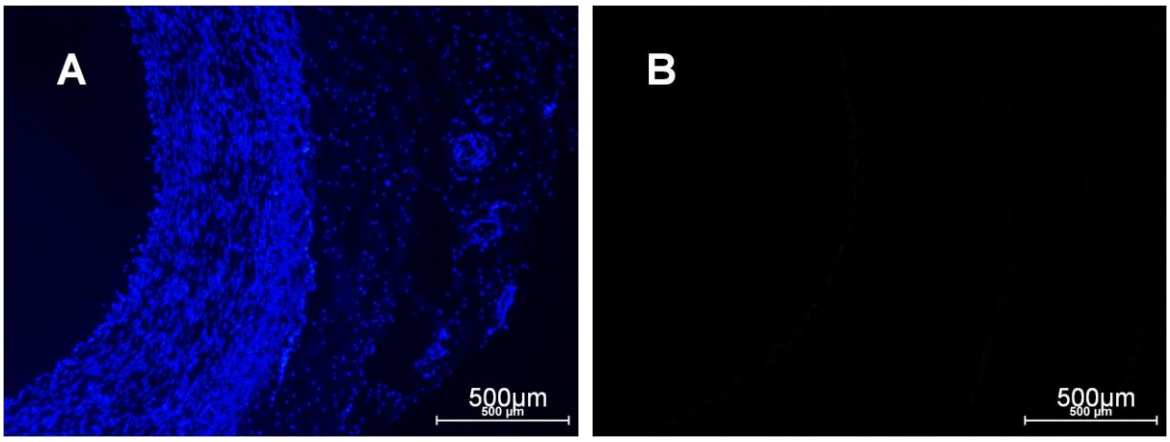

Figure 23: Cross section of a porcine carotid artery incubated with DAPI (A) respectively a CD31 antibody (B) after 30 min incubation with antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax) and static cultivation at 37 °C in 90 ml of culture medium in a cell culture flask. No degradation of endothelium was observed.

After dynamic cultivation changes on elastin fibres were observed (see figure 24) and endothelium was absent as indicated by a missing CD31 staining (figure 25). This might be due to the flow of the medium. *Membrana elastic interna* and elastic fibres showed straightening. Fragmentation of elastic fibres was observed. Smooth muscle cells were evenly distributed in the media. Cells in adventitia showed less red staining of the cell plasma then the cells in native tissue. Some cells in the adventitia showed no staining. No dilation of the artery was observed (see figure 26)

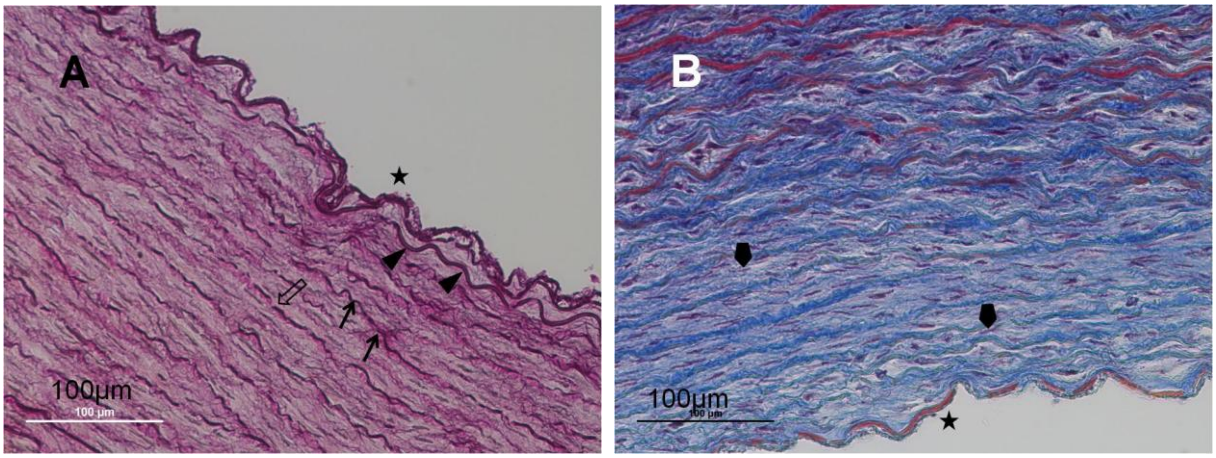

Figure 24: : Elastica van Gieson (A) and Masson Trichrome staining (B) of a cross section of a porcine common carotid artery after dynamic cultivation. Stars indicate the lumen of the artery. A: *Membrana elastic interna* **(triangels) detached and showed straightening. Elastic fibres showed straightening (two arrows) and degradation (empty arrow). B: Appearance of smooth muscle cells (pentagons indicate red nuclei of cells) was not affected by the cultivation. After 30 min incubation with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax) a dynamic cultivation (262 ml/min culture medium) in the bioreactor system at 37 °C was performed..**

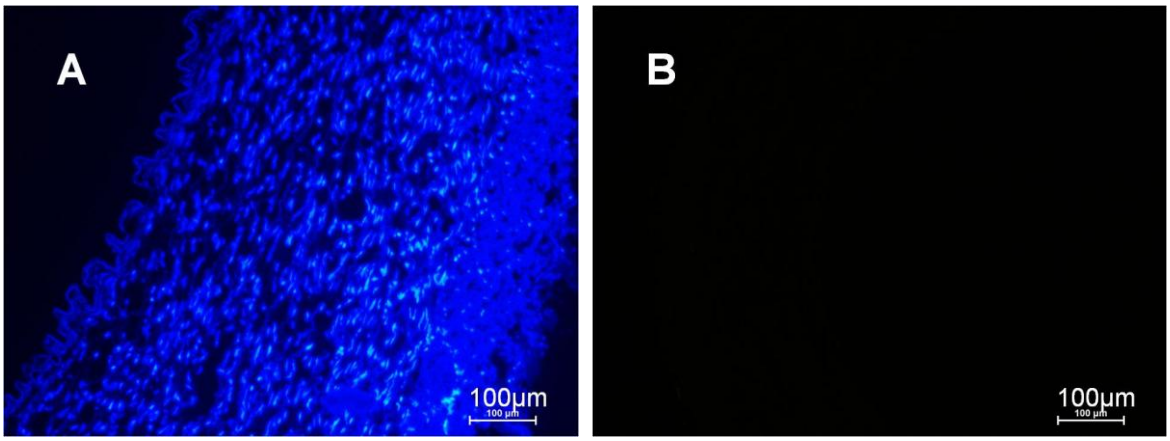

Figure 25: Cross section of a porcine carotid artery incubated with DAPI (A) or a CD31 antibody (B) after 30 min incubation with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax) and dynamic (262 ml/min culture medium) cultivation in the bioreactor system at 37 °C. No endothelium was observed.

Figure 26: Vessel unit of the bioreactor system with an artery cultivated for 5 days. Circle show the postion of the artery.

4.4.2. Cultivation with Enzyme treatment

An agarose gel with collagenase (380 CDU/ ml) and elastase (75 U/ml) was used to partially coat the intima of arteries. After gel application, arteries were covered with 2 ml of culture medium and incubated for four hours at 37 °C. After a washing step (45 min HBSS with 10 % FBS) arteries were treated with antibiotic solution for 30 min. Disinfected arteries were used for cultivation.

The medium of dynamic cultured arteries stayed clear. Sterility tests showed a contamination of arteries after the dynamic and static cultivation, but not after the disinfection step. No significant decrease of viability after enzyme treatment and antibiotic treatment was detected (see figure 27). Chosen cultivation parameters keep constant

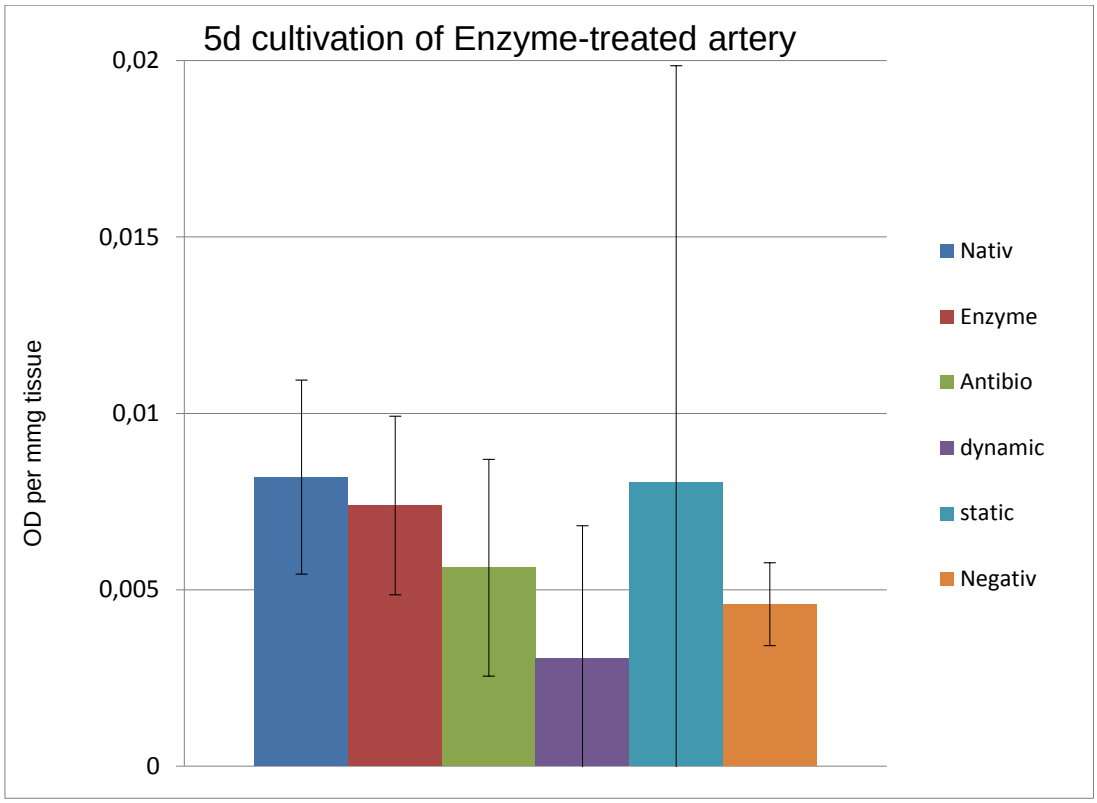

Figure 27: Viability after enzyme treatment and 5 days of cultivation. A MTT assay was performed to determine the viability of 50 ± 10mg tissue. Tissue incubated with the enzymes did not show a significant decrease of viability in comparison to native tissue. All samples showed no significantly different values in comparison to the negative control. Porcine carotid arteries were taken from the local abattoir, treated with an enzyme gel (LMP agarose with 380 CDU/ml collagenase and 75 U/ml elastase) for 4 h at 37 °C and disinfected with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax) for 30 min. Afterwards the samples were put in the bioreactor system for dynamic cultivation with a flow rate of 262 ml/min culture medium respectively in a cell culture flask with 90 ml of culture medium for static cultivation at 37 °C. Tissue incubated for five days in 10 % NBF at 37 °C was used as a negative control. Native: tissue viability of native tissue; Enzyme: Tissue viability after enzyme treatment; Antibio: tissue viability after disinfection. Dynamic: tissue viability after 5 days of cultivation under dynamic flow; Static: tissue viability after 5 days of cultivation in a cell culture flask. Negative: tissue viability after 5 days of incubation in formalin.

