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„Cultivation and characterisation of porcine endothelial cells for toxicity analysis by flow cytometry“

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ABSTRACT

Linear polyethylenimine (LPEI) is a golden standard reference in gene transfection for a broad range of cell lines- and types, including tumour cells, and is already used in human clinical trials for gene therapy approaches in cancer patients. Despite its high transfection efficiency, cytotoxicity seems to be a limiting factor. Further in vitro studies need to be performed to investigate the molecular basis of its cytotoxicity. Here I optimized the in vitro cultivation and characterization of primary porcine endothelial cells (pEC) in order to facilitate cytotoxicity studies. At first endothelial cells where isolated from porcine thoracic aorta, seeded on culture dishes, and maintained with specialized culture medium. However, after five to six passages, primary isolated cells were contaminated with fibroblast-like cells. The overgrowth of contaminating cells circumvented first experiments as pECs rapidly died off. Therefore, a long-term maintenance system of pEC was established. The effect of lower concentrations of FCS in medium on cell viability and proliferation rate was investigated. Further, gelatine-coating ensured stable culture conditions and the importance of early splitting before complete confluency was determined. Thereby pECs were characterized by analysing morphology under a microscope and expression of endothelial specific marker CD31 by fluorescent-activating cell sorting (FACS) determined. A long-maintenance up to passage 22 was performed with over 90% of CD31 positivity and typical cobble-stone shape until passage 19. Consequently, a cytotoxicity assay with LPEI with different molecular weights and increasing concentrations was realised. Obtained results provide insights into the toxic effect of LPEI compared to the alkaloid camptothecin. With this study the foundations have been laid for long-term maintenance of highly pure primary porcine endothelial cells, which represent a suitable cell culture system for further in vitro cytotoxicity assays of LPEI.

ZUSAMMENFASSUNG

Lineares Polyethylenimin (LPEI) ist eine “Goldstandard”-Referenz der Gentransfektion für eine große Breite an verschiedenen Zelllinien- und typen (Tumorzellen eingeschlossen). Es wird bereits in humanen, klinischen Versuchen zur Gentherapie von Krebspatienten angewendet. Das Polykation weist neben einer hohen Transfektionseffizienz jedoch auch eine hohe Zelltoxizität auf. Weitere in vitro Studien sind notwendig um die molekularen Grundlagen der Zelltoxizität zu ermitteln. In diesem Zusammenhang optimierte ich die in vitro Kultivierung und Charakterisierung von primären Schweine-Endothelzellen um Zelltoxizitätstest zu ermöglichen. Die Endothelzellen wurden hierbei aus der Schweineaorta entnommen, in Zellkulturschalen ausgelegt und mit speziellem Kulturmedium gezüchtet. Jedoch musste festgestellt werden, dass die isolierten Primärzellen, nach 5-6 Passagen mit Fibroblasten-ähnlichen Zellen kontaminiert waren. Die Verunreinigung durch kontaminierte Zellen verhinderte erste Experimente, da die Endothelzellen rasch abstarben. Daher wurden langfristige Erhaltungssysteme für primäre Endothelzellen entwickelt. Der Einfluss niedriger Konzentrationen von FCS im Medium auf die Zellviabilität und Proliferationsrate wurde untersucht. Weiters ermöglichte eine Gelatinebeschichtung stabile Kulturbedingungen. Auch die Notwendigkeit frühzeitiger Subkultivierung noch vor einer kompletten Konfluenz wurde ermittelt. Hierbei wurden die Endothelzellen durch mikroskopische Analyse ihrer Morphologie und Expression endothel-spezifischer Marker mittels Durchflusszytometer charakterisiert. Eine Langzeitkultivierung bis Passage 22 wurde durchgeführt mit über 90% CD31 Positivität und typischer Pflasterform bis Passage 19. Folglich wurde ein LPEI-Zytotoxizitätstest mit unterschiedlichen Molekulargewichten und unterschiedlichen Konzentrationen realisiert. Die erhaltenen Resultate liefern einen Einblick in den toxischen Effekt von LPEI verglichen mit dem Alkaloid Camptothecin. Diese Arbeit schafft Grundlagen für die Langzeitkultivierung von hochreinen primären Schweine-Endothelzellen, welche ein geeignetes Zellkultursystem für weitere Zytotoxizitätstests von LPEI darstellen.

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1. INTRODUCTION

The figures speak for themselves: According to the World Health Organization (WHO), within the next 15 years, more than 17 million cases of cancer will be diagnosed every year. Eventually in year 2030 cancer will be the world´s number one cause of death, ahead of cardiovascular diseases. But cancer constitutes not only a burden of an individual, but also places a tremendous strain on the healthcare system [1]. In the US in 2011 direct medical costs (total of all health care costs) for cancer amounted to $88.7 billion as estimated by The Agency for Healthcare research and Quality (AHRQ) [2].

Cancer is a disease of the genome caused by mutations and chromosomal aberrations. DNA molecules in living organism are confronted with a vast number of endogenous and exogenous genotoxic attacks every day. Estimates suggest that every cell faces up to 10^5 DNA lesions every day. Endogenous damage manifests in hydrolysis or alkylation of DNA base. Radical oxygen species (ROS) which emerge during mitochondrial respiration damage DNA base by oxidation. Mutations also arise from erroneous incorporation of deoxyribonucleotides during the process of replication.

Physical factors like ultraviolet (UV) light, ionizing radiations (IRs), and thermal disruption as well as chemical factors like chemotherapeutic drugs, industrial chemicals, and cigarette smoke are noxious for DNA molecules and can lead to formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidone photoproducts (6-4PPs). Recognition and removal of the incurred lesions are the crucial tasks of the distinct DNA repair system. If damages remain irreparable DNA damage checkpoints stop cell cycle, induce cellular senescence or apoptosis. Hence genome can be preserved and transmitted safely across generations. Erroneous DNA repair on the contrary can result in mutations and or chromosomal aberrations [3].

Oncogenes, tumor suppressor genes and stability genes play a decisive role thereby. Oncogenes can be set on by gain-of-function mutations and loss-of-function mutations result in inactivation of suppressor genes. Furthermore, mutations inactivate stability genes, essential for mitotic recombination and chromosomal segregation. As a consequence uncontrolled tumorous cell growth occurs. P53 is a suppressor gene, widely known as the “guardian of the genome”. Its protein has major influence on cell cycle, DNA repair and

apoptosis in response to cellular stress. In consequence of DNA damage, oncogene activation and hypoxia it can either induce a G_1 growth arrest or apoptosis by inducing *bax*-1, leading to elimination of damaged cells. Mutation of p53 causes a loss of chromosomal integrity. In roughly 50% of all human cancers p53 is mutated [3]–[7].

Gene therapy appears a promising candidate for cancer treatment. Unlike conventional treatment, which often lack selectivity for tumour cells, gene therapy allows for the targeting of the therapeutic transgene directly the malignant cells, thus preventing normal tissue toxicity [8]. Here gene therapy aims to modify expression patterns by delivering genetic-based material to specific cells. In order to slow down or stop the progress of malignancy plasmid DNA (pDNA), micro RNA (miRNA) or interference RNA (RNAi) are introduced into cells. Exogenous DNA interacts with specific mediators enabling expression of a gene of interest or inhibition of mRNA translation.

MiRNAs are small non-coding RNA molecules, which function as key regulators of gene expression. They intervene in fundamental processes like cell signalling, proliferation, differentiation, survival and death causing gene silencing by targeting mRNA. They induce inhibition of translation and degradation of mRNA.

Best-known RNAis are short-interfering RNA (siRNA) and short-hairpin RNA (shRNA). SiRNAs, double-stranded RNA molecules with a length of 19-23 base pairs, function by causing site-specific split leading to destruction of messenger RNA (mRNA). Simplest method of RNAi appears transfection of chemically synthesized siRNAs directly into the cytosol. Unlike shRNA and gene therapy based in pDNA delivery siRNA prove effective without the need to reach the nucleus. This fact appears advantageous as nucleus uptake implies a complex intracellular trafficking. The second form of RNAi, shRNA, consists of two complementary 19-22 bp RNA sequences. Both are linked by a short hairpin of 4-11nt. While siRNA migrate into the cytosol, shRNA possesses the ability of DNA integration.

Thus gene therapy focuses not on treatment of symptoms but rather elimination of the cause of various disorders at the genetic level [9]–[12].

Yet delivering nucleic acids to its target is challenging. Nucleic acids are highly hydrophilic macromolecules, relatively large in size with negative charges. These physical properties restrict a binding to the cell surface and they do not readily diffuse across the lipophilic cell membrane. Furthermore chemical modifications are crucial to improve stability in vivo as

nucleic acids are sensitive towards degradation by plasma and tissue nucleases. A good transport system should therefore meet the following criteria: 1) protect nucleic acid from degradation, 2) accumulation in target and 3) enable cellular uptake [13], [14].

Protection from degradation – long circulation time

The delivery of unmodified nucleic acids like siRNA is limited by many barriers. They are hardly taken up by cells and are rapidly degraded by nucleases and eliminated by phagocytic immune cells when administered intravenously. Furthermore immunogenicity, renal clearance, poor endosomal release and untoward side effects act as obstacles that need to be overcome [10].

Gene vectors, whether virus based or self-assembling complexes, also lack efficient intravenous delivery. Synthetic vectors are frequently found unstable in blood system. Viral vectors too have poor circulation properties. They are rapidly recognized by antibodies or other plasma proteins and cleared into liver and spleen. Coating the agent with hydrophilic polymer appears a practical solution. Positively charged hydrophilic polymers (polycations) condense with negatively charged polynucleotides. These nano-sized non-viral vectors self-assemble due to strong coulombic forces, hydrogen bonds, hydrophobic and van der Waals interactions. Similarly system delivery of genes with viral vectors can be improved by surface coating. Physical properties can be modulated enabling 'stealthing' during the delivery phase. Hydrophilic polymer coating improves systemic circulation properties endowing better access to disseminated targets. But simple coating with polycations show its limit. Particle aggregation can be prevented due to electrostatic repulsion. But positive surface charge facilitates interactions with negatively charged groups of plasma proteins, vessel endothelium and blood cells. Large aggregations are formed which accumulate in very tiny capillaries of the lung. As aggregate stability is low, most complexes travel back to the circulating blood. Components of the immune system are activating leading to elimination of the aggregation through reticuloendothelial system (RES). Subsequently they accumulate in in the liver, kidney and spleen. Here improved steric stabilisation is crucial to prevent disintegration within the bloodstream [15], [16].

Intratumoral accumulation mediated by EPR-effect

Malignant tissue is characterized by several distinctive properties: limitless replicative potential, sustained angiogenesis, avoidance of apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals and tissue invasion and metastasis as well as evasion of immune destruction and reprogramming of energy metabolism [6]. These properties induce over expression of certain transmembrane receptors, increasing vascular permeability and interstitial pressure or changes in pH. Characteristics that can be harnessed for specific delivery approaches [17]. A growing solid tumor presents an abnormal vasculature with irregular architecture and poor lymphatic drainage. Furthermore resistance to blood flow and paracellular permeability is increased. This leaky tumor vasculature is the key to systemic delivery and enhances the uptake of intravenously administered anticancer drug in a solid tumor, an implication described as the Enhanced Permeation and Retention (EPR) effect [18].

Gene delivery

The first strategy for gene delivery consists of direct injection of naked DNA to the tumor site. This simple handling produces high levels of gene expression leading to a number of experimental protocols. Yet this strategy is only feasible by easy accessible tissues such as the skin and muscles, where direct injection is possible. Systemic application of free DNA on the contrary leads to quick degradation due to the presence of serum nuclease [19]. Hence for successful systemic delivery a number of gene transfer vehicles, known as vectors, have been developed. They aim to introduce therapeutic genes into target somatic cells and can broadly be divided into two groups: viral and non-viral vectors [20].

Viral vectors consist of genetic material surrounded by either a capsid or an envelope. The capsid comprises proteins and the envelope is lipidic. This shell enables binding on cell surface through specific receptors. Once inside the cell the virus shell protects the therapeutic gene from lysosomal degradation and provides it into the nucleus. The method of gene delivery via a virus is termed transduction. Viral vectors are very efficient at the transfer of gene and achieve high expression of the transgenic protein. For the purpose of gene transfection viral vector lack viral genes in their vector backbone that are required for replication within the host cell. They can be further classified into integrating and non-

integrating vectors. Some viruses like lentivirus or onco-retrovirus integrate into the host chromosome, their vectors are named integrating vectors. This method ensures stable propagation of genes to the daughter nuclei. Besides this benefit the risk of genotoxicity remains. Insertional mutagenesis can occur due to random integration. Furthermore the integrated construct can affect the expression level of surrounding genes to an extent, which cannot be predicted. On the other hand non-integrating vectors do not integrate into the tumor cell genome, transgene maintain episomal. This reduces the risk of insertional oncogenesis. These vectors however are unsuitable for gene transfer into rapidly dividing cells such as haematopoietic stem cells as episomally retained DNA is lost with each cell division. They can however be utilized very effectively for post-mitotic cells that are incapable of proliferation like differentiated neurons and muscle cells that comprise the brain, heart and skeletal muscle. Here they can be stably maintained for longer time [5], [19]–[22].

Adenoviral (Ad) vectors belong to the class of non-integrating vectors. They are double stranded (ds)DNA vectors devoid of an envelope. Cellular uptake is accomplished by interaction of the fiber protein with coxsackie-adenovirus receptor (CAR). Endocytosis proceeds clathrin-mediated followed by nucleus uptake and efficient transduction. Efficacy has been largely improved as early Ad vectors contained residual viral genes in the vector backbone. Genes activated T-cell mediated immune system inducing elimination of gene-modified cells. Latest adenoviral vectors lack residual adenoviral genes and are so called gutless vectors. Thereby their ability to induce inflammation has been reduced and simultaneously their cargo capacity rose to 30kb. Still, a risk of immune system activation remains [21]. The challenge for Ad vector-mediated cancer gene therapy constitutes the promiscuous tropism of Ad vectors, which can lead to transduction of non-target cells. Fiber protein not only binds with the CAR, but interacts also with scavenger receptor A of Kupffer cells, blood factors and neutralizing antibodies. In consequence Ad vectors are uptake by the liver and other off-target sites of delivery [23]. In addition a significant fraction of cancer cells show deficiency of CAR, the docking site for Ad. It results in a relative resistance of target tissues to Ad vectors. For the enhancement of the vector-to-host affinity and internalization to the host cell fiber protein can be modified genetically [24].

Adeno-associated viral vectors (AAV) as well show distinct tissue tropism due to their capsid protein structure. They are single stranded (ss) DNA vectors. They achieve persistant

transgene expression with their genome retained episomally. Therefore they belong to the group of non-integrating vectors. Compared to Ad vectors AAV vectors trigger much less inflammation which makes them of interest for further gene therapy applications [21].

Lentiviral vector is a single stranded (ss) RNA vector. The genome contains of gag, pol and env genes. These genes encode capsid proteins, the enzymes reverse transcriptase, protease and integrase as well as envelope glycoproteins. Additionally a packaging signal and an exogenous promoter, necessary for transgene expression, are included. All this is flanked by long terminal repeats (LTRs) facilitating genome replication and integration. Genome usually contains also accessory genes, yet they are expendable in recombinant vectors. The genome capacity is limited to 8-10 kb. Lentiviral vectors belong to the group of integrating vectors. However, integration can be prevented by removal of the integrase from the packaging construct. Hence transgene remains episomal. For spatial restriction of gene expression several methods are provided. The simplest process appears to be pseudotyping. Here glycoproteins which define viral tropism are derived from another enveloped virus. Thereon pseudotyped lentiviral vectors possess the tropism of the originating virus. This method enhanced transduction of certain cell types and tissues. The second method takes advantage of the fact that promoter control gene expression. Tumor-specific promoter should assure gene expression limited to tumor cells. Yet the specificity of these promoters is not absolute. Endogenous promotors also consist of enhancer and insulator elements. These parts are absent in lentiviral vectors. Simplified promotor sequences are used on account of small vector size. This in turn compromises the specificity. Various cancer/tissue specific promoters such as human telomerase reverse transcriptase (hTERT) promoter or carcinoembryonic antigen (CEA) promoter have been developed, yet hTERT is the only successfully used in clinical trials. As a third method genetic aberration in cancer cells can be harnessed to assure viral replication selectively in tumor cells. The suppressor gene p53 induces growth arrest or apoptosis after a DNA virus infection (see above). To enable viral replication the adenoviral E1b-55k gene product binds p53 and inactivates it. As a consequence an adenovirus devoid of E1B-55k is unable to bind p53 and therefore replication is impossible. On the other hand, tumor cells lacking p53 function grant viral replication [4], [17], [25]–[28].

The great advantage of viral vectors is their high and stable gene expression. But major safety problems remain. Mutagenicity, carcinogenicity, immunologic and inflammatory reactions are still obstacles that need to be overcome. In addition problems in their production and purification stages are not clarified yet [29].

Non-viral vectors provide a real alternative. Their major advantage is its biosafety. However, non-viral gene transfer has been ignored in the past on account of poor efficiency of delivery and low transient expression of the therapeutic gene. Progress in efficiency, specificity, gene expression duration and safety finally led to an increase of clinical trials with non-viral vectors from 2004 to 2013. Various delivery system had been developed, which can be broadly divided into two categories: physical and chemical methods. The simpler method appears to be the physical one. Here physical force is employed to surmount the membrane barrier and provide gene delivery. A **syringe needle** enables gene delivery without any carrier. Genetic material of interest can be injected directly into tissue (e.g. muscle, skin, liver, cardiac muscle and solid tumours) or intravenously into the blood system. As mentioned above this method is ineffective, because of rapid degradation by serum nucleases and clearance by mononuclear phagocyte system. A method used in gene therapy research in ovarian cancer is named gene gun or **ballistic DNA**. Here target tissues are crossed by DNA coated heavy metal particles at high speed. Usually gold, tungsten or silver particles are utilized with a diameter of 1μm. Sufficient speed is facilitated by high voltage electronic discharge, spark discharge or helium pressure discharge. This method allows precise delivery of DNA doses. **Electroporation** is technique where an electric field is applied which through different effects increases cell permeability. Pores are formed allowing molecules to pass. Depending on the field strength and pulse duration these pores are reversible or remain on the cell surface, thus leading to cell death. Irreversible electroporation is a reliable physical method to destroy cancer cells. Reversible electroporation on the contrary is used for delivering plasmid DNA. This method can be applied intradermally, intramuscularly or intratumoural. Another technique enhancing cell permeability is named **sonoporation**. Here micro bubbles containing genetic material are administered into blood system. Simultaneously ultrasound is applied external. The ultrasound waves involve microbubbles in target tissue and eventually facilitate transfection of transgenes. This method can be used not only in brain, cornea and kidney, but also

peritoneal cavity, muscle and heart tissues. Similar level of transgene expression appears to have a technique called **photoporation**. As the title suggests single laser pulse creates pores on a cell membrane enabling cellular uptake. Yet concrete evidences for this physical method are not provided. **Magnetofection** is a transfection method that is well documented. This site specific technique utilizes therapeutic gene-magnetic particle and high gradient magnets. Particles are administered into systemic circulation, while magnets are placed external fixing particle complex at target. Subsequently genetic material is released into the cell. When a large volume of DNA solution is injected at a high infusion rate generated hydrodynamic pressure increased permeability of cell membrane. Transient pores support the uptake of the therapeutic gene of interest in the cell. The term for this mechanism of hydrodynamic delivery is **hydroporation**. The last physical method presented is highly controversial. It is alleged that **mechanical massage** of the liver harms cell membrane for a very short time. Still long enough to introduce plasmid DNA into the cell by diffusion. Studies on this technique are scarce [30].

Chemical non-viral nucleic acid delivery systems can be divided into 3 categories:

1) DNA/Cationic lipid (lipoplexes)
2) DNA/cationic polymer (polyplexes)
3) DNA/cationic polymer/cationic lipid (lipopolyplexes)

Lipids developed for gene transfer generally consist of a positively charged hydrophilic head, a linker structure and a hydrophobic tail. The positively charged head binds with negatively charged phosphate groups in nucleic acid building a complex called lipoplex. Lipids coating genetic material build a protective layer against extracellular and intracellular nucleases. At the same time the positive molecular shell facilitates cell internalisation due to interaction with negatively charged glycoproteins and proteoglycans of cell membrane. The surface charge also has a drawback. It leads to extremely fast elimination from blood stream. Various strategies like polyethylene glycol (PEG) shielding, PEG shielding with anionic PEG and postgrafting have been developed to reduce the overall charges and prolong the half-life. PEG-coated particles may achieve long circulation time, an impact on accumulation into tumor is yet not proven. In addition PEG coating can inhibit cellular internalization of nanoparticles. Lipoplexes exhibit low toxicity until the ratio of lipid: DNA exceed 1:3 [30]–[32].

Polyplexes are nanosized complexes of cationic polymers combined with DNA. In comparison to lipoplexes these particles are more stable. Polymers can generally be divided into natural proteins, peptides and polysaccharidespolymers on one side and synthetic polymers like polyethylenimine (PEI), dendrimers, and polyphosphoesters on the other side. Among cationic polymers **PEI** is the most effective transfection agent. It is considered as a gold standard for polymeric gene delivery. These molecules have high density amino groups which can be partially protonated at physiological pH. This high buffering capacity exerts protein sponge effect. Acidification of endosomal pH is inhibited, inducing chlorid influx, increase of osmotic pressure and finally leading to disruption of the endosomal membrane. Inherent toxicity of PEI is based on strong positive charge resulting in mitochondria and/or cell membrane interaction. Membrane disruption occurs, leading to pore formation. Modifications of the polymeric backbone are necessary to reduce positive charge of PEI and further cytotoxicity. PEI can come in 2 different forms: linear (LPEI) and branched (BPEI). Polyethylenimine in its linear form shows higher transfection efficiency and cell viability. But transfection efficiency can vary depending on the molecular weight (e.g. 25-800kDa) and amount of the polymer available for polyplex formation. Large PEI chains have a superior effect on efficiency with simultaneously higher cytotoxicity. This limits their application [30], [33]–[36]. Polyplexes can also be built up by **chitosan**, a natural polysaccharide. It is derived by deacetylation of chitin and is composed of glucosamine. Non-viral vectors with chitosan show low toxicity even at high concentration. On the contrary they are affected by poor transfection efficiency. This is due to a number of factors: “1) low solubility at physiological pH; 2) lack of buffering amines with reduced endosomal escape effect; 3) strong pDNA and siRNA condensing ability with resulting inefficient unpacking of transgene in cytoplasm.” Combination of chitosan with PEG, as exerted for lipoplexes, can improve biocompatibility, solubility as well as cellular internalization compared to unmodified chitosan [30], [37]. A study recently published revealed the positive impacts of PEI-chitosan conjugates. Toxicity increased when nanoparticles contained PEI-conjugated chitosan compared to PEI or chitosan separately [38]. Chitosan/DNA polyplexes are used in wide-range applications. On account of their mucoadhesive properties they are perfectly suitable for oral and nasal gene therapy. **Dendrimers** are a relatively new class of compound. They are highly symmetric and possess terminal group functionality. The positively functional groups exposed on their surface interact with the phosphate groups in nucleic acids [30], [37].

Lipopolyplexes are combinations of lipids with peptids or polymers. Although lipoplexes and polyplexes predominate in the non-viral vector field, lipopolyplexes are drawing increasing attention due to their multifunctionality and high transfection efficiencies. Peptide-DNA (PD) polyplex form spherical, cationic complexes. They show high cellular uptake and simultaneously poor transgene expression. These agents become entrapped in endosomes, lacks membrane trafficking properties and are consequently degraded by specific enzymes. At the same time strong peptide-DNA interaction impedes release from the complex leading to low plasmid DNA availability. Lipid/DNA lipoplexes function exactly opposite to polyplexes. Their cellular uptake is lower to those of peptide-DNA complex and their transgene expression is more efficient. Lipoplexes possess greater ability to escape the endosomal pathway. Lipopolyplexes combine the optimal features of both, polyplexes and lipoplexes, with efficient cellular uptake and enhanced transfection efficiency [39]–[41].