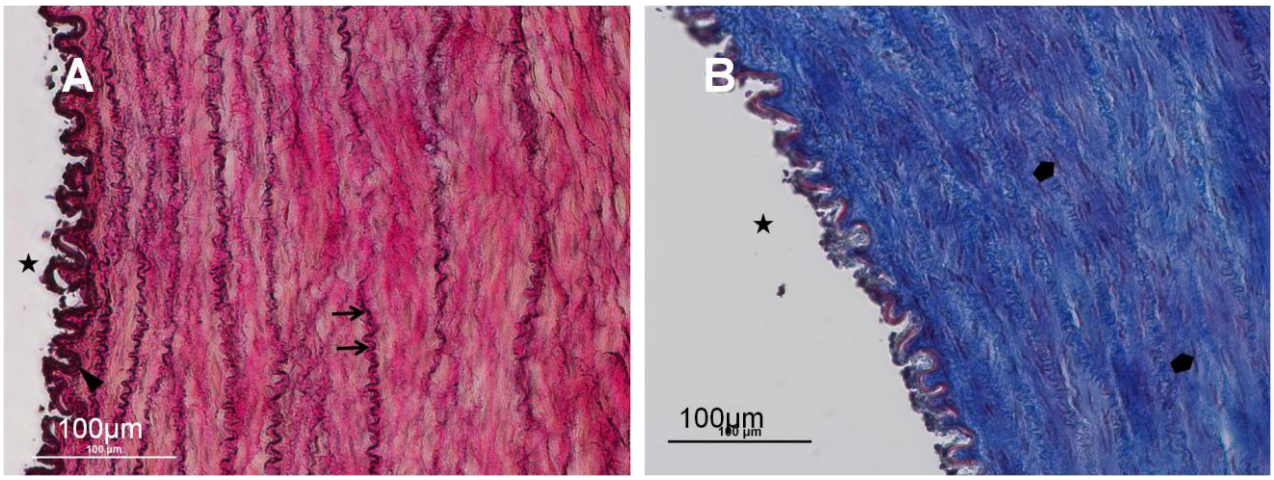

Figure 28: Elastica van Gieson (A) and Masson Trichrome staining (B) of a cross section of a porcine common carotid artery. Stars indicate the lumen. A: Elastic fibres (two arrows) and *membrana elastic interna* (triangle) were intact. B: Collagen matrix (blue) and smooth muscle cell (red cell nuclei indicated by pentagons) showed no abnormal structures.

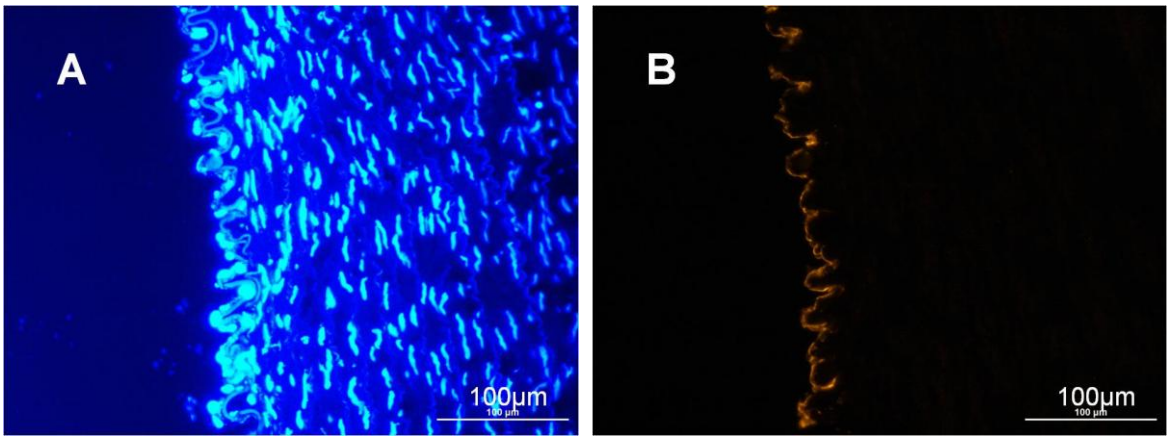

Figure 29: Cross section of a porcine carotid artery incubated with DAPI (A) respectively a CD31 antibody (B). The endotheliumwasintact. The endothelium was intact.

Native arteries showed an intact structure of collagen and elastin matrix and an intact endothelium as shown in figure 28 and figure 29.

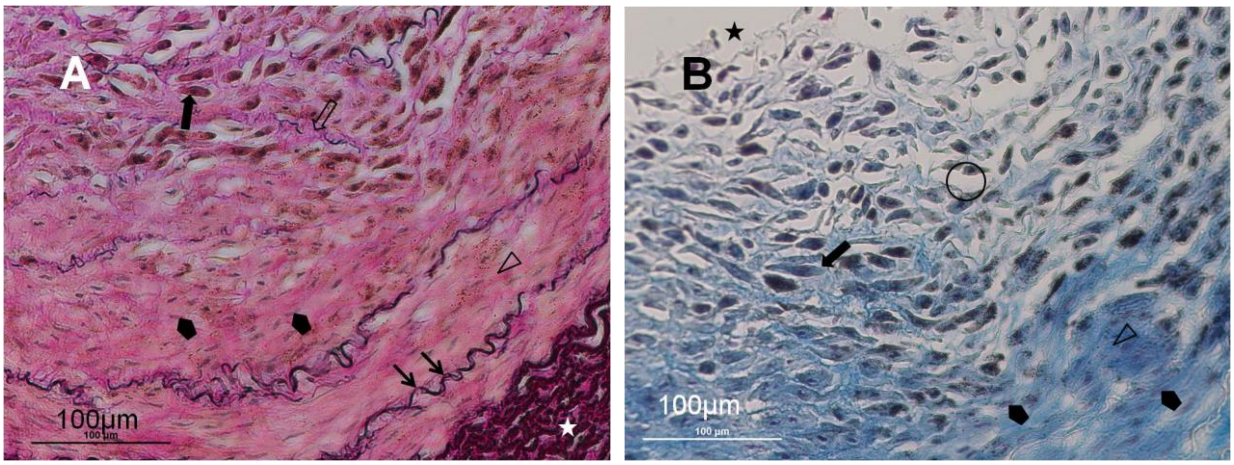

Figure 30: Elastica van Gieson (A) and Masson Trichrome staining (B) of a cross section of a porcine common carotid artery incubated for 4 h with an enzyme gel (LMP agarose with 380 CDU/ml collagenase and 75 U/ml elastase) at 37 °C. A: The star indicates the adventitia. Detached Smooth muscle cells (filled arrow) and degraded elastic fibres (empty arrow) were observed near the lumen. At the adventitia smooth muscle cells (black nuclei indicated by pentagons) were continuously distributed. The collagen matrix was intact (empty triangle) and elastic fibres (two arrows) showed partial straightening but no degradation. B: The star indicates the side of the lumen. Next to the lumen areas without collagen were observed (circle). Smooth muscle cells near the lumen detached (filled arrow). Next to the adventitia the collagen matrix was intact (empty triangle) and smooth muscle cells showed a native like shape (empty triangle).

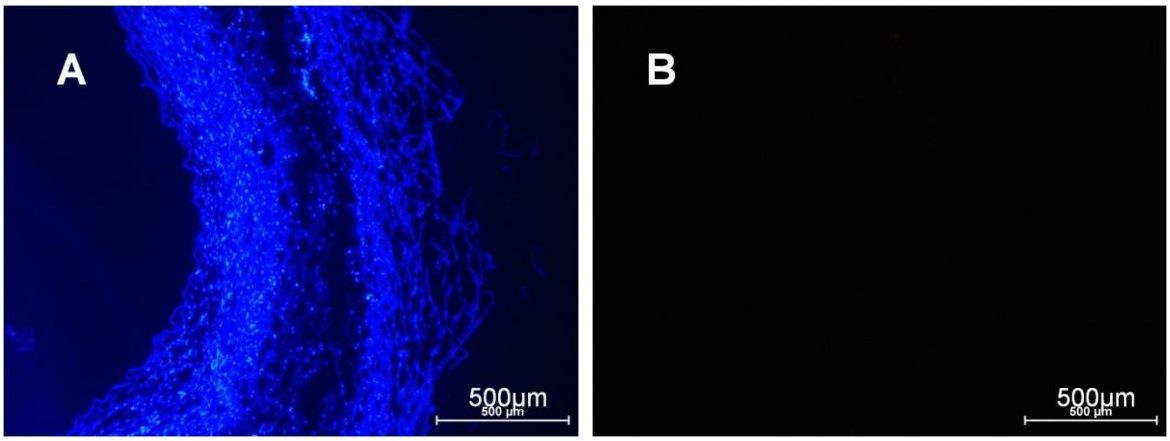

Figure 31: Cross section of a porcine carotid artery incubated with DAPI (A) respectively a CD31 antibody (B) The artery was treated for 4 h with an enzyme gel (LMP agarose with 380 CDU/ml collagenase and 75 U/ml elastase) at 37 °C. No endothelium was observed.

After the enzyme treatment a complete loss of endothelium was observed (see figure 31). Collagen matrix and elastin fibres of the tissue were degraded and cells of the media detached (figure 30). Elastic fibres were almost absent. However fragments of elastic fibres were observed near the lumen. Near the adventitia straightened elastic fibres were still

present. Smooth muscle cells near the lumen detached or were completely absent but next to the adventitia collagen matrix and smooth muscle cells showed a native shape. Areas without collagen were observed near the lumen. The integrity of the adventitia was not affected by the enzyme treatment. In the adventitia nuclei of cells were observed but the red staining of the cell plasma was less intense than in native tissue.

Treatment with the antibiotic solution did not affect the architecture of the artery (see figure 32 and figure 33).