Cytotoxicity

Nonviral vectors, such as cationic lipids or polymers are utilized to improve the transfection efficiencies of pDNA. Yet their chemotoxicity remains a criticial challenge. Polycations like PEI are toxic and prone to aggregation when administered in vivo. In addition their lack of degradability inevitably leads to potential accumulation in the body. Toxicity can be noticed at two different levels. At the cellular level strong positive charges of PEI induce interaction with cell membrane resulting in cell death. At the systemic level DNA/PEI polyplexes aggregate and interact with blood components. As a consequence capillary embolism occurs. A previous study by Chollet et al. (2002) describes the side-effects of a systemic injection of LPEI/DNA complexes in mice. These include high selectivity for lung cells and especially pneumonocytes as LPEI/DNA complexes aggregate with blood components because these aggregates are then blocked mechanically in the narrow lung capillaries. A second limitation appears to be the very transient expression of the transgene. Expression is not sustained for more than 1 day. In this regard an increase of LPEI/DNA dose contributes in augmentation of lung endothelium activation but is also associated with liver necrosis and death. This study demonstrates the necessity of toxicity assays for development of safe pharmaceutical formulations with wide therapeutic index and low toxicity profiles [42]–[44]. At the same time it is widely accepted that animal testing should be reduced, refined or replaced as far as possible. In vitro tests present a preliminary method to evaluate cytotoxicity. Thereby a wide

selection of in vitro cell types is offered. It is essential that these cells mimic the phenotype of cells within the human target tissue [45], [46]. Cells used in cell culture can be divided into two major groups: cells freshly derived from explants, named **primary cells** and **established cell lines**. Primary cells differ from cell lines in 5 main points: firstly they have a finite life-span. On the contrary cell lines have become immortalized. Secondly, primary cells can be diverse as they can come from a variety of donors. Cell lines often come from one single donor. Furthermore primary cells can change in culture. Therefore working with earlier passages is recommended. Unlike immortalized cell lines which are often genetically manipulated either with cancer genes, viruses or other inducible modifications which may alter their phenotype, native functions and their responsiveness to stimuli primary cells have not been modified. And finally primary cells are more sensitive and tend to be more difficult to maintain in culture. Both cell groups have comparative advantages. Primary cells present a better simulation of the clinical situation, whereas established cell lines provide a pure population of cells which allow better comparison of inter-laboratory results. Still immortal cell lines do not completely mimic primary cells and thus key experiments should also be replicated in primary cells [47], [48], [49].

The species of animals used in cell culture are widespread. Comparative genome studies reported that DNA sequence in human is about 70-90% the same in mice, 75% in dogs and 71% in pigs. In this context swine are increasingly used as an alternative to the dog or monkey as the choice of nonrodent species in preclinical toxicologic testing. Human and swine share similar anatomic and physiologic characteristics involving cardiovascular system. Here porcine endothelial cells present an appropriate in vitro model for vascular injury after intravenous application of drug candidates [50]–[52].

Endothelium cells built the inner surface of the blood vessel wall. It establishes a multifunctional semi-permeable cellular barrier at the blood-tissue interface. Typical morphology is a cobblestone appearance and growth in monolayer. Cultivated porcine aortic endothelial cells are positive for CD31 and CD54 and Ac-LDL-Dil uptake [53].

2. AIM OF THE THESIS

Polyethylenimine is a nonviral cationic macromolecule, which is widely used for gene delivery. This polycation, however, is cytotoxic. In the needs of finding better materials to increase transfection efficiency and decrease toxicity, in vitro cell culture studies are vital [35]. The present work aimed at establishing a protocol which enables easy cultivation of porcine endothelial cells, which can further be used for toxicity tests. Special attention was thereby given to long-term maintenance, high purity and well characterization of the porcine primary endothelial cells. The effect of gelatine coating of cell culture surfaces, different amounts of FCS and various harvesting methods were tested to optimize cell growth and culture conditions. Subsequently an initial toxicity assay with pECs at different molecular weights was executed to study the relationship between molecular weight and cytotoxicity. Necrosis and apoptosis was thereby determined using FACS (fluorescence-activated cell sorting) analysis [54].

3. MATERIALS AND METHODS

Product name	Detailed information	Company	Order number
Dulbecco´s phosphate buffered saline	PBS	Sigma Aldrich	D5545
TC Flask T25	Cell culture flask	Sarstedt	83.3910.002
TC Flask T75	Cell culture flask	Sarstedt	83.3911.002
Amphotericin B	Antibiotic solution	Sigma Aldrich	A2942
96 Well Cell culture Microplates	PS, F-Bottom	Greiner bio-one	55098
Collagenase 2	from clostridium histolyticum	Sigma Aldrich	C6885
$CaCl_2$ solution	for molecular biology	Sigma Aldrich	21115
M199-medium	With Earle's salts and sodium bicarbonate	Sigma Aldrich	M2154
Centrifuge	Heraeus Megafuge 16R	Thermo Scientific	
Cell scraper	25cm multi position blade	VWR	734-2602
CO_2 incubator	HeraCell 150i	Thermo Scientific	
DMSO	Dimethyl sulfoxide	Sigma-Aldrich	D5879-500ML
FCS	Fetal calf serum	Sigma Aldrich	F7524
Cryogenic store vials	External-thread 2D coded universal system cryogenic tubes	Thermo Scientific	374502
Freezing container	Mr. Frosty™	Thermo Scientific	5100-0036
Freezer -80°C	REVCO ExF	Thermo Scientific	
Freezer -150°C	REVCO	Thermo Scientific	
L-glutamine	200mM	Sigma Aldrich	G7513

TrypLE	TrypLE Express (1x)	Gibco by Life Sciences	12605-010
CD31 antibody	Purified Mouse Anti-Rat CD31	BD Pharmingen	557273
Centrifuge tube 50ml		LabStar	E1450-0200
Isotype	Purified mouse Ig G1 K isotype control	BD Pharmingen	555025
Antibody 2	Alexa fluor 488 goat anti-mouse Ig G (H+L)	Abcam	Ab150113
Propidium iodide	PI	Sigma Aldrich	P4170
Flow cytometer	MACS Quant Analyser	Milteny Biotec	130-096-343
Flow Jo_V10	Single cell analysis software	FlowJo Enterprise	
Gelatine	From bovine	Sigma Aldrich	G99391-100G
Cellstar® Multiwell plate 24	Culture plate, sterile, with lid	Sigma Aldrich	M8812
Microscope	AE31 Elite Trinocular	Motic	
camera	Moticam 3.0MP	Motic	
Serological pipettes 5ml, 10ml,25ml		Sarstedt	86.1253.001
Annexin V-Alexa-488	For apoptosis assay	Life Technologies	A13201
(S)-(+)-Camptothecin	Cytotoxic alkaloide	Sigma Aldrich	C9911
Biological Safety Cabinet	HeraSafe KS	Thermo Scientific	51030130
Penicillin-Streptomycin	Pen/Strep antibiotic	Sigma Life Science	P0781
Syringe filter	Sterile, 0,2µm	VWR	12465897
LPEI Mw (weight average molecular weight) 3kDa, 8kDa, 10kDa	linear polyethylenimine	Supplier: Mag. Alexander Taschauer (Department for Pharmaceutical Chemistry,	

		Laboratory of Macromolecular Cancer Therapeutics, University of Vienna)

3.1. Primary isolation of porcine endothelial cells

The aorta was aseptically obtained from freshly slaughtered Landrace pigs (Sus scrofa) and immediately translated to laboratory in Dulbecco´s phosphate buffered saline supplemented with 5% Pen/Strep and 2.5mg/l amphotericin B. Fat and connective tissues were removed carefully and aorta opened longitudinally. Opened segments of aortic tissue of approximately 0.25 cm2 were placed intimal side down on a sterile plate followed by rinsing with PBS. It was then incubated with PBS supplemented with 0.1% trypLE, 0,1% collagenase 2 and 1% 0,01 M $CaCl_2$ solution for 20 min at 37°C. After removing the solution 5ml of M199-medium were added. Medium was pipetted up and down several times and endothelial cells were gathered by gentle abrasion with a cell scraper. The medium containing detached EC was collected in a 15ml Greiner tube and the cell suspension was centrifuged at 800g for 8 min. The cell pellet was washed, suspended in M199-medium and placed in a 75 cm^2 culture dish. The dish was then maintained at 37°C in an incubator with a humidified atmosphere containing 5% CO_2/95% air. After 2 days medium was changed. Once the cells reached 80% confluence they were subcultured [53] [55].

3.2. Cryopreservation of cultured cells

Cells with different passages (2-6) were amplified and frozen as seed- and working stocks. Therefore DMSO was sterile filtered with a 0.2µm syringe filter and 1:10 solutions of DMSO with FCS were prepared. Freezing medium was then stored at 2° to 8°C until use. Cells were harvested and centrifuged at 200g for 5 min. Supernatant was removed entirely and the cell pellet was resuspended in cold freezing medium. Then cell suspension was dispensed into cryogenic store vials. Carefully cryotubes were transferred to a freezing container, which achieves a rate of cooling very close to -1°C/minute, the optimal rate for cell preservation. After storing the freezing apparatus at –80°C overnight, cryovials were transferred into the -150°C freezer.

3.3. Resuscitation of frozen cells

As the cryoprotectant DMSO is toxic above 4°C, frozen cells were thawed quickly in order to maintain the viability of the cells. Therefore the cryovial containing the frozen cells was removed from the -150°C freezer and immediately placed into a 37°C water bath. The vial was thereby gently swirled in the water bath until there was just a small block of ice left in the vial. After whipping the outside of the vial with 70% ethanol thawed cell suspension was transferred into a culture flask containing pre-warmed complete growth medium. Since endothelial cells are delicate cells centrifugation to remove cryoprotectant was omitted. Culture medium effectively diluted the cryoprotectant reducing the toxicity of DMSO. After 24 hours medium was removed and fresh pEC medium comprised of Medium 199 containing 20% fetal bovine serum, 5% Pen/Strep, 50mg/l L-glutamine and 2.5mg/l Amphotericin B was added.

3.4. Initial maintenance of porcine endothelial cells in vitro

Primary cultures were harvested and plated on cell-culture dishes without coating. They were grown in pEC-medium which was changed every 2-3 days until cells reached 90-100% confluence. Cell splitting process started by aspirating medium from the cell culture vessel. Cells were then harvested by treatment with TrypLE. After 5 min incubation at 37°C cells were examined under the microscope. When cells were fully detached TrypLE was inactivated with fresh medium. After cell count was determined cells were routinely passaged at a constant 1: 10 split ratio [56].

3.5. Characterization of porcine endothelial cells with CD31 antibody staining

Platelet endothelial cell adhesion molecule-1 (PECAM), also known as cluster of differentiation 31 (CD31) is a transmembrane glycoprotein expressed on the surface of platelets, leukocytes and endothelial cells. As a specific component of EC junctions, it participates in regulation of EC interaction with blood components [57], [58].

Evaluation of surface expression of CD31 molecules on pECs was performed by indirect staining. Therefore 2µl of CD31 antibody Purified Mouse Anti-Rat CD31 were put into an Eppendorf tube and diluted with 398µl of ice-cold PBS (supplemented with 10%FCS). 2µl of

isotype Purified mouse Ig G1 K Isotype control were diluted with 398µl of ice-cold FCS/PBS and 2µl of antibody 2 Alexa fluor 488 goat anti-mouse Ig G (H+L) were added to 998µl of ice-cold FCS/PBS. All three tubes were stored at 4°C for 1h. Meanwhile 3 x 10^5 cells were harvested in an 1,5ml Eppendorf tube and serum components were removed by washing the cells twice with isotonic PBS buffer (supplemented with 10% FCS) by centrifugation at 300g for 5min. Supernatant was aspirated and cell pellet was resuspended in 100µl of FCS/PBS and 100µl of CD31 antibody solution (5µl/ml). In parallel, 3 x 10^5 cells were stained with 100µl of isotype solution (5µl/ml), as negative control. After 45 min incubation at 4°C, cells were washed once and then 100µl of FCS/PBS and 100µl of second antibody solution (2µl/ml) were added. Cells were incubated in a freezer for 45 min at 4°C. After washing and centrifugation cell pellet was resuspended in 200µl FCS/PBS and 3µl of propidium iodide solution in concentration of 1mg/ml were added for flow cytometric exclusion of dead cells. Both samples were then analysed on the flow cytometer and received data were evaluated using software package Flow Jo_V10 [59].

3.6. Optimization of maintenance

In order to optimize viability, growth and purity of cultured cells the following modifications were tested and compared:

3.6.1. Coating of the culture dish surfaces with gelatine

The extracellular matrix of endothelial cells is composed of various proteins such as fibronectin, collagen, laminin and glycosaminoglycans. These components enable anchorage of cells to the matrix and activate various intracellular signalling pathways that direct proliferation, differentiation and cell viability. Therefore to improve cell adhesion and function of cells in tissue culture, flasks were coated with gelatine. Gelatine is a translucent substance extracted from animal collagen. The use of gelatine-coated dishes improves cell attachment for certain types of primary cells. The objective of the study was to evaluate cell growth at two different matrices, gelatine-coated and uncoated culture flasks. At first 500ml of a 0.5% gelatine solution was prepared. Therefore 2.5g of gelatine were added to a 500 ml glass bottle. Demineralised water was poured on the top of the gelatine until 500ml were reached. It was then sterilized by autoclaving at 121°C, 2 bar for 15 min. The culture surface of a 24 well-plate was coated with 160µl of cooled gelatine solution per well and dried for 30

minutes. Meanwhile porcine endothelial cells, passage 2 were thawed. After 30 min gelatine solution was removed and cells were seeded. After 2 days cells reached 90% confluence and were split with a split ratio of 1:2. Half of the cells were put in uncoated wells, the other half in gelatine coated wells. This was performed with 4 confluent wells. After 24 h cell density was compared utilizing the microscope equipped with a camera [60], [61].

3.6.2. Inhibition of proliferation of contaminating fibroblasts

In endothelial cell cultures several contaminating types of cells can occur, in particular fibroblasts. While resting endothelial cells display typical cobblestone morphology phenotype can reversibly change to a sprouting when confluence is low, resembling the angiogenic cells involved in the formation of new vessels. Fibroblasts on the other hand are disjoint and scattered when they have to cover a large space. When confluence is high they are often locally aligned in parallel clusters. Unlike endothelial cells, which are restricted by an attachment to a basal lamina forming a flat monolayer, fibroblasts show tendency to overlap or overgrow one another forming a multilayer. To prevent fibroblast overgrowth a method of selective detachment was chosen. It is based on the fact that fibroblasts can detach from the substrate faster [62], [63].

Porcine endothelial cells, passage 5 were thawed and transferred into two 25 cm2 flasks (flask A and flask B). At confluency cells were passaged. Therefore cell culture medium was removed and discarded from the culture vessels. After rinsing cells twice with PBS wash solution was removed and 2 ml of dissociation reagent TrypLE were added to cover the cell layer completely. Instantly 1.5 ml of TrypLE were removed and both vessels were put in the incubator for 3 minutes. Detachment was observed under the microscope. At this point flasks were treated differently.

- Flask A: **Without** tapping the vessel supernatant was removed and transferred to a new 25 cm^2 flask. Pre-warmed complete growth medium was added and new flask was put in the incubator. Meanwhile flask A with remaining cells attached on the cell layer surface was returned back to the incubator for 2 more minutes. After this duration the vessel was tapped to expedite the detachment. Pre-warmed complete growth medium was added and dispersed by pipetting over the cell layer surface several times. Cells were transferred to a new 25 cm^2 flask and put in the incubator.

- Flask B: With **slightly tapping** the vessel supernatant was removed and transferred to a new 25 cm2 flask. Further steps were performed as in flask A (see above).

Percentage of confluency and percentage of fibroblasts within the culture flask were analysed at all 4 obtained flasks. Thereby every flask was organized into 4 equal squares. Using a microscope the centre point of every square was focused with a 10x objective. Confluency and amount of fibroblasts were estimated and note down by two independent examiners. For evaluation the average of both values was used. The procedure was continued until passage 8, subculturing only flasks with 5 min trysinization. Flasks with cells gained from supernatant were discarded after analysis.

3.6.3. Long-term maintenance of porcine endothelial cells

Most scientific papers involving porcine endothelial cells recommend cell experiments at low passage number. Frequent cell splitting increases chances of morphological, phenotypical and functional alterations. Proliferative capacity tends to decrease from the 5^{th} passage and CD31 expression disappears rapidly [55].

To analyse function and characteristics of pEC during long-term culture cells passage 10 were taken from a previous experiment. When confluency reached 90-100% cells were split with a ratio of 1:10. During cell culture morphology was analysed under the microscope. Characterization of pEC was carried out by staining with CD31 antibodies (platelet/endothelial cell adhesion molecule, PECAM).

3.6.4. Effect of fetal calf serum concentration on cell growth

Fetal bovine serum (FBS) or fetal calf serum (FCS) is commonly used in cell culture media as a nutritional serum supplement. It contains a mixture of important basic proteins such as growth factors and hormones which promote cell growth and stimulate proliferation of cell [64], [65]. To study the effect of different levels of FCS on cell growth in vitro pECs passage 5 were thawed and seeded in a $25cm^2$ gelatine-coated flask with fresh complete medium containing 20% FCS. After confluency reached 90% cells were trypsinized and counted with a flow cytometer. Subsequently 100 000 cells were plated in each of four 25 cm^2 gelatine-coated flasks in fresh medium consisting of four different concentrations of FCS (0%, 5%, 10% and 20%). Images of the cells were captured with a microscope. Additionally cells were stained with antibodies for CD31 for endothelial cell characterization [66].

3.6.5. Time of antibody dilution

Stock solution of primary antibody, isotype and second antibody was stored at-20°C to ensure stability. Dilutions with FCS/PBS were freshly made on the same day of usage for CD31 antibody staining (procedure see above). Manufacturer recommends preparing fresh working solutions for each study to receive consistency of results. Storage overnight at -4°C is possible but fixation might decrease due to instability of proteins. Microbial growth is also a concern when storing antibody dilution at 4°C for re-use [67]. To analyse the influence of production time of antibody dilution on CD31 positivity pECs passage 10 were stained with freshly made antibody solution and dilution made 48h before. Analysis was performed using MACSquant cell analyser and flow jo_10.

3.6.6. First toxicity test

In this current study the cytotoxic impacts of different molecular weights (MWs) of LPEIs in pECs were investigated. In addition LPEI polymers at different concentration were applied to gain more information about its cytotoxicity. Analysis of apoptosis was performed using an Annexin kit. Necrosis was determined by double-staining with propidium iodide. In comparison to the LPEI toxicity cells were also exposed to campothecin, a cytotoxic alkaloid. At first pECs passage 8 were seeded and cultured up to 90% confluency in a 25 cm^2 flask. After 48h cells were seeded in two 75 cm2 flasks to increase cell number. At complete confluency cells were counted using a flow cytometer. Cell suspension with 20 000 cells was added in each well of a gelatine-coated 96 well plate. Remaining cells were stained with CD31. 81.22% of primary cells expressed CD31. Hence LPEIs at different molecular weights and increasing concentrations were added to the wells (see fig. 1). For better comparison some wells were left untreated and 8 wells were treated with camptothecin as a positive control.

										cell type:	
ToxAssay	**24 h**			20.000 cells/well			**pEC**				

	1	2	3	4	5	6	7	8	9	10	11	12
A	UT			UT			UT			Camptothecin 2 µg/ml		
B	3 kDa 0.5µg/ml			8 kDa 0.5µg/ml			10 kDa 0.5µg/ml			Camptothecin 4 µg/ml		
C	3 kDa 2.5µg/ml			8 kDa 2.5µg/ml			10 kDa 2.5µg/ml			Camptothecin 6 µg /ml		
D	3 kDa 5µg/ml			8 kDa 5µg/ml			10 kDa 5µg/ml			Camptothecin 8 µg /ml		
E	3 kDa 12.5µg/ml			8 kDa 12.5µg/ml			10 kDa 12.5µg/ml			Camptothecin 10 µg /ml		
F	3 kDa 25µg/ml			8 kDa 25µg/ml			10 kDa 25µg/ml			Camptothecin 15 µg /ml		
G	3 kDa 50µg/ml			8 kDa 50µg/ml			10 kDa 50µg/ml			Camptothecin 20 µg /ml		
H	UT			UT			UT			Camptothecin 25 µg /ml		

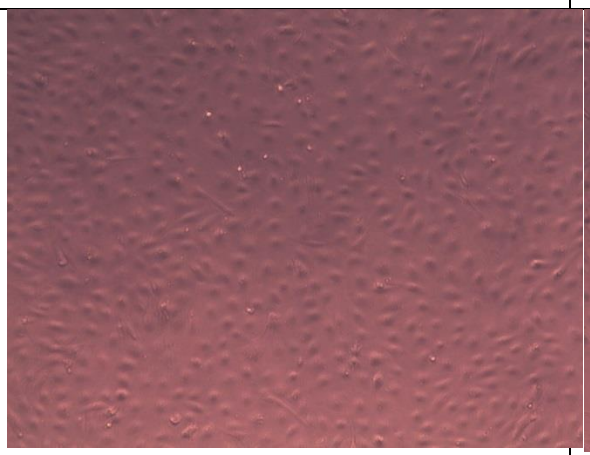
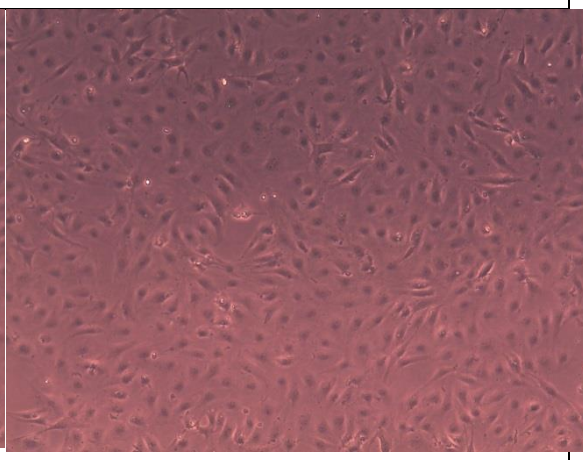
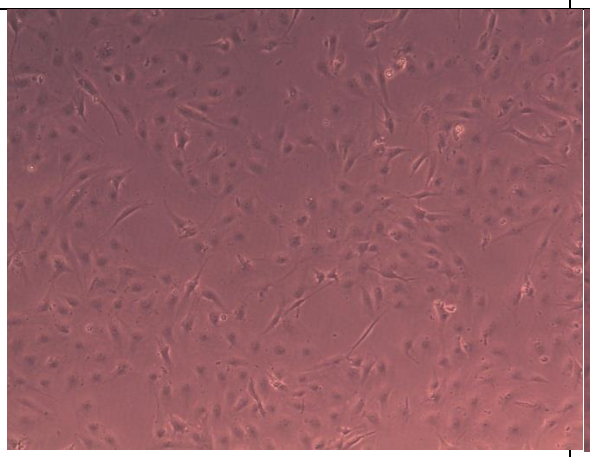
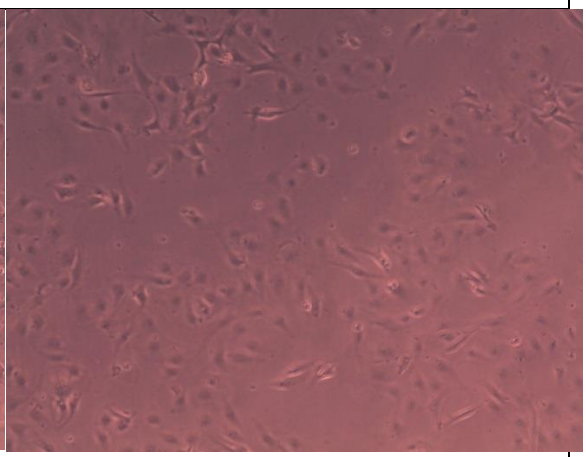
Fig. 1: 96-well plate plan for cell seeding and LPEI/camptothecin treatment.