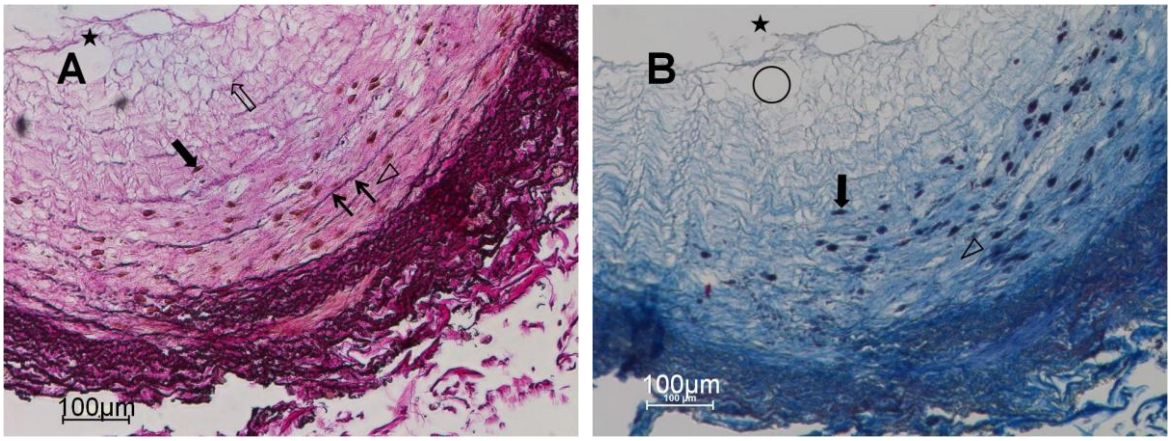

Figure 32: Elastica van Gieson (A) and Masson Trichrome staining (B) of a cross section of a porcine common carotid artery after enzyme treatment and disinfection. No changes in comparison to the arteries treated with the enzyme were observed. Stars indicate the side of the endothelium. A: Near the lumen, elastic fibres were degraded (empty arrow). Smooth muscle cells detached (filled arrow). Next the adventitia elastic fibres showed straightening (two arrows), collagen matrix was intact (empty triangle). B: Smooth muscle cells showed a round shape (filled arrow) Near the lumen areas without collagen were observed (circle). Next to the adventitia the collagen matrix were intact (empty triangle) .The samples were incubated for 4 h with an enzyme gel (LMP agarose with 380 CDU/ml collagenase and 75 U/ml elastase) at 37 °C and 30 min with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax).

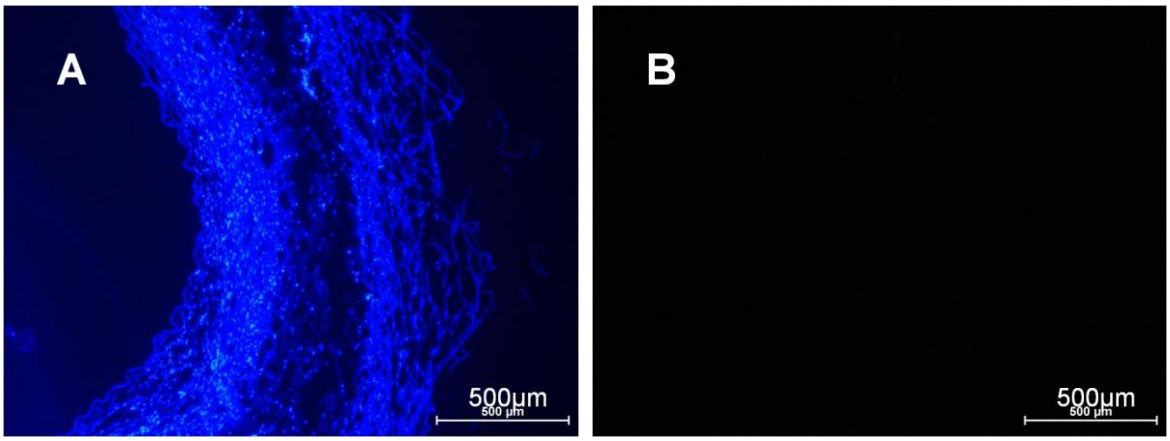

Figure 33:Cross section of a porcine carotid artery incubated with DAPI (A) respectively a CD31 antibody (B) after enzyme treatment and disinfection. Endothelium was absent. The artery was treated for 4 h with an enzyme gel (LMP agarose with 380 CDU/ml collagenase and 75 U/ml elastase) at 37 °C and 30 min with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax).

After static cultivation, only remnants of the media were observed (see figure 34 and figure 35). At the lumen the elastin fibres had detached and a fragmentation of the elastic filaments was observed. The elastin fibres at the adventitia were not affected. Collagen destruction showed an incline of detached areas with less collagen and more collagen matrix at the adventitia. Next to the adventitia the tissue showed small areas devoid of collagen. Cell nuclei but no cell plasma was observed in the adventitia in the Masson Trichrome staining.

Arteries cultivated under dynamic conditions are shown in figure 36 and figure 37. Elastic fibres were fragmented near the lumen but intact at the andventia. Degradation of the collagen matrix showed a gradient of destroyed and detached tissue with areas devoid of collagen at the lumen to intact tissue at the adventitia. Parts of the media showed evenly distributed cells but other parts showed no cells there. The smooth muscle cells showed a rounded shape. Cells in the adventitia were not stained red in the Masson Trichrome staining but black nuclei were observed. It is possible that these cells might have lost their cell plasma. An endothelium was not observed. A dilation of the artery was observed.

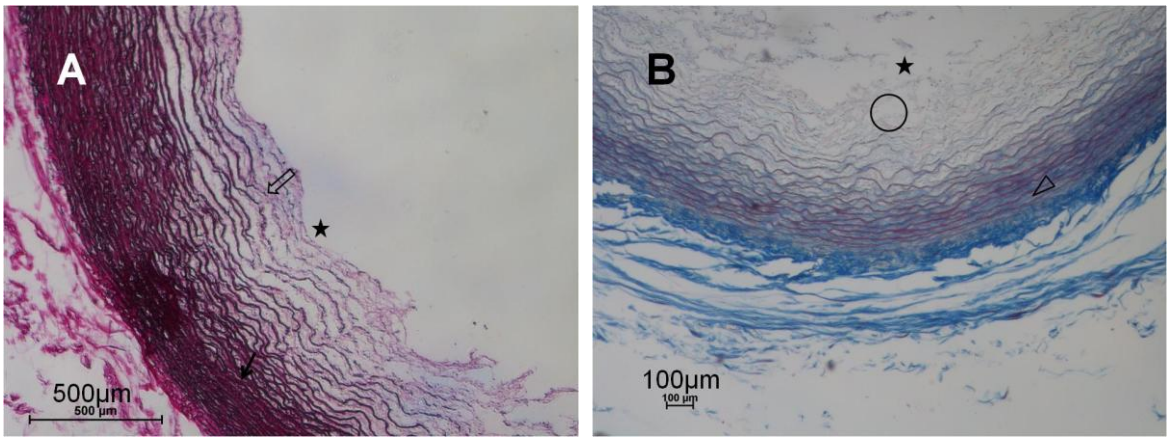

Figure 34: Elastica van Gieson (A) and Masson Trichrome staining (B) of a cross section of a porcine common carotid artery after static cultivation. Stars indicate the lumen. A: Near the intima elastic fibres showed fragmentation (empty arrow), next to the adventitia elastic fibres were not affected. B: Near the lumen areas devoid of collagen (circle) were observed. Next to the adventitia the collagen matrix was intact (empty triangle) The tissue was incubated for 4 h with an enzyme gel (LMP agarose with 380 CDU/ml collagenase and 75 U/ml elastase) at 37 °C and 30 min with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax). After disinfection the artery was incubated for 5 days with 90 ml of culture medium at 37 °C in a cell culture flask.

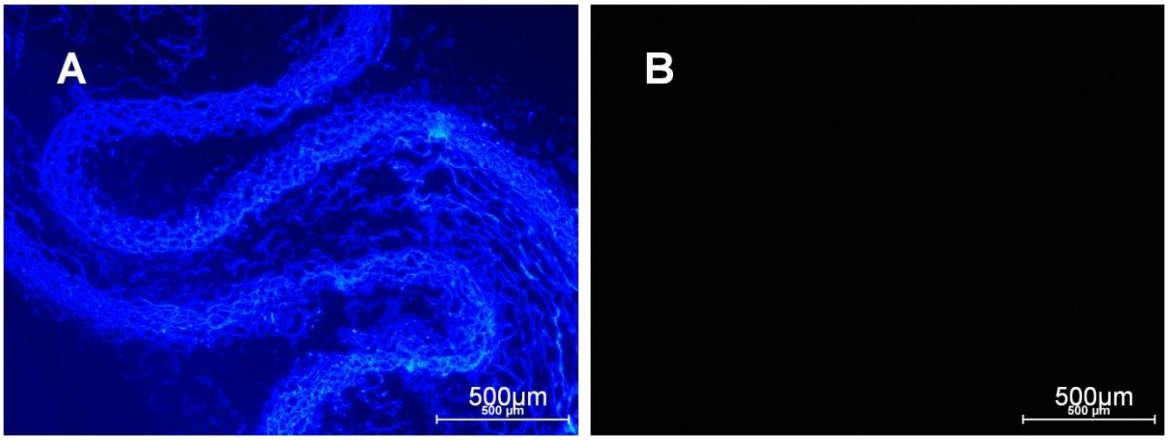

Figure 35: Cross section of a porcine carotid artery incubated with DAPI (A) respectively a CD31 antibody (B) after static cultivation. No endothelium was observed. The artery was treated for 4 h with an enzyme gel (LMP agarose with 380 CDU/ml collagenase and 75 U/ml elastase) at 37 °C and 30 min with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax). After disinfection the artery was incubated for 5 days with 90 ml of culture medium at 37 °C in a cell culture flask.

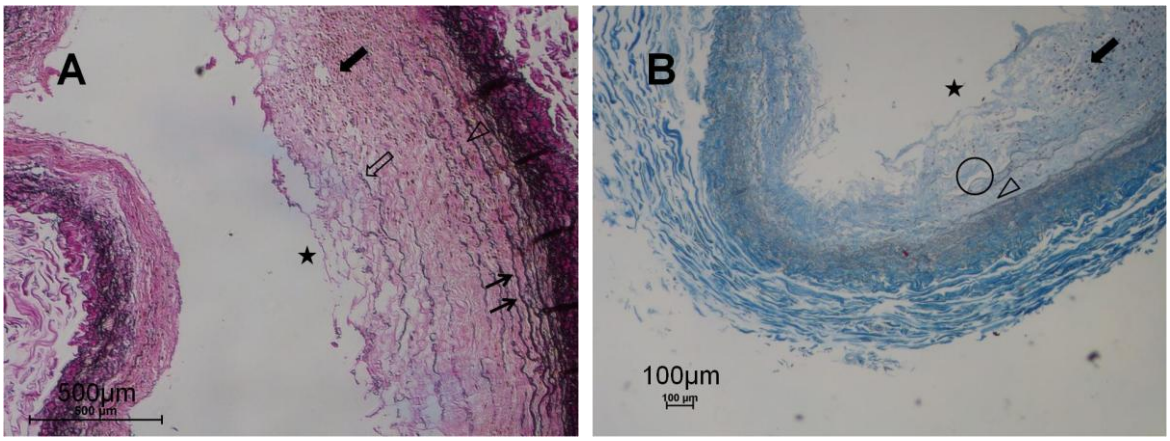

Figure 36: Elastica van Gieson (A) and Masson Trichrome staining (B) of a cross section of a porcine common carotid artery after dynamic cultivation. Stars indicate the lumen. A: Fragmenation of elastic fibres (empty arrow) were observed near the lumen. Next to the adventitia elastic fibres showed straightening (two arrows). Smooth muscle cells showed a rounded shape (filled arrow). Near the adventitia, the collagen matrix was intact (empty triangle). B: Areas devoid of collagen (circle) were seen in the artery wall. Next to the lumen a small area was not degraded (empty arrow). Smooth muscle cell showed a round shape (filled arrow). The tissue was incubated for 4 h with an enzyme gel (LMP agarose with 380 CDU/ml collagenase and 75 U/ml elastase) at 37 °C and 30 min with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax). After disinfection the artery was put in a bioreactor and cultivated under a medium flow of 262 ml/min culture medium for 5 days at 37 °C.