Cells were then incubated at 37°C for 24h in a CO_2-incubator. Medium was removed and cells were washed once with PBS. After detaching with TrypLE cells were transferred into a V-bottom 96 well plate followed by centrifugation for 5min at 200g. Supernatant was removed and a 50µl mixture of staining solution consisting of 10µl of a readily prepared Annexin V-Alexa-488 conjugate solution and 2µl of a 1µg/µl propidium iodide solution dissolved in 988µl Annexin Binding Buffer was added to each well. Cells were then incubated for further 30 min at room temperature and transferred to the MACSquant cell analyser. Thereafter, the whole plate was analysed successively using a robotic arm on a cooled plate.

4. RESULTS

4.1. Effect of gelatine coating on endothelial cell growth

Comparison of porcine endothelial cells seeded on two types of matrices illustrates that on gelatine coated well pECs spread well in 24h reaching a confluency of almost 100% and achieving cobblestone morphology. On uncoated wells spontaneous proliferation and spreading were decreased and cells showed a more stretched morphology as cells were growing slower. Cells from field 1A and B were originally taken from well no.1, field 2A and 2B from well no.2, field 3A and 3B from well no.3 and field 4A and 4B from well no.4.

	A gelatine coated well	B uncoated well
field 1		
field 2		

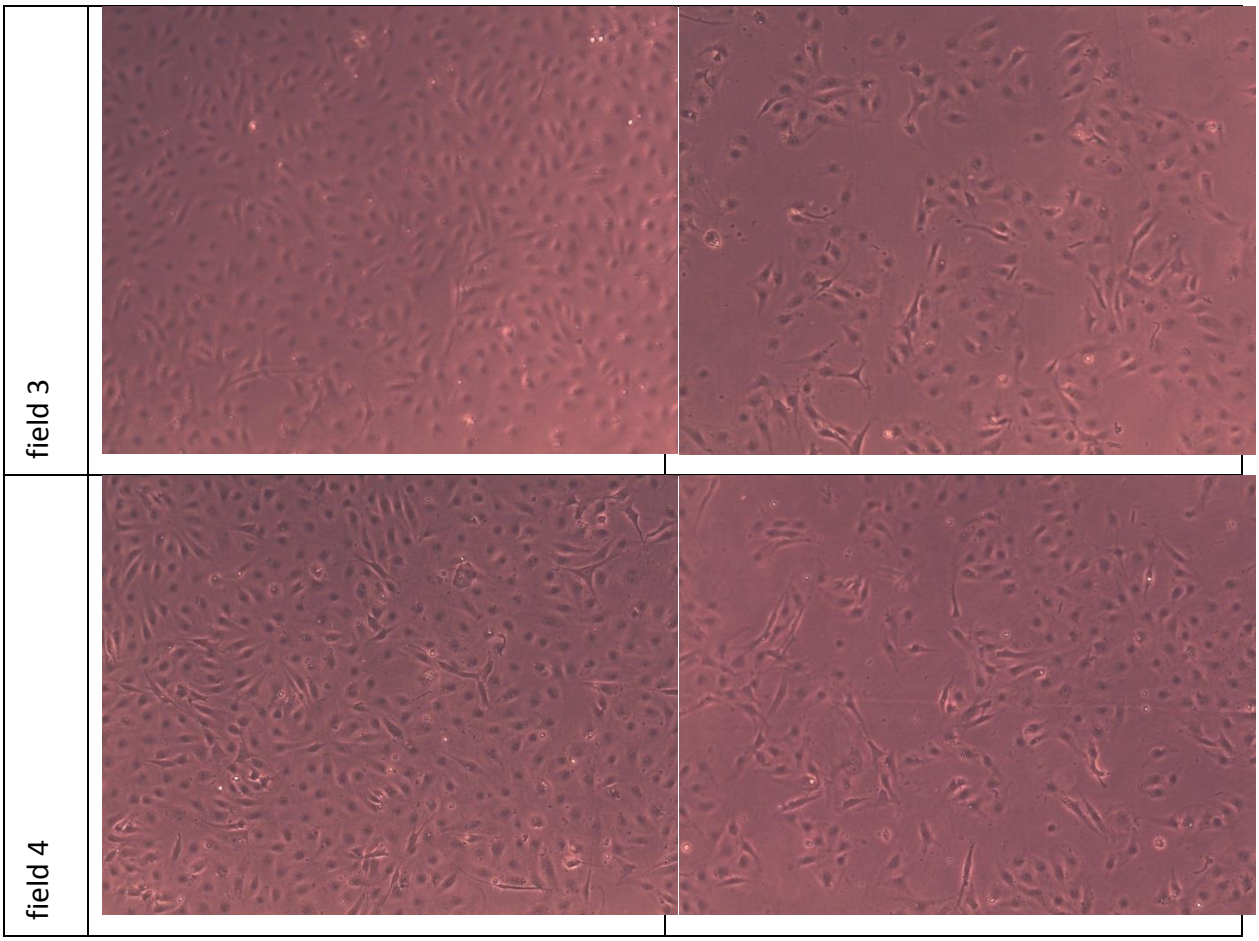


Fig.2: pECs on study matrices: (1A), (2A) (3A) and (4A) are representative fields of pEC on gelatine-coated surfaces, 24h after seeding. pEC on uncoated plastic are in (1B), (2B), (3B) and (4B). All cells used for seeding were from the 3rd passage. Original magnification 10x.

4.2. Elimination of fibroblast overgrowth

To investigate whether contamination fibroblasts detach easier by enzymatic treatment with TrypLE than endothelial cells, flask A was treated with TrypLE for 3 minutes. Without tapping the vessel supernatant was removed. The flask was put back to the incubator for 2 minutes. Remaining cells were transferred to a new flask. Both flasks were analysed visually, only flask with remaining cells was subcultured. Fig. 3 shows percentage of confluency and fibroblasts at passage 5-8. Because supernatant was removed without gently tapping the vessel back and forth only a very low number of cells detached and confluency in flask with supernatant remained low even after 2 days of cultivation. At this point endothelial cells and fibroblasts

showed similar morphology which made it significantly difficult to distinguish both cell types. Still percentage of fibroblasts tended to be higher in supernatant.

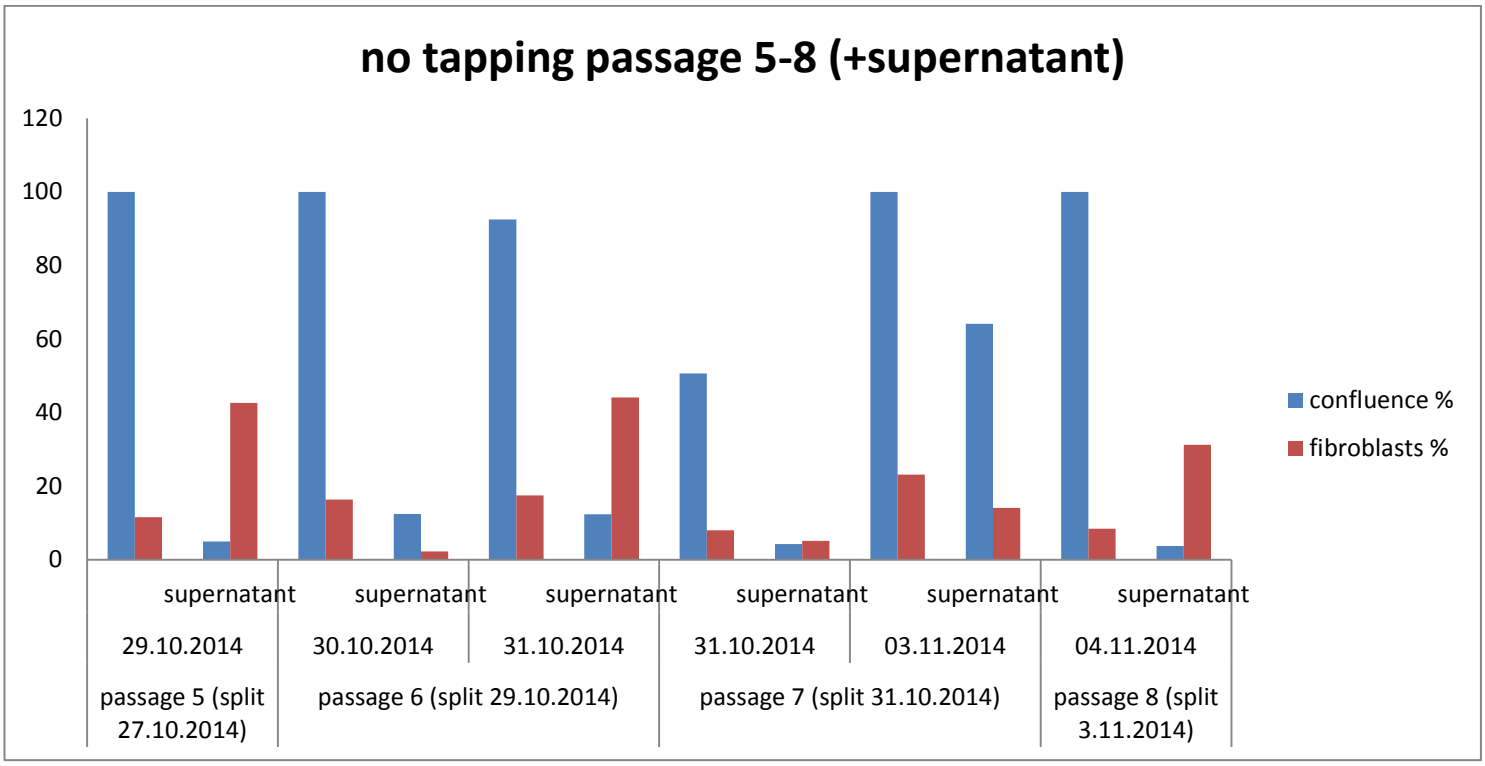


Fig. 3: Visual analysis of confluence and percentage of fibroblasts during endothelial cell cultivation, supernatant was removed without any agitation.

Confluency and contamination with fibroblasts in cell cultures where supernatant was removed by tapping the flask is presented in fig. 4. Here more cells appeared in the supernatant. Fibroblasts were observed in both cultures at all passages, yet the claim to find more fibroblasts within the supernatant could not be confirmed clearly. Furthermore endothelial cells showed a tendency to growth quicker than fibroblasts at low confluency. When flask surface was completely covered with cells, pECs stopped proliferating and fibroblasts started to overgrow the culture flask.