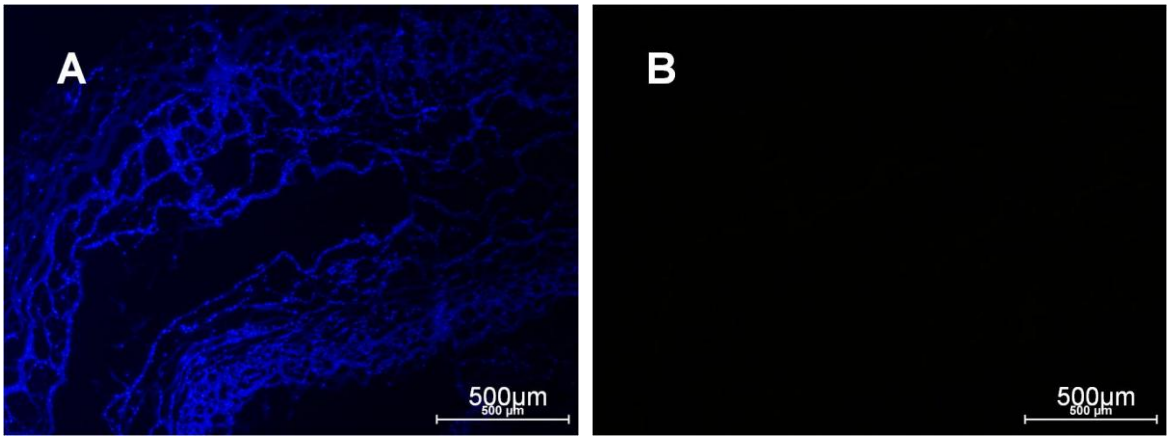

Figure 37: Cross section of a porcine carotid artery incubated with DAPI (A) respectively CD31 antibody (B) after dynamic cultivation. No endothelium was observed. The artery was treated for 4 h with an enzyme gel (LMP agarose with 380 CDU/ml collagenase and 75 U/ml elastase) at 37 °C and 30 min with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax). After disinfection the artery was put in a bioreactor and cultivated under a medium flow of 262 ml/min culture medium for 5 days at 37 °C.

After 5 days of cultivation under dynamic conditions a dilation of the artery was observed (figure 38)

Figure 38: Vessel unit of the bioreactor system with an collagenase and elastase treated artery cultivated for 5 days. Circle show the postion of the dilation of the vessel wall.

4.4.2. Cultivation with Laser treatment

Arteries were isolated from pigs from at local abattoir and turned inside out. After transport to the laser system, the arteries were cut. A disinfection step followed. Disinfected arteries were cultivated under dynamic and static conditions

Viability tests showed no statistically significant difference between tissue cut with a laser and the negative control (see figure 39). A long time delay of five hours between getting tissue from the abattoir and the laser treatment might be an explanation. An increase of the

viability of tissue after disinfection detected by the MTT assay might be an indicator for a contamination and an inadequate disinfection. The sterility test showed a contamination of the arteries after the disinfection step. A decrease of the oxygen concentration during the cultivation was observed which indicate a contamination.

Native tissue used for this experiment showed an intact endothelium collagen matrix and elastin fibres (see figure 40and figure 41). Cells with cell plasma were evenly distributed in the media. In the adventitia cells with cell plasma were detected.

The tissue was successfully removed from the cut as can be seen in figure 42. The tissue under the cut was not affected; collagen matrix and elastic fibres were intact. Holes in the media might be due to the procedure that used to turn the artery inside out. Cells were evenly distributed in the media and intact cells with cell plasma were observed in the adventitia. figure 43 shows that the endothelium was absent after laser treatment.

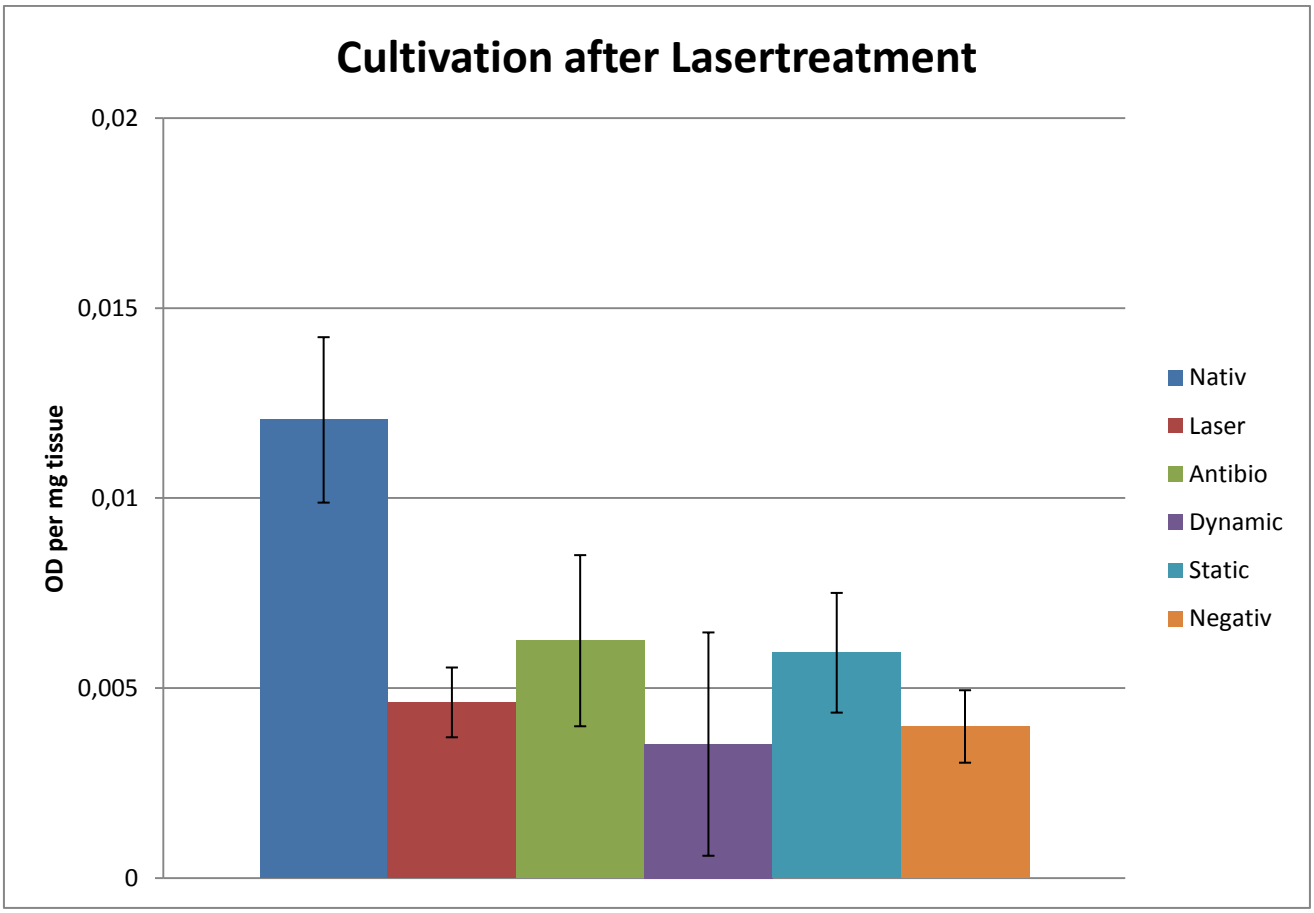

Figure 39: Viability after laser treatment and 5 days of cultivation. The tissue viability of arteries after laser treatment did not show a significant difference to the negative control. A MTT assay was performed to determine the viability of 50 ± 10mg tissue. Porcine carotid arteries were taken from the local abattoir, cut with the laser system (300 nJ, 2546 mW in a depth of 150 µm two times) and disinfected with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax) for 30 min. Afterwards the samples were put in the bioreactor system for dynamic cultivation with a flow rate of 262 ml/min culture medium or in a cell culture flask with 90 ml of culture medium for static cultivation at 37 °C. Tissue incubated for five days in 10 % formalin at 37 °C was used as a negative control. Native: tissue viability of native tissue; Laser: Tissue viability after lase treatment; Antibio: tissue viability after disinfection. Dynamic: tissue viability after 5 days of cultivation under dynamic flow; Static: tissue viability after 5 days of cultivation in a cell culture flask. Negative: tissue viability after 5 days of incubation in formalin.

.

Following static cultivation a loss of collagen matrix was observed (see figure 44). In the area next to the cut holes appear in the media. The greatest decrease of collagen content was most near the lumen. At the adventitia in the area near the laser cut less areas devoid of collagen and elastin were observed. Elastic fibres detached near the lumen. The tissue in the not-dissected area showed areas devoid of collagen but elastic fibres were not affected. The *membrana elastic interna* was disrupted. Cells were evenly distributed in the

media similar to native tissue. They showed a spindle like shape. Cells in the adventitia showed no difference to the cells in native tissue. The endothelial layer was absent after static cultivation (figure 45).

Endothelium was absent after dynamic treatment as shown in figure 47. Elastic fibres in the media were straightened, the collagen contend was decreased. A loss of cells in the media was observed. Near the lumen almost no cells were observed in the media, at the side of the adventitia the cells in the collagen matrix where not affected. Cells in the adventitia showed a loss of cell plasma. More tissue was removed compared to the amount removed after laser treatment (figure 46). A flap with adventitia was seen. These results might indicate that the stress induced by the flow of the medium leads to tissue erosion. Following cultivation under dynamic flow, the medium get cloudy.A dilation of the blood vessel was observed (see figure 48).

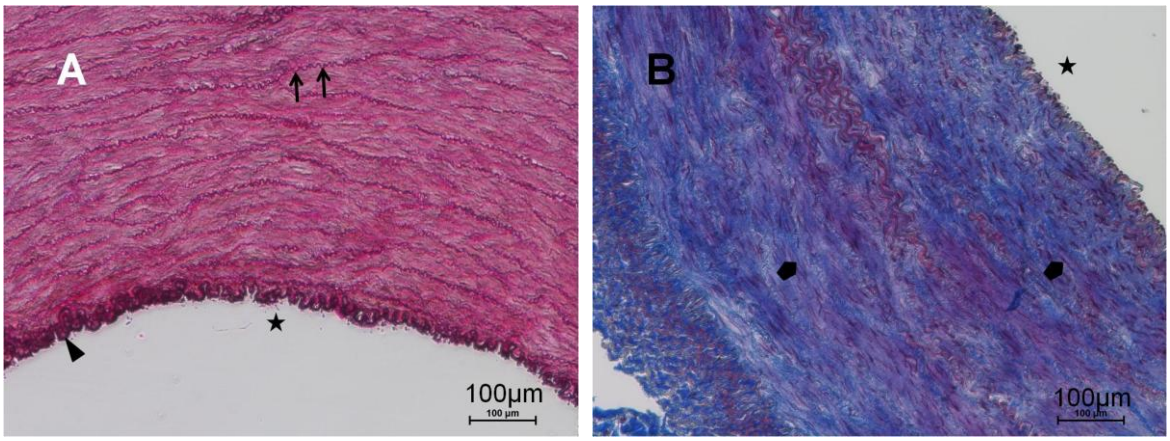

Figure 40: Elastica van Gieson (A) and Masson Trichrome staining (B) of a cross section of a porcine common carotid artery before laser treatment. Stars indicate the lumen. A: Elastic fibres (two arrows) and *membrana elastic interna* (triangle) were intact. B: The collagen matrix did not show empty areas, smooth muscle cells (red nuclei indicated by pentagons) were continuously distributed.

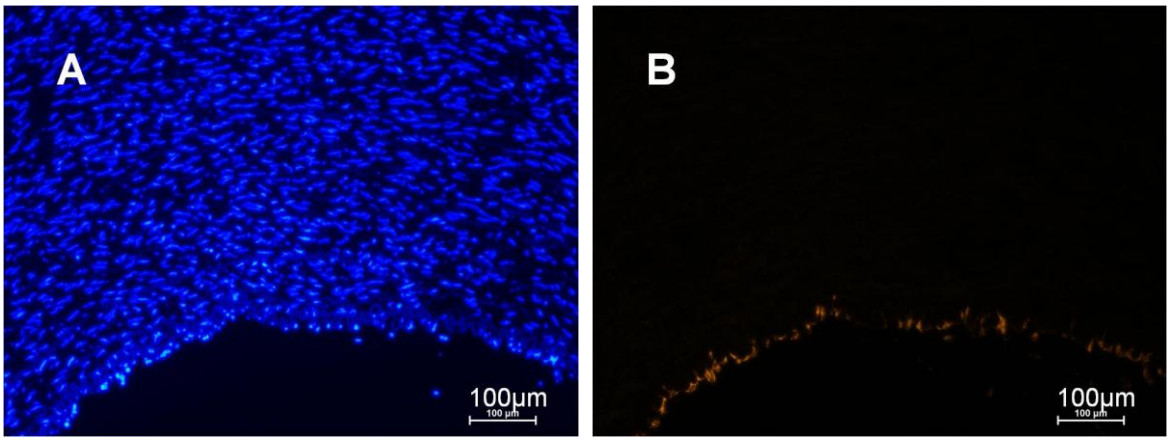

Figure 41: Cross section of a porcine carotid artery incubated with DAPI (A) repectively a CD31 antibody (B) before laser treatment. The endothelium was intact.

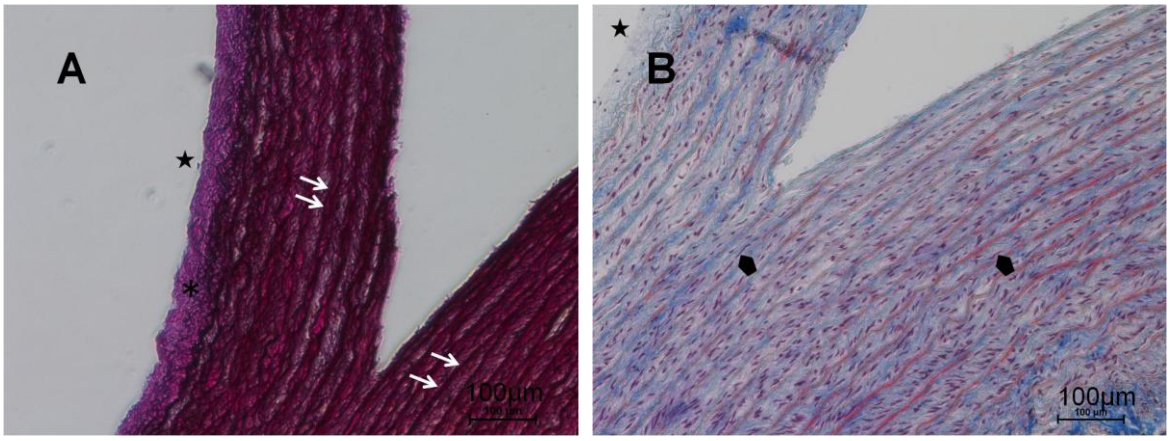

Figure 42: Elastica van Gieson (A) and Masson Trichrome staining (B) of a cross section of a porcine common carotid artery cut with a laser. Stars indicate the lumen of the tissue. A: Elastic fiber next to the cut were intact (two arrows). At the endothel a zone without elastic fibres was seen (asterix). B: Smooth muscle cells (red nuclei indicated by pentagons) were continuously distributed in the tissue and collagen matrix was intact. The tissue was cut with 300 nJ and 2546 mW in a depth of 150 µm and incubated with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax).

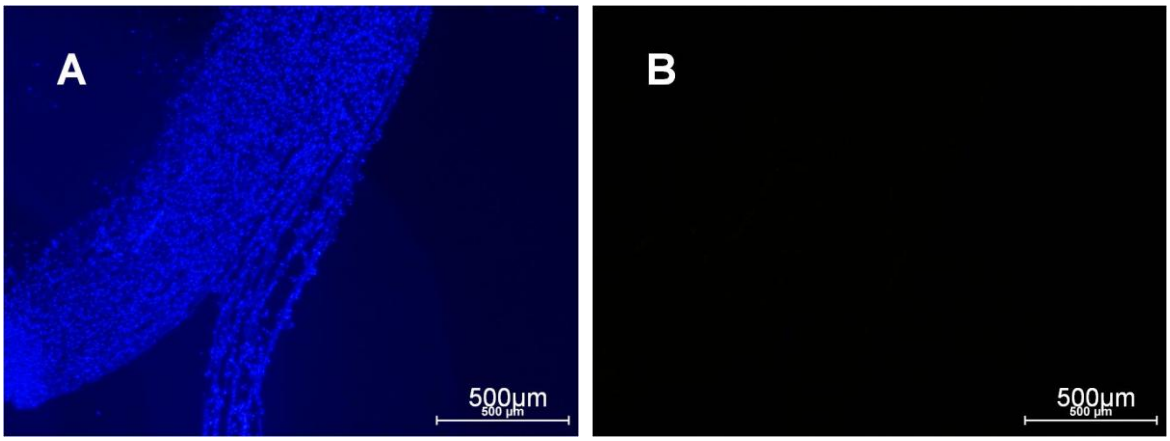

Figure 43: Cross section of a porcine carotid artery incubated with DAPI (A) respectively a CD31 antibody (B) after laser treatment. No endothelium was observed. The artery was cut with a laser (300 nJ, 2546 mW) in a depth of 150 µm and incubated with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax).

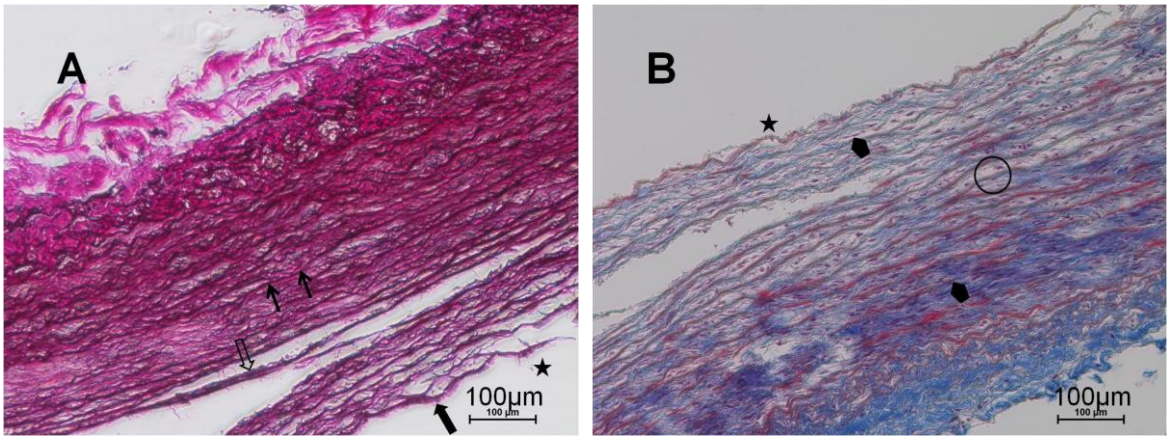

Figure 44: Elastica van Gieson (A) and Masson Trichrome staining (B) of a cross section of a porcine common carotid artery cut with a laser and static cultivation. Stars indicate the lumen. A: Next to the cut elastic fibres were detached (empty arrow). The *membrana elastic interna* (filled arrow) showed disruption. About 100 µm away from the cut elastic fibres were not affected (two arrows). B: Areas free of collagen were observed in the artery wall (circle). Smooth muscle cells were continuously distributed in the media and the nuclei showed a spindle like structure (red nuclei indicated by pentagons). The arteries were cut with 300 nJ and 2546 mW in a depth of 150µm and incubated with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax). After disinfection the artery was incubated for 5 days with 90 ml of culture medium at 37 °C in a cell culture flask.

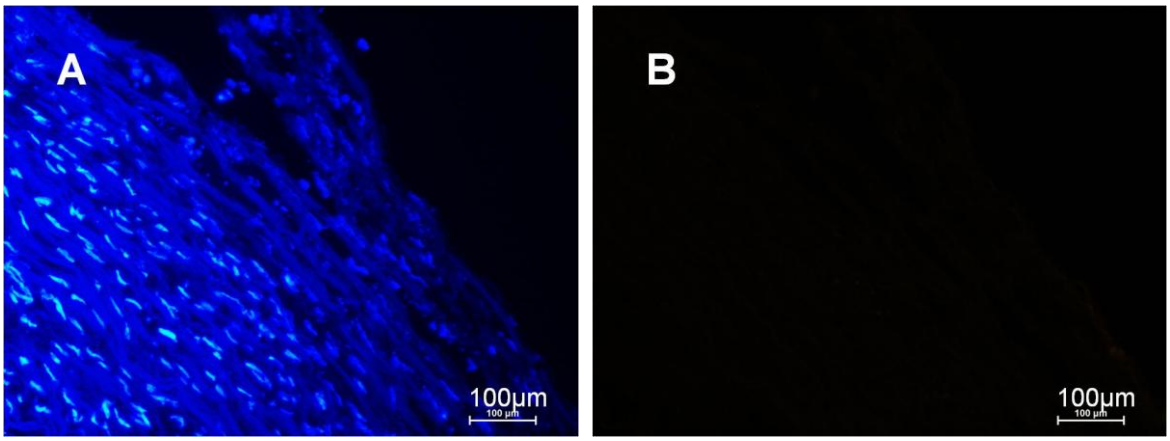

Figure 45: Cross section of a porcine carotid artery incubated with DAPI (A) respectively a CD31 antibody (B) after laser cutting and static cultivation. No endothelium was observed. The artery was cut with a laser (300 nJ, 2546 mW) in a depth of 150 µm and incubated with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax). After disinfection the artery was incubated for 5 days with 90 ml of culture medium at 37 °C in a cell culture flask.