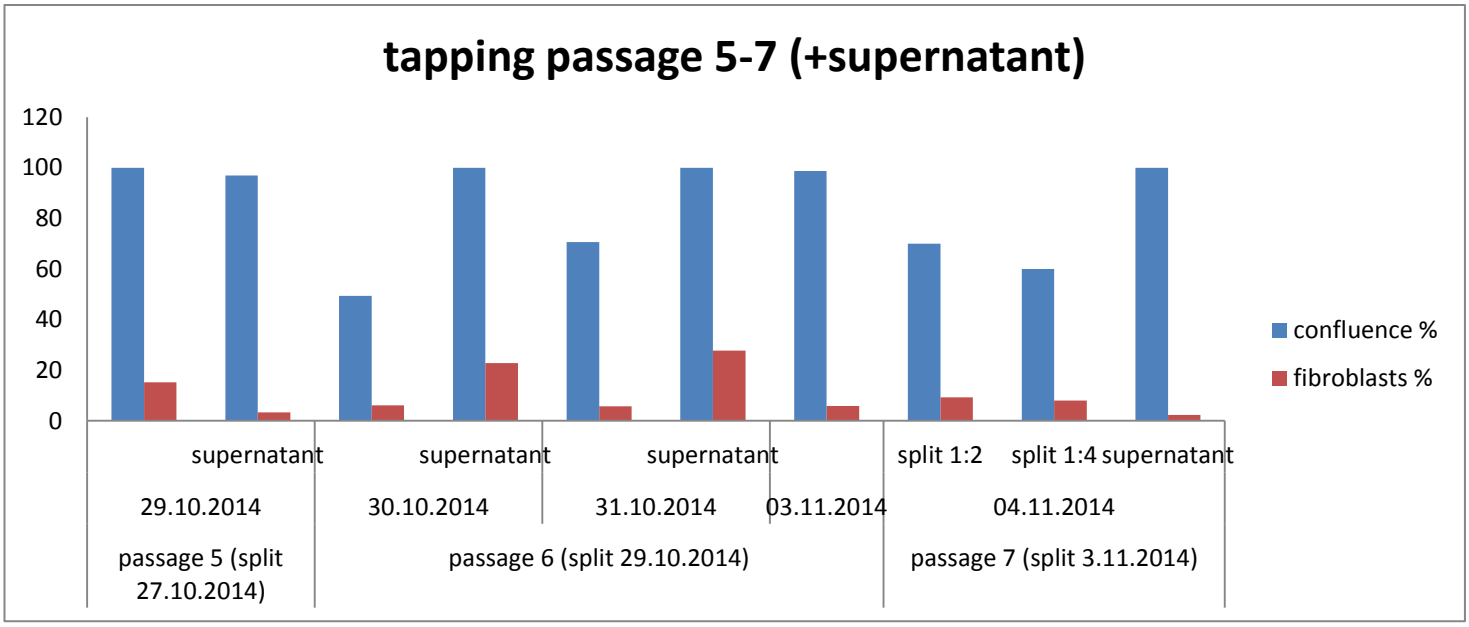


Fig. 4: Visual analysis of confluence and percentage of fibroblasts during endothelial cell cultivation, supernatant was tapped, than removed.

4.3. Long-term cultivation of pEC

To quantify the purity and function of the primary endothelial cells, cells were labelled with CD31 antibodies. Passage 10 was split after 3 days, passage 11 after 3 days, passage 12 after 3 days, passage 13 after 4 days, passage 14 after 4 days, passage 15 after 4 days, passage 16 after 3 days due to a holiday, passage 17 after 5 days exceptionally with a ratio of 1:5, passage 18 after 4 days. At this point cells were frozen due to Christmas holidays. After 4 weeks cells were defrosted again, passage 19 was split just one day after thawing. Passage 20 was split after 9 days, passage 21 after 8 days, passage 22 after 6 days although they were not confluent yet. Remaining cells from every split were used for CD31 measuring.

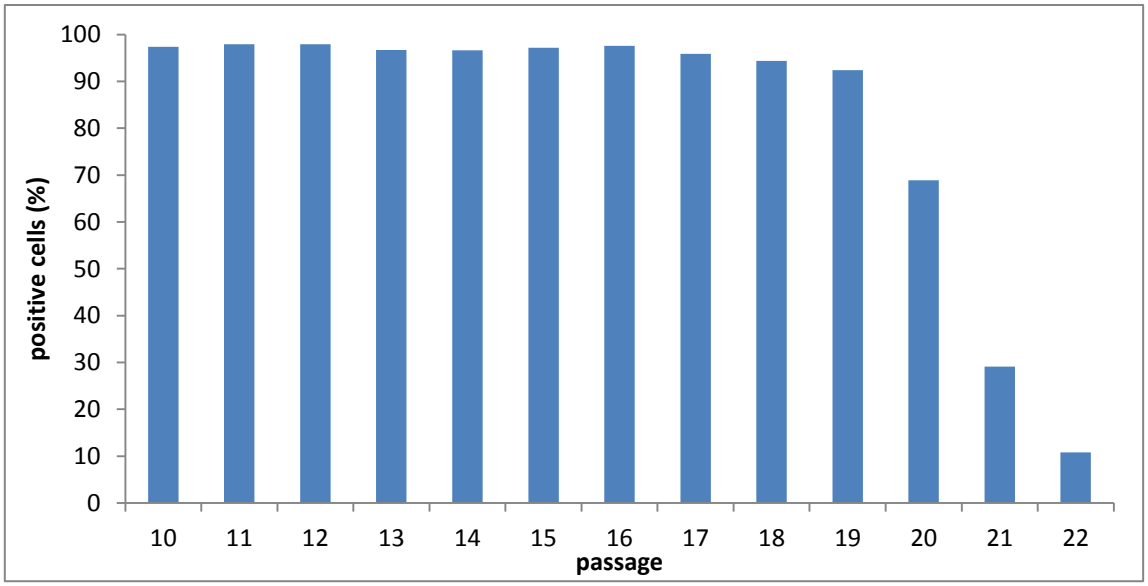


Fig. 5: CD31 positivity of pECs at different passages. Endothelial cells were labelled with CD31; determination was carried out by flow cytometry.

Figure 5 illustrates the high percentage of CD31 positive cells up to passage 19. At this time pECs demonstrated the characteristic cobblestone morphology as described and shown in photographs previously. Due to Christmas holidays passage 19 was frozen. From passage 20 on cells started to grow much slower. 8 days and more passed by before cells reached complete confluency and were ready for splitting. At the same time CD31 positivity greatly decreased. Interestingly at passage 20 no fibroblast overgrowth was observed under the microscope and also morphology of pEC remained unmodified. At passage 21 endothelial cells started to disappear and more and more fibroblast occurred. Finally at passage 22 no endothelial cells were found under the microscope and cells with spindle-shape morphology, which is characteristic for fibroblasts, overgrew the bottom of the flask. We stopped our experiment at this point. FACS analysis showed that 10.78 per cent of cells were positive for surface antigen CD31.

4.4. Relationship between endothelial cell growth and the concentration of FCS

Using primary pECs, this study compared the effects of different levels of fetal calf serum (FCS) (0%, 5%, 10% and 20%) on cell growth in vitro. The morphology of cells treated with 0% FCS changed considerably. Cells shrivelled, rounded and detached from the gelatine-coated plate. Fibroblasts in cell culture were scattered and disjointed and equally detached. At day 5

medium was changed. Quantitative analysis of CD31 staining was impossible due to lack of cells.

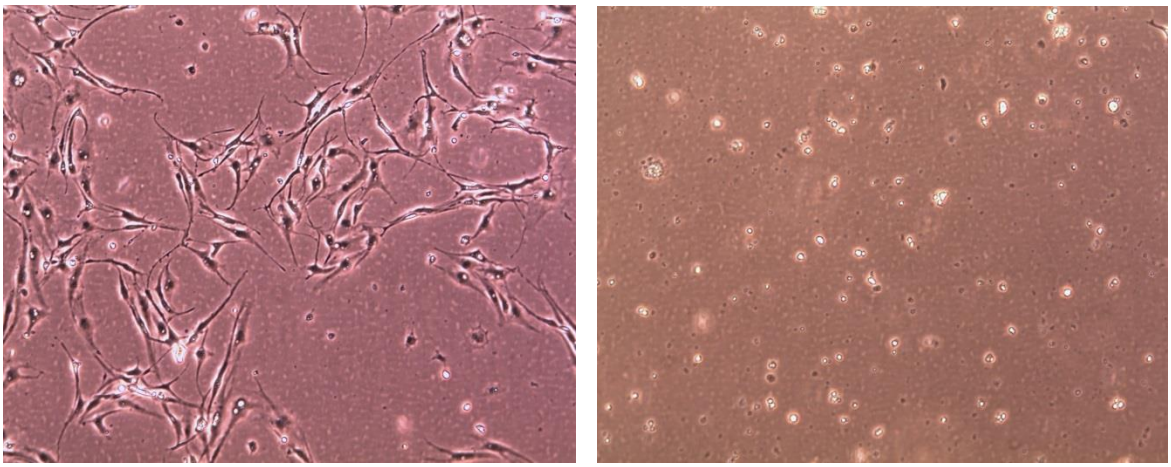


Fig. 6: Morphology of pECs under 0% FCS concentration: Image left was taken on day 5. Fibroblasts were scattered and disjointed, pECs were shrivelled and rounded. Image right was taken at day 15. Medium was changed, detached cells were washed out. Magnification 10x

For organizational reasons both, 10%- and 20%-FCS treated cultures were split at the same time at a constant ratio of 1:10. They were passaged after 4-6 days, when 20% FCS-cells reached 100% of confluency and 10% FCS-cells 95% of confluency. 20% FCS treatment showed higher proliferation rate with qualitatively more cells than 10% FCS treatment. Medium was changed every 2-3 days. Expression level of CD31 was similar in both cultures, ranking relatively stable at almost 100%. This finding is consistent with the observation of morphology under the microscope. Confluent endothelial cells formed a monolayer with typical cobblestone pattern with minimum of fibroblast contamination.

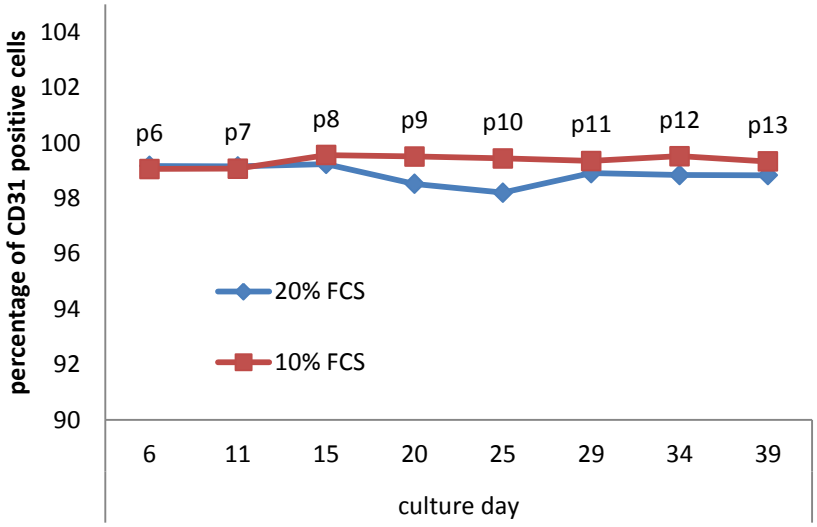


Figure 7: Effects of FCS on expression of CD31 on pECs. The results showed that expression levels of CD31 appeared similar in 10% and 20% FCS-treated cells. At passage 10 in the 20% FCS condition number of CD31+cells dropped for the short term. Here cells were overgrown with confluency above 100%. Development was observed through 7 passages.

Cells showed lower proliferative potential with stimulation with 5% FCS. Due to organizational reasons cells were split even at low percentage of confluency, which additionally extended time of cultivation as split ratio remained at 1:10. Interestingly the expression level of CD31 was not significantly different between 5%, 10% and 20% FCS-treated groups, but only cell proliferation rate decreased with lower FCS concentration.