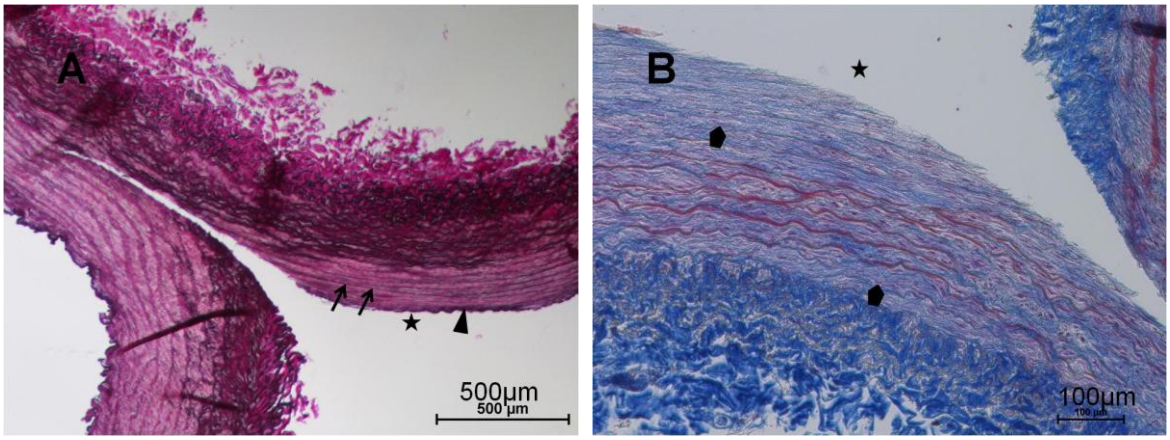

Figure 46: Elastica van Gieson (A) and Masson Trichrome staining (B) of a cross section of a porcine common carotid artery cut with a laser after dynamic cultivation. Stars indicate the lumen. A: A flap with adventitia was disrupted from the artery. Next to the dissected area, *membrana elastic interna* (triangle) and elastic fibres (two arrows) showed no degradation. B: Smooth muscle cells (red nuclei indicated by pentagons) were continuously distributed in the media and nuclei showed a spindle structure. The collagen matrix was intact. The tissue was cut with 300 nJ and 2546 mW in a depth of 150 µm and incubated with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax). After disinfection the artery was incubated for 5 days at 37 °C in the bioreactor system at a medium flow rate of 262 ml/min.

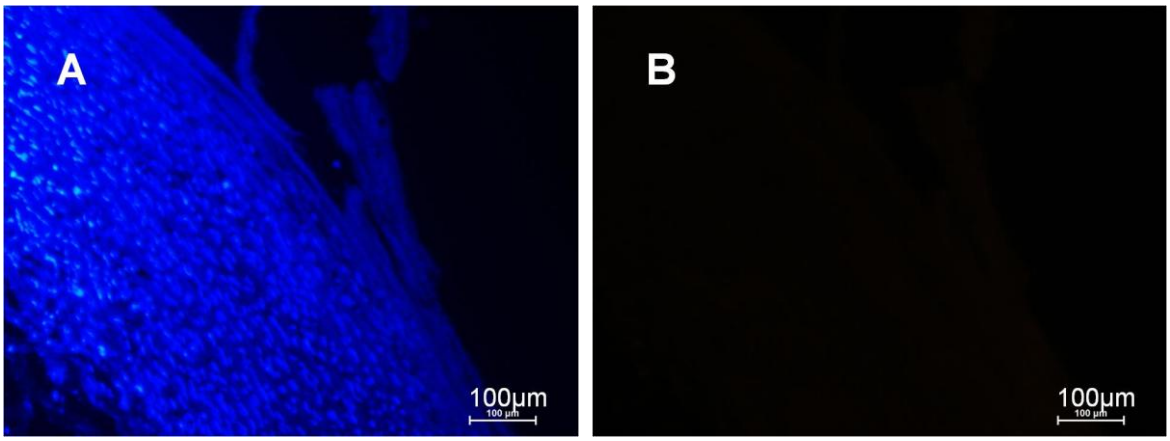

Figure 47: Cross section of a porcine carotid artery incubated with DAPI (A) respectively a CD31 antibody (B) after laser cut and dynamic cultivation. No endothelium was observed. The artery was cut with a laser (300 nJ, 2546 mW) in a depth of 150 µm and incubated with an antibiotic solution (0.5 mg/ml Gentamycin; 0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 10 % FBS and 1 % Glutamax). After disinfection the artery was incubated for 5 days at 37 °C in the bioreactor system at a medium flow rate of 262 ml/min.

Figure 48: Vessel unit of the bioreactor system with a laser treated artery cultivated for 5 days. Circle show the postion of the artery. The cloudy medium indicate a contamination.

5. Discussion

5.1. Disinfection of Arteries

For the disinfection three different antibiotic solutions were tested, including 1) 250 µg/ml Amphotericin B and 0.5 mg/ml Gentamycin in DMEM with 10 % FBS and 1 % Glutamax as suggested by Jentsch, (2014) 2) a solution with 0.5 mg/ml Gentamycin,0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in PBS or 3) 0.5 mg/ml Gentamycin,0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in DMEM with 1 % Glutamax and 10 % FBS . After 30 min incubation with 250 µg/ml Amphotericin B and 0.5 mg/ml Gentamycin the arteries were not sterile. This was consistent with previous work of that lab that used this protocol with equine carotid arteries and did not get a sterile result, too. An incubation time of 1 h with this antibiotic solution showed an effective disinfection of the arteries, but had a significant effect on the viability of the tissue (28). Arteries treated with 0.5 mg/ml Gentamycin,0.2 mg/ml Polymixin B and 0.05 mg/ml Vancomycin in PBS were sterile, but showed a statistically significant decrease of viability. This decrease might be due to a lack of culture medium, which would prevent dying of the tissue. Using the same antibiotics in DMEM with 10 % FBS and 1 % Glutamax did not show a significant decrease of viability..

5.2. Effect of Enzymes

5.2.1. Collagenase

Degradation of collagen is a feature of aneurysms. Collagenase can mimic this degradation. To investigate the effect of collagenase degradation on arterial walls histological changes were examined. Treatment with collagenase showed detachment but no loss of cells in the media. Degradation of the collagen matrix was observed. (21) reported a loss of smooth muscle cell integrity after a 12 day cultivation of a porcine carotid artery treated with collagenase. Loss of smooth muscle cells and collagen disruption and degradation were observed in a porcine *in vivo* model using an elastase and collagenase mixture (10). An *in vitro* study of porcine arteries treated with elastase also showed a reduction of smooth muscle cell concentration (9). Thus the loss of smooth muscle cells might be an effect of the elastase treatment. Degradation of collagen by collagenase can mimic the imbalance of degradation and synthesis of collagen in aneurysmatic tissue (11). In human a elevated levels of the collagenase MMP-1wereobserved (9), thus treatment with collagenase can simulated the degradation of collagen caused by this enzyme.

Arteries digested by collagenase applied at the intima showed more degradation of the media than when the collagenase was applied at the adventitia. The adventitia might have acted as a barrier for enzyme diffusion. In ruptured abdominal aneurysms in cattle a destruction of the media and intima was observed (29), thus applying the gel at the intimal side and digesting the intima and media seems more capable of mimicking a native aneurysm.

5.2.2. Elastase

Because elastin loss is a feature of aneurysms the effect of elastase treatment on arteries were investigated.Fragmentation of the elastin fibres, degradation of the *membrana elastic interna* and a loss of smooth muscle cells were observed in arteries treated with elastase. Wills, *et al*. (1996) noticed a reduction in smooth muscle cells and a degradation of elastin in an *in vitro* model of a porcine thoracic aorta treated with 100 U/ml elastase for 24 h in an *in vitro* model of a procine thoracic aorta for aneurysm. Riches, *et al.*(2013) reported loss of elastin fibrils in porcine carotid arteries following enzyme treatment with elastase and cultivation for 14 days in a porcine carotid. In porcine arteries perfused with a collagenase and elastase mixture disorganization, disruption and degradation of the elastin fibres was seen (10) . Aneurysm induction by $CaCl_2$ in the mouse model aneurysm showed a fragmentation of elastic fibres (30). In a rabbit model of aneurysm the right common carotid artery of rabbits was infused with elastase and showed no elastin in the artery after treatment (31). These findings are consistent with other *in vitro* and *in vivo* studies. A study of ruptured aneurysms in cattle showed disorganized and fragmented elastin fibres (29).In patients with Marfan syndrome elastin fragmentation and areas devoid of elastin has been observed , making these patients vulnerable to aneurysm formation (11).In addition loss of smooth muscle cells has also been seen in patients with aneuryms (11). Loss of smooth muscle cells and elastin fragmentation are features of natural aneurysmatic tissue and can be induced by elastase treatment. MMP-9 is an elastase secreted by macrophages. Elevated activity levels of this enzyme were reported in aneurysmatic tissue (8). Applying an elastase has the potential to mimic the elastolytic effect of macrophage infiltration seen in aneurysms.

Applying the elastase at the adventitia of the artery showed no effect. The adventitia might act as a barrier for the enzyme. In ruptured aneurysms, a degradation of the intima and adventitia have been reported (29). Therefore a treatment of the intima might produce the desired effect. A minimal effect on the structure was seen when using a concentration of 25 U/ml and therefore an increase of the elastase concentration is would be a logical

improvement. For the cultivation the arteries were treated with an elastase concentration of 75 U/ml as described by Riches, *et al.*(2013) and covered with medium to keep the tissue as viable as possible but decrease the dilution effect of the medium.

5.3. Effect of Laser treatment

In atherosclerotic aneurysms a plaque inside the media destroys the artery. A cut inside the media with a laser might mimic that effect. Therefore the effect of laser treatment on arteries was investigated. The laser cut the artery in depth of 200 µm without affecting the surrounding tissue. Collagen matrix and elastin fibres stayed intact and smooth muscle cells did not show differences to the native tissue. Van Leeuwen, *et al.*, (1992) described a necrotic zone of 40 µm when using a XeCl excimer (λ=308 nm) with a pulse of 115 ns and 39 mJ/mm^2. (32) report about thermal injury of tissue using a 1 µsec pulsed laser with wavelength of 465 nm and 15.9 ± 2.2 J/cm^2. The laser used in this study worked at a wavelength of 1030 nm with a pulse of 300 fs. No thermal injury or charring were observed. Excimer lasers used for tissue ablation emit a wavelength primary absorbed by proteins; the laser used in this study emits a beam with 1030 nm, which is absorbed from water and not from proteins. Thus the laser used for this study can penetrate the tissue without destroying proteins, which explains the absence of charring. Because of the high power input in a short time (300 nJ in a pulse of 300 fs) tissue evaporates but cannot transmit the heat. The thermal relaxation time of tissue is longer than the pulse, so the heat cannot be transmitted.

5.4. Effect of Cultivation

5.4.1. Cultivation of untreated arteries

The effect of cultivation on the viability and architecture arteries was investigated. Tissue viability decreased significantly after antibiotic treatment. The arteries showed no significant difference in viability compared to the negative control after dynamic and static cultivation. During the cultivation, a turbidity of the culture medium was observed. Microscopic investigation showed the formation of crystals. These crystals might had an effect on the viability of the tissue. Jentsch, 2014 observed similar results for static cultivation, the tissue did not show a significant different viability value than the negative control. For the dynamically cultivated arteries, Jentsch observed a significant decrease in viability, but also a significant difference compared to arteries after dynamic cultivation and the negative control. During the cultivation, a turbidity of the culture medium was observed, wheras microscopic investigation showed the formation of crystals. Theses crystals might had an effect on the viability of the tissue.