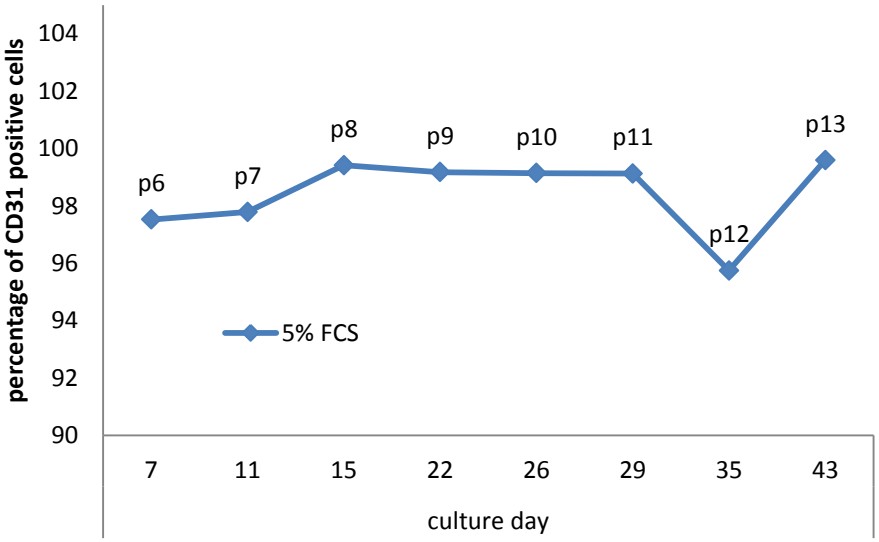


Fig. 8: CD31 antibody staining in endothelial cells cultures supplemented with 5% FCS. The drop in the graph at passage 12 is due to the disruption of the cell pellet during CD31 staining. Most cells were aspirated with the supernatant. The percentage of CD31 positive cells is not representative at this point. Development observed through 7 passages.

4.5. Freshly diluted and preserved antibody solutions in flow cytometry analysis

Two pEC samples were stained with CD31 antibody solutions prepared at different dates to evaluate the effects of production time of solution on CD31 positivity. Therefore freshly prepared antibody dilution was compared to stock solution diluted 48h before.

CD31 positivity (fresh antibody solution): 97.95%

CD31 positivity (2 days old antibody solution): 97.85%

The difference was considered not significant. Hence freshly prepared working stocks were stored at 4°C and used within 48h.

4.6. Cytotoxic impacts of LPEI in pECs

Flow cytometry analysis was performed to detect the induction of apoptosis / necrosis phenomena in LPEI treated pECs at different molecular weight and concentration in comparison with untreated cells and cells treated with camptothecin. As shown in fig. 9 concentrations of camptothecin were 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml, 10 µg/ml, 15 and 20 µg/ml, respectively. The viability of the cells incubated with camptothecin was around 40% compared to untreated cells, showing similar toxicities at all concentrations.

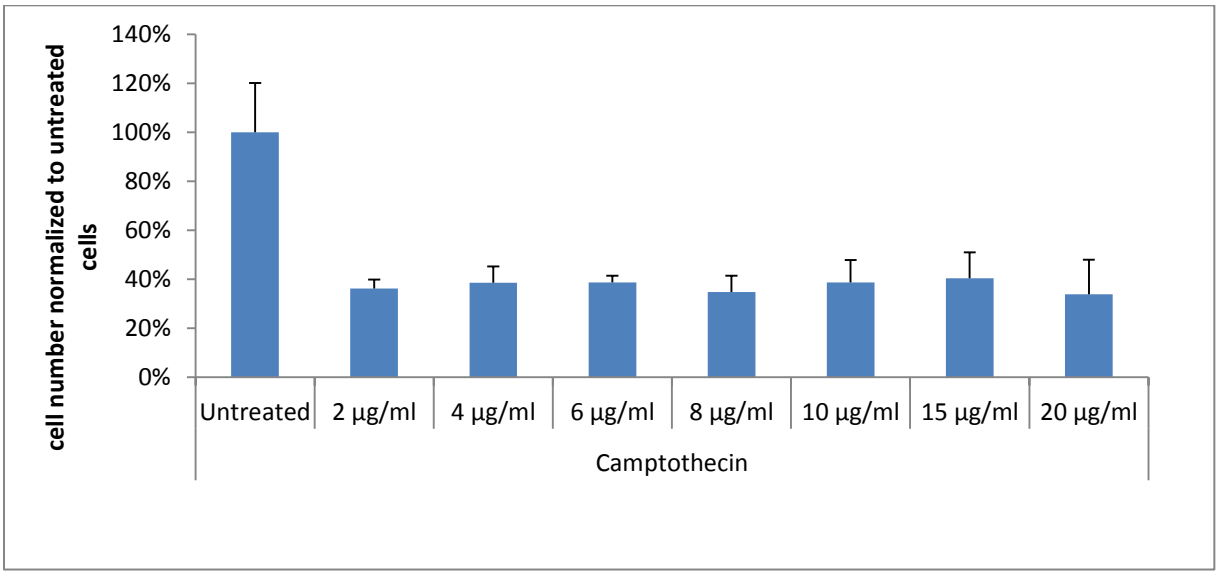


Fig. 9: cell viability of pECs 24h after treatment with increasing concentrations of camptothecin compared to untreated pECs.

The differentiation of dead and apoptotic cells was undertaken to assess the apoptotic potential of camptothecin. Upon treatment with camptothecin apoptosis appeared to be largely dependent on concentration (Fig. 10).

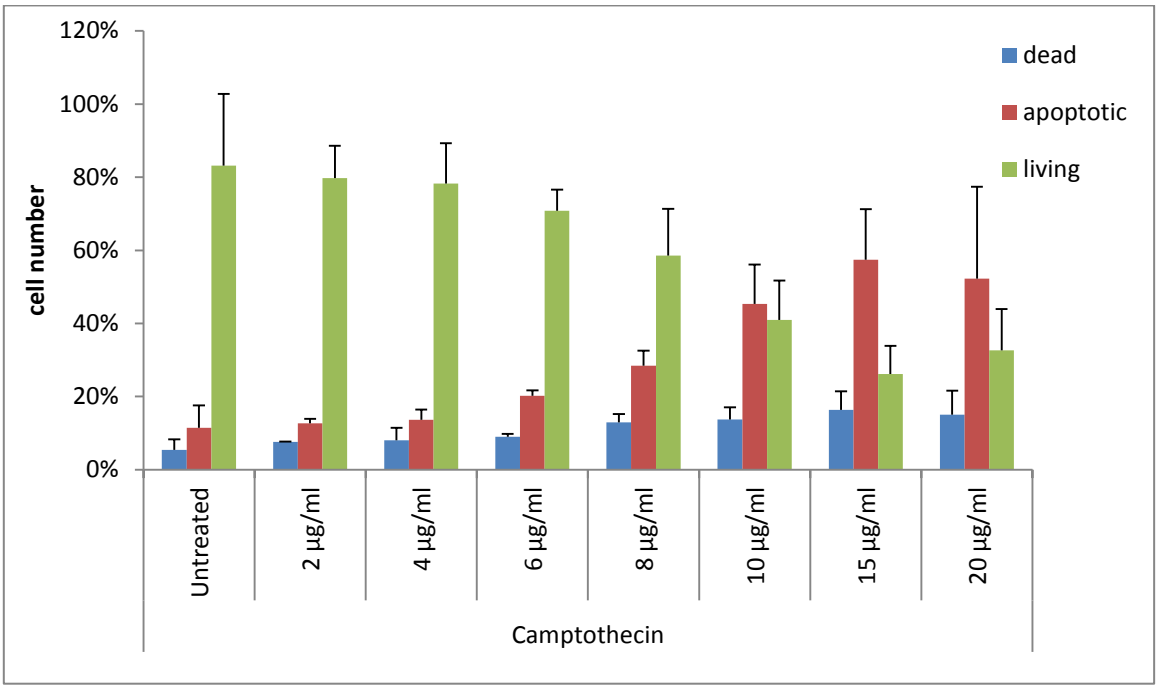
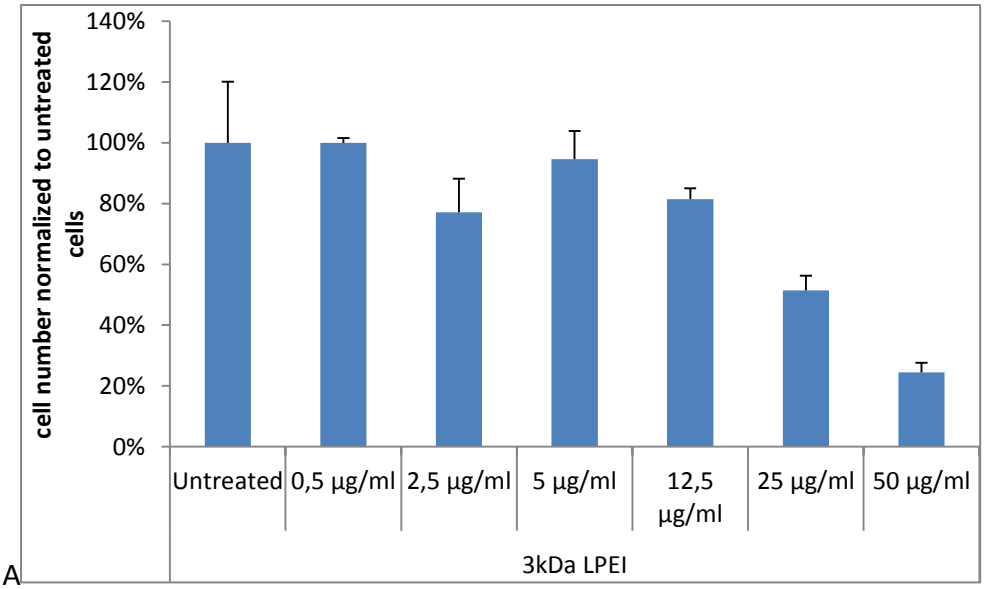
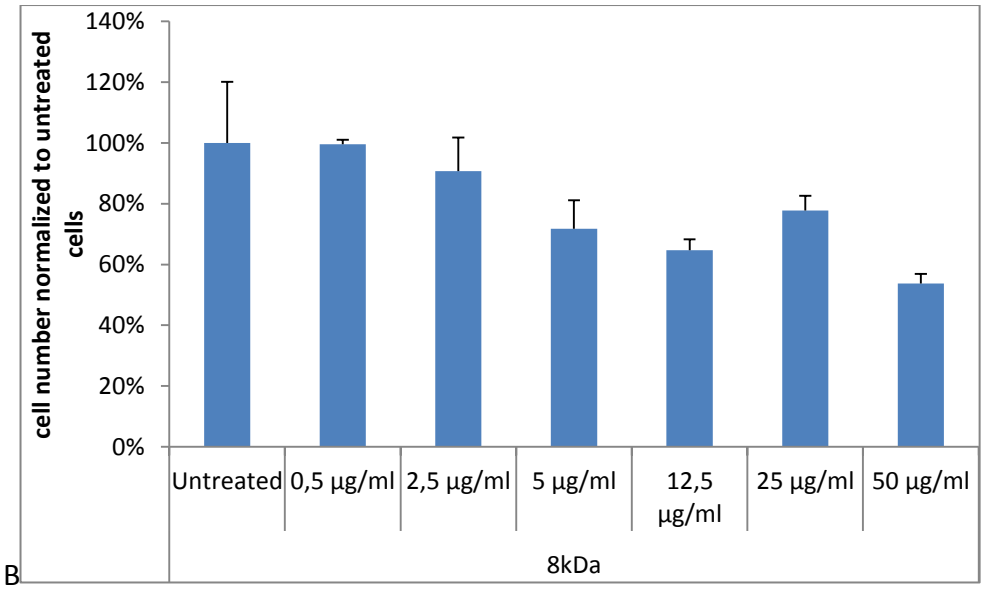


Fig. 10: Live/dead staining. Cell number of living, dead and apoptotic pECs 24h after treatment with increasing concentrations of camptothecin compared to untreated cells.