Following static cultivation a straightening of the *membrana elastic interna* and elastin fibres in the media was observed. The endothelium and collagen matrix did not show differences compared to native tissue. The effects of dynamic cultivation were loss of endothelium, straightening and degradation of *membrana elastic interna* and elastin fibres in the media and a loss of collagen matrix. Static and dynamic cultivation did not affect the smooth muscle cells. Jentsch (2014) reported that arteries show "uniformly distributed smooth muscle cells" after static and dynamic cultivation, whereas an intact endothelium after static cultivation. After dynamic cultivation Jentsch reported a degradation of the endothelium and a degradation of the *tunica interna*. In contrast Wills, *et al.* (1996), reported that porcine aortas did not show changes in collagen and elastin content after 14 days of static cultivation. Riches *et al*(2013) also reported that there were no histological changes after 12 days of cultivation under dynamic conditions.

Internal MMP's might have degraded the elastin fibres of the tissue. (9) observed a continued degradation of elastin fibres after removal of the elastase and hypothesised the presence on endogenous internal MMP's that continue to degrade the tissue after initial elastase treatment. During cultivation messengers provided by the organism are absent. These messenger might regulate the balance of degradation and synthesis of collagen and elastin. Without these messengers, tissue degrades and the generated peptides might activate MMP's (9).

5.4.2. Cultivation of arteries treated with Enzymes

Enzyme treatment with collagenase and elastase can mimic the degradation of the arterial of aneurysms. The medium flow can simulate the shear stress caused by the blood flow. The aim of this experiment was to show, if it is possible to create a living arterial aneurysm *in vitro* by using enzymes for degradation of the vessel wall.

The disinfection of arteries after enzyme treatment was successfull, but after static and dynamic cultivation the samples were contaminated. A contamination of the bioreactor system can explain that. The tissue viability showed no significant difference to the negative control after static and dynamic cultivation. This finding is consistent with the results of the cultivation of untreated arteries. The aim to create a **living** aneurysm was not achieved.

Following the application of collagenase and elastase only remnants of the media layer of the artery were observed, whereas the endothelium was absent. The collagen matrix was degraded near the lumen and the smooth muscle cells were detached. The elastin fibres near the lumen were also fragmented. Riches *et al* (2013) observed a "clear loss of elastin fibres" and "a loss of smooth muscle integrity" in arteries treated with a combination of elastase and collagenase after a cultivation period of 12 days. They also reported on a flattened and rhomboid shape of smooth muscle cells in comparison to a spindle like shape in native arteries.

In vivo studies showed similar results. Hynecek, *et al.* (2007) reported loss of endothelium ; "elastin disorganization, disruption and degradation" and "lamellar collagen disruption and degradation". In this study they also observed that the smooth muscle cells were shrunken and irregular after enzyme treatment. Hoh, *et al*.(2004) reported on the thinning of the artery and elastin degradation in a rabbit model of aneurysms, induced by elastase perfusion. Destruction of elastin fibres were also seen in a mouse model of aneurysm induced by $CaCl_2$ (30).

A study of ruptured aneurysms in cattle showed that *tunica intima* and parts of the *tunica intima* were absent in native late stage aneurysms. In this study loss and fragmentation of elastin was observed (29). Thus the histological changes observed in this study might represent the end stage of an aneurysm.

5.4.3. Cultivation of arteries treated with Laser

A cut in the arterial wall can mimic the destruction of the tissue by an aneurysmatic plaque. The medium flow can simulate the shear forces occurring on endothelial cells of blood vessels. Combination of laser treatment and cultivation under dynamic conditions might mimic the situation in aneurysms. The effect of laser treatment and following cultivation was investigated. The tissue viability of arteries after laser treatment showed no significant difference to the negative control. A long delay between getting the tissue from the abattoir and the cut might be the reason. Disinfection of the arteries was not successful. The non sterile conditions during the cut process are a source of contamination and might be the reason for the contamination and not successful disinfection.

Aneurysm formation was observed in patient after laser angioplasty (32). Dissection also appeared after laser treatment (33). Laser injury and cultivation under dynamic conditions might be an option to mimic the situation in these patients. The laser used in that study did not affect the structure of the media between the laser and the cut. Thus the laser cut might

cause the same damage as an atherosclerotic plaque in the early stage of an atherosclerotic aneurysm. Removing the tissue can mimic the rupture of the plaque.

The arteries cut with the laser showed loss of smooth muscle cells after dynamic cultivation, a feature of aneurysm formation. Also straightening of elastic fibres were observed as reported in other studies (10). Arteries showed dilation after five days of cultivation. This model might be used as a simulation for the early stages of aneurysm formation.

6. Summary and Outlook

The hypothesis of this project was that it is possible to generate a living arterial aneurysm *in vitro*. Porcine carotid arteries were degraded with either enzymes or a laser. A cultivation under continuous medium flow was performed, afterwards. Enzyme degradation of the arterial wall showed loss and detachment of smooth muscle cells and degradation of elastin fibres and collagen. Following cultivation under flow in a vascular bioreactor dilation of the vessel wall was observed. Laser treatment produced a clear defined cut in the arterial wall without charring or heat damage of the tissue next to the cut. The tissue between the cut and the *intima* was removed. Following cultivation of the tissue no dilation of the vessel wall was observed. The medium flow removed parts of the arterial wall. Both, arteries degraded by enzymes or cut with the laser system were contaminated after the cultivation. Therefore, the bioreactor system was contaminated. The aim to create a living *in vitro* model was not achieved. All samples showed a decrease of tissue viability up to the level of the negative control.

Future work can address the evaluation of an adequate disinfection method for the bioreactor system or development of a new bioreactor system. A high variation of viability between arteries and within one artery was seen. The use of a vascular graft sodded with cells might result in a more consistent value of tissue viability. A limitation of the study is the low number of replicates per cultivation. More cultivations need to be done.

Bibliography

1. **Rohen, J and Lütjen-Drecoll, E.** *Funktionellle Histologie.* Stuttgart : F. K. Schattauer Verlagsgesellschaft mbH, 2000. Vol. 4.

2. **Lüllman-Rauch, R.** *Taschenlehrbuch Histologie.* Stuttgart : Georg Thieme Verlag, 2006. Vol. 2.

3. **Bob, Alexander and Bob, Konstantin.** *Innere Medizin.* Stuttgart : Georg Thieme Verlag, 2009. Vol. 2.

4. **Renz-Polster, H, Krautzig, S and Braun, J.** *Basislehrbuch Innere Medizin.* Münnchen : Elsevier GmbH, 2004. Vol. 3.

5. **Boecker, D and Moch, H.** *Pathologie.* Muenchen : Elsevier GmbH, 2008.

6. **Classen, M, Diehl, V and Kochsiek, K.** *Innere Medizin.* Münnchen : Urban & Fischer, 2004. Vol. 5.

7. **Brisman, Jonathan, Song, Joon and Newell, David.** Cerebral Aneurysms. *new england journal of medicine.* 2006, Vol. 355.

8. **Kurosawa, Koji, Matsumura, Jon S and Yamanouchi, Dai.** Current Status of Medical Treatment for Abdominal Aortic Aneurysm. *Circulation Journal.* 2013, Vol. 77.

9. **Wills, Andrew, Thompson, Matthew M., Crowther, Matthew, Brindle, Nicholas P., Nasim, Ahktar, Sayers, Robert D. and Bell, Peter R. F.** Elastase-induced matrix degradation in arterial organ cultures: An in vitro model of aneurysmal disease. *JOURNAL OF VASCULAR SURGERY.* 1996, Vol. 24, 4.

10. **Hynecek, Robert L:, DeRubertis, Brian G., Trocciola, Susan M., Zhang, Honglei, Prince, Martin R., Ennis, Terri L., Kent, K. Craig and Faries, Peter L.** The creation of an infrarenal aneurysm within the native abdominal aorta of swine. *Surgery.* 2007, Vol. 142.

11. **Kumar, Vinay, Abbas, Abul K., Fausto, Nelson and Aster, Jon C.** *ROBBINS AND COTRAN PATHOLOGIC BASIS OF DISEASE.* Philadelphia : Saunders, 2010.

12. **Seibert, Brad, Tummala, P. Ramachandra, Chow, Ricky, Faridar, Alireza, Mousavi, Seyed A. and Divani, Afshin A.** Intracranial aneurysms : review of current treatment options and outcomes. *frontiers in Neurology.* Review article, 2011, Vol. 2.

13. **Aggarwal, Sourabh, Qamar, Arman, Sharma, Vishal and Sharma, Alka.** Abdominal aortic aneurysm: A comprehensive review. *Experimental & Clinical Cardiology.* 2011, Vol. 16, 1.

14. **Brown, S.** New techniques in laser therapy. *British medical journal.* 1998, Vol. 316.

15. **Abrahamse, Heidi.** Regenerative Medicine, Stem Cells, and Low-Level Laser Therapy: Future Directives. *Photomedicine and Laser Surgery.* 2012, Vol. 30.

16. **Massai, Diana, Cerino, Giulia, Gallo, Diego, Pennella, Francesco, Deriu, Marco A, Rodriguez, Andres, Montevecchi, Franco M, Bignardi, Cristina, Audenino, Alberto and Morbiducci, Umberto.** Bioreactors as Engineering Support to Treat Cardiac Muscle and Vascular Disease. 2013, Vol. 4, 3.

17. **Rattcliffe, A and Niklason, L.** Bioreactors and Bioprocessing for Tissue Engineering. *Ann. N.Y. Acad. Sci.* 202, Vol. 961.

18. **Williams, C and Wick, T M.** Perfusion Bioreactor for Small Diameter Tissue-Engineered Arteries. *Tissue Engineering.* 2004, Vol. 10.

19. **Hoffmann, Stefan A., Müller-Vieira, Ursula, Biemel, Klaus, Knobeloch, Daniel, Heydel, Sandra, Lübberstedt, Marc, Nüssler, Andreas K., Andersson, Tommy B., Gerlach, Jörg C. and Zeilinger, Katrin.** Analysis of Drug Metabolism Activities in a Miniaturized Liver Cell Bioreactor for Use in Pharmacological Studies. *Biotechnology and Bioengineering.* 2012, Vol. 109, 12.

20. **Darnell, Malin, Ulvestad, Maria, Ellis, Ewa, Weidolf, Lars and Andersson, Tommy B.** In Vitro Evaluation of Major In Vivo Drug Metabolic Pathways Using Primary Human Hepatocytes and HepaRG Cells in Suspension and a Dynamic Three-Dimensional Bioreactor System. *THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS.* 2012, Vol. 343.

21. **Riches, Kirsten, Angelini, Timothy G, Mudhar, Gurprit S, Kaye, Jean, Clark, Emily, Bailey, Marc A, Sohrabi, Soroush, Korossis, Sotirios, Walker, Peter G, Scott, D Julian A and Porter, Karen E.** Exploring smooth muscle phenotype and function in a bioreactor model of abdominal aortic aneurysm. *Journal of Translational Medicine.* 2013, Vol. 11.

22. **Touroo, Jeremy S. and Williams, Stuart K.** A tissue-engineered aneurysm model for evaluation of endovascular devices. *JOURNAL OF BIOMEDICAL MATERIALS RESEARCH.* 2012, Vol. 100A, 12.

23. **Trollope, Alexandra, Moxon, Joseph V., Moran, Corey S. and Golledge, Jonathan.** Animal models of abdominal aortic aneurysm and their role in furthering management of human disease. *Cardiovascular Pathology.* 2011, Vol. 20.

24. **Deplano, Valérie, Knapp, Yannick, Bailly, Lucie and Bertrand, Eric.** Flow of a blood analogue fluid in a compliant abdominal aortic aneurysm model: Experimental modelling. *Journal of Biomechanics.* 2014.

25. **Bonnema, Garret T., O'Halloran Cardinal, Kristen, Williams, Stuart K. and Barton, Jennifer K.** An automatic algorithm for detecting stent endothelialization from volumetric optical coherence tomography datasets. *PHYSICS IN MEDICINE AND BIOLOGY.* 2008, Vol. 53.

26. **Piotin, Michel, Mandai, Shinya, Murphy, Kieran J., Sugiu, Kenji, Gailloud, Philippe, Martin, Jean-Baptiste and Rüfenacht, Daniel A.** Dense Packing of Cerebral Aneurysms: An In Vitro Study with Detachable Platinum Coils. *American Journal of Neroradoiology.* 2000, 21.

27. **Baráth, Krisztina, Cassot, Francis, Rüfenacht, Daniel A. and Fasel, Jean H. D.** Anatomically Shaped Internal Carotid Artery Aneurysm in Vitro Model for Flow Analysis to Evaluate Stent Effect. *American Journal of Neuroradiology.* 2004, 25.

28. **Jentsch, Julian-Alexander.** *Investigation of the effect of different flow rates on the cell viability of fresh carotid arteries in vitro.* 2014.

29. **Lamm, Catherine G., Guard, Charles L., Erb, Hollis N. and Njaa, Bradley L.** Characterization of rupture of abdominal artery aneurysm in dairy cattle. *Journal of Veterinary Diagnostic Investigation.* 2007, Vol. 19.

30. **Longo, G. Matthew, Xiong, Wanfen, Greiner, Timothy C., Zhao, Yong, Fiotti, Nicola and Baxter, B. Timothy.** Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. *The Journal of Clinical Investigation.* 2002, Vol. 110, 5.

31. **Hoh, B. L., Rabinov, J. D., Pryor, J. C. and Ogilvy, C. S.** A modified technique for using elastase to create saccular aneurysms in animals that histologically and hemodynamically resemble aneurysms in human. *Acta Neurochirugica.* 2004, Vol. 146.

32. **Prince, Martin R, Deutsch, Thomas F, Shapiro, Ascher H, Margolis, Randall J, Oseroff, Allan R, Fallon, John T, Parrish, John A and Anderson, R Rox.** Selective ablation of atheromas using a flashlamp-excited dye laser at 465 nm. *Proceedings of the National Academy of Science of the United States of America.* 1986, Vol. 83.

33. **Van Leeuwen, Ton G, Van Erven, Lieselotte, Meertens, John H, Motamedi, Massoud, Post, Mark J and Orst, Cornelius.** Origin of Arterial Wall Dissections Induced by Pulsed Excimer and Mid-Infrared Laser Ablation in the Pig. *Journal of the American College of Cardiology.* 1992, Vol. 19, 7.

34. **Allaire, Eric, Muscatelli-Groux, Béatrice, Guinault, Anne-Marie, Pages, Carine, Goussard, Audrey, Mandet, Chantal, Bruneval, Patrick, Méllière, Didier and Becquemin, Jean-Pierre.** Vascular Smooth Muscle Cell Endovascular Therapy Vessel Mimics for the Evaluation of Endothelialization of Intravascular Devices. *Annals of Surgery.* 2004, Vol. 239, 3.

35. **Yan, Lei, Shi, Wan-Yin, Wang, Dan, Zhu, Yue-Qi, Tan, Hua-Qiao and Li, Ming-Hua.** Application of the Willis covered stent using the telescopic technique for the treatment of fusiform aneurysm in a canine model. *Neurology India.* 2013, Vol. 61.

36. **Morticelli, Lucrezia.** *Guided Functional Re-Engineering of the Mitral Valve Leaflet.* 2013.

Appendix

Product	Name	Company
50ml Tubes	Röhre 50ml	Sarstedt
6 well plate	Tissue culture plate 6-well Flat bottom	Sarstedt
96-well plate	Tissue culture plate 96-well Flat bottom with Lid	Sarstedt
Acetic acid	acetic acid 100 %	AppliChem
Aceton	Aceton >99,7 % ,reinst	Roth
Agarose gel	Agarose low gelling temperature	Sigma Life Science
Air filter	Midisart 200	Satorius
Aluminium foil	aluminium folie OMNILAB Labor	OMNILAB
Amphotericin B	Amphortericin B	Biochrom
Aniline blue solution	Aniline blue solution	Sigma Aldrich
Anti mouse antibody	Alexa Flour 555 goat anti-mouse IgG	invitrogen
Anti rabbit antibody	Alexa Flour 555 goat anti-rabbit IgG	Invitrogen
Autoclave	Tuttnauer 5075 ELVC	Systec
Biebrich scarlet-acid fuchsin solution	Biebrich scarlet-acid fuchsin	Sigma Aldrich
Biostat	Biostat B-DCU II	Satorius Stedin
Brush	12, 4 and 8	Rico Desing Hobby
BSA	Albumin bovine Serum, Protease free	VWR
Cable Strep	UB 200C-N	Carl Roth GmbH
CD31 Antibody	PECAM-1 (M-20 Sc 150R rabbit polyclonal IgG)	Santa Cruz Biotechnology
Cell culture flask	Tissue Culture Flask 150 cm^2 with reclosable lid	TPP
Centrifuge	HERAEUS Fresco 17	Thermo Scientific
Cold plate	Cop 20	Leica
Collagenase	Collagenase from Clostridium histolyticum	Sigma Life Science
Control station	BioPAT DCU Tower station	Satorius Stedin
Cover slips	Menzel Gläser	Menzel
Cryo microtom	HM560	Microm
DMEM	Bio Whittaker DMEM	Lonza
DMSO	Dimethyl sulfoxide cell culture grade	AppliChem
Elastase	Elastase,Porcine Pancreas, High	Calbiochem

	Purity, Crystallized	
Embedding casette	Rotilabo-embedding cassetes, Macro	Roth
Embedding station	Typ 72000	Microm
Ethanol	Ethanol ca 99 % vergällt Typ 642	Büfa
FCS	Fetal Borvine Serum	Biochrom
Formalin	Formalin solution , neutral buffered 10 %	Sigma
Freezer	Liebherr premium	Liebherr
Fridge		Liebherr
Gentamycin	Gentamycin sulfate	Biochrom
Glas pipette tips	Pasterpipetten aus Glas	Brand
Gloves	Vasco Nitril White	Braun
Glutamax	GlutaMAX	gibco
Water bath	Wasserbad WNB 7-45	Memmert
HBSS powder	Hanks balanced Salt Solution	AppliChem
Heate plate	Typ 12800	Microm
Ice maschien	MF 30	Scotsman
Image J	Image J	
Incubator	HERAcell 240, CO2 incubator	Thermo Scientific
Incubation hood	CERTOMAT	Satorius Stedin
Isotype control mouse	Mouse IgG1	abcam
Isotype control rabbit	Normal rabbit IgG	Calbiochem
Potassium chloride	Potassium choride	Sigma
Korsolex	Korsolex plus	Bode Chemie
Laser system		Rowiak
LIVE/DEAD assay	LIVE/DEAD viability/cytotoxicity kit	invitrogen
MHC antibody	Myosin heavy chain antibody	Thermo Scientific
Microscope	Nikon inverted Microscope Eclipse TE300	Nikon
Microtom	2040	Reichert-Jung
Microtom blade cryotom	SEC 35 low Profile Blade	MICROM
Microtom blade microtom	Feather Microtom BLADE stainless stell R35	FEATHER
Mounting Immunohistochemistrie	Roti-Mount Flou Care DAPI	Roth
Mounting Histology	Corbit Balsam	Hecht
MTT powder	Thiozyl Blue Tetrazolium Bromid	Sigma
Mucasol	Mucasol	Merz
Oven	9010-001	BINDER
Parafin warming pot	Typ 43900	Medax
PBS with Ca and Mg	Dulbecco's phosphat buffered salini (1x) with Ca and Mg	Lonza
PBS without Ca and Mg	PBS Dulbecco	Biochrom

Pennicilin-Streptomycin	Pennicilin-Streptomycin	Lonza
pH probe	Easy Ferm Plus	???
Phosphomolybdic acid solution	Phosphomolybdic acid	Sigma Aldrich
Phosphotungstic acid solution	Phosphotungstic acid	Sigma Aldrich
Picrofuchsin solution according to van Gieson	Picrofuchsin	Merck
Pipette boy	accu jet pro	Brand
Plastic sleeves	Schutzärmel aus PE	Hygonorm
pO2 probe	Oxyferm FDA	???
Polimxin B	Polimixin B sulfate	Sigma
PPG	Polypropyleneglycol 200	VWR
Pump	ISMATEC	BVP
Quick connectors	Schnell Verschlusskupplung	Medinet GmbH
Safety cabinet	SAFE 2020	Thermo Scientific
Siringe	Single use siringe 2 piede 10 ml	Braun
Slide	Superfrost UltraPlus	Thermo Scientific
Sodium Chloride	Sodium Chloride	Roth
Sodium hydrogen Carbonate	Sodium bicarbonat	Sigma Life Science
Soy Casein powder	CASO Boillon	Roth
Spectrometre	Multiscan FC	Thermo Scientific
Sterile filter 400ml	500ml Bottle Topfilter	Sarstedt
Sterile gloves	Latex Biogel Surgeons	Mölnycke Healthcare
Sterile petri dish	Petrie dish 92x 16 mm with cams	Sarstedt
Sterile pipette tips	sterile pipette tips	Sarstedt
Sterile scalpel	Feather dispsable scalpel No 21	Feather
Sterile tissue	OP-towel	Mölnycke Healthcare
Continuous stirred tank reactor	G-13587	Satorius Stedin
Surgical sutures	Prolene polypropylen or MERSILENE polyester	Ehticon
Temperature probe	PN237540	Oxyferm
Thermo Mixer	Thermomixer C	eppendorf
OCT	Tissue Tek	Sakura
Tris	Tris Pufferan > 99,9 %	Roth
Tween 20	Tween 20	Roth
Vancomycin	Vancomycinchloride	Aldrich
Vortexer	IKA Vortex Genius 3	IKA
Water bath	Typ 249000	Microm
Water filtering System	arium 611	Satorius Stedim
Weigert's solution A alcoholic hematoxylin solution	Weigert's solution A	Merck

Weigert's solution B hydrochloride iron (III) nitrate solution	Weigert's solution B	Merck
Xylene	Xylol	Roth

The candidate confirms that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Ich versichere hiermit, dass ich die Masterarbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Das bearbeitete Thema wurde nicht schon früher im Rahmen einer anderen Arbeit behandelt und/oder anderswo als Prüfungsarbeit eingereicht.