Fig. 11 shows the results obtained by the means of the cytotoxicity assay for pECs treated with LPEI at different concentrations. No difference of cell viability was observed within untreated cells and those treated with 0.5µg/ml of 3kDA LPEI and 8kDa LPEI. Except for some irregularities cell viability decreased constantly with higher concentration.


cell number normalized to untreated cells
140%
120%
100%
80%
60%
40%
20%
0%
Untreated
0,5 µg/ml
2,5 µg/ml
5 µg/ml
12,5 µg/ml
25 µg/ml
50 µg/ml
3kDa LPEI

A


cell number normalized to untreated cells
140%
120%
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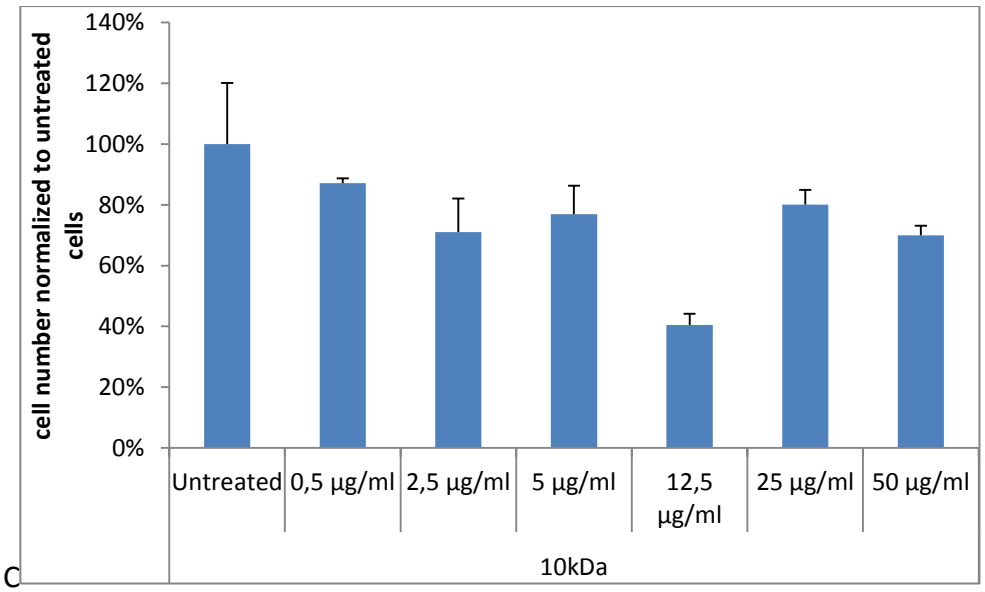
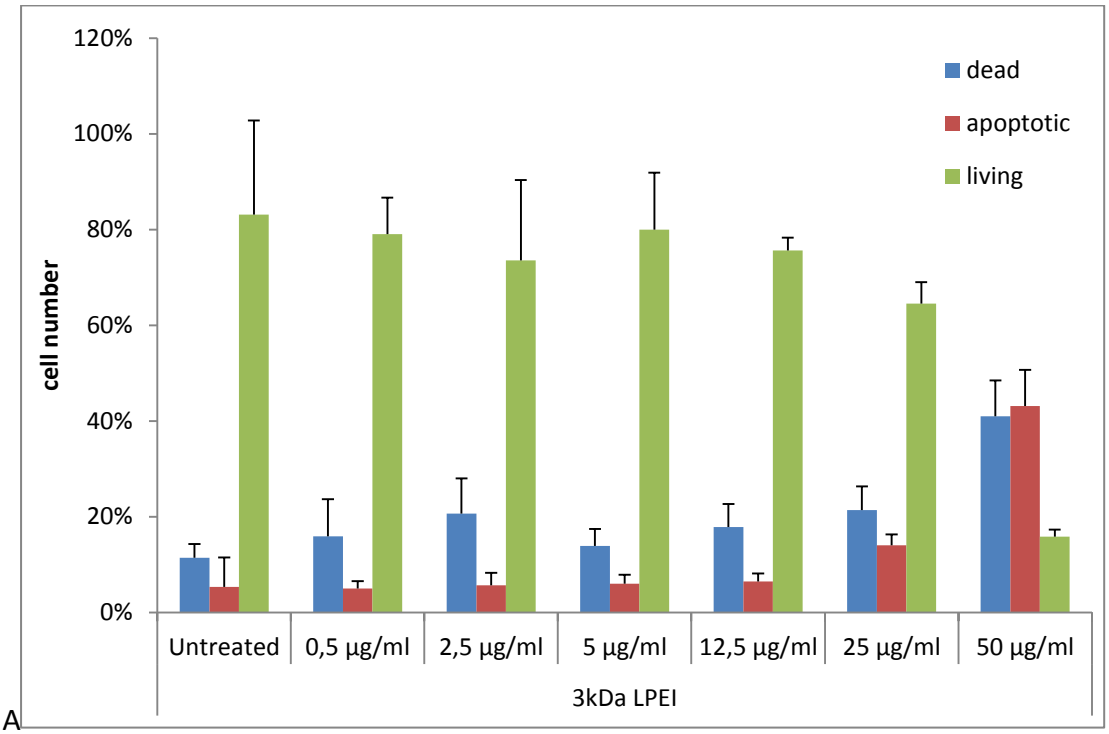

cell number normalized to untreated cells
140%
120%
100%
80%
60%
40%
20%
0%
Untreated
0,5 µg/ml
2,5 µg/ml
5 µg/ml
12,5 µg/ml
25 µg/ml
50 µg/ml
10kDa

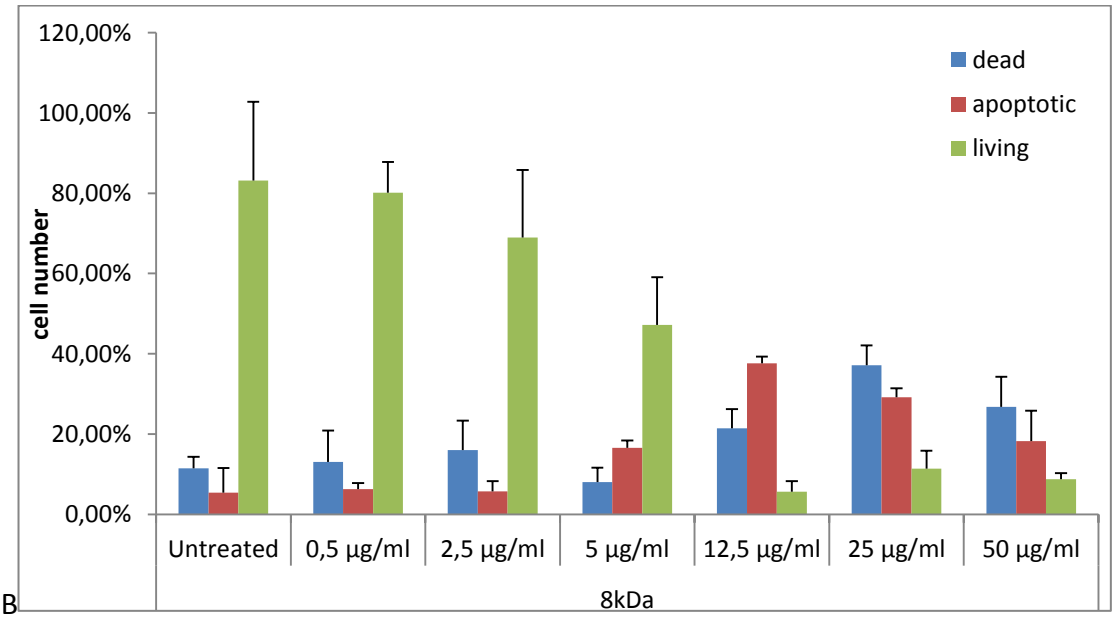
C

Fig. 11: cell viability of pECs 24h after treatment with increasing concentrations of 3kDA (A), 8kDa (B) and 10kDa (C) LPEI compared to untreated pECs.

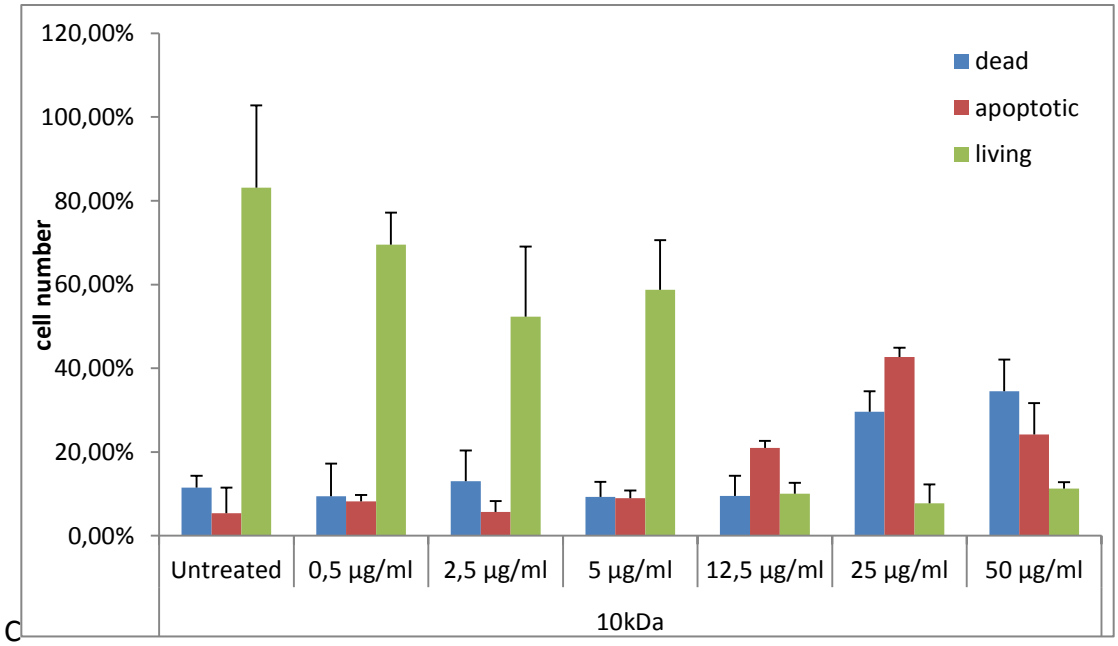
Fig. 12 demonstrates that apoptosis of cells increases gradually along with the concentration. Additionally LPEI treated cells showed significant increases in proportion of dead cells. Errors in application led to irregular results.



A



B



C

Fig. 12: Live/dead staining. Cell number of living, dead and apoptotic pECs 24h after treatment with increasing concentrations of 3kDa (A), 8kDa (B) and 10kDa (C) LPEI compared to untreated cells.

5. DISCUSSION

The work was carried out with the aim to establish culture conditions for maintaining primary porcine endothelial cells for cytotoxicity studies of LPEI. Previous cultivation has been hampered by the rapid appearance of fibroblasts and fast occultation of PECs. The fibroblast overgrowth was basically characterized by accumulation of disjointed and scattered cells covering the layer of endothelial cells. This observation led to the assumption fibroblasts were to be eliminated by constant removing of superficial cells through slightly tapping the flask during the splitting process and discarding supernatant. This assertion is not clearly confirmed by the present study. Visual analysis was impeded as fibroblasts and endothelial cells show similar morphology during the spreading process and cell number was mostly too low for a quantitative analysis of CD31 staining. Yet the study has shown that fibroblasts accelerate cell growth when culture flask is confluent. Compatible with these findings, Pelisek et al. (2001) demonstrated that growth to confluence is sufficient for ECs to achieve quiescence. Cell – cell contact resulted in efficient inhibition of cell proliferation. Even several days following confluence the cell number remained constant without any detectable toxic effects. In contrast, smooth muscle cells (SMC) continued to replicate when confluence was reached growing on top of each other. It can be suspected that fibroblast act similar to SMC. This finding suggests that cell splitting before achieving confluence can stem fibroblast growth.

Further, differences between gelatine-coated culture plates and plastic culture plates in endothelial cell proliferation were evaluated. Relou et al. (1998) reported that spontaneous proliferation of EC was enhanced in gelatine-coated wells compared to non-coated wells. This might be explained by the higher attachment rate of pECs onto the gelatine-treated plates. The images of the present study lead to the same outcome. More cells were found in gelatine-coated plates than in bare plastic plates. Still, long-term effects of gelatine were not examined. Chennazhy et al. (2005) discovered that endothelial cells easily lose its proliferation potential and turn apoptotic when they are cultured on gelatine. They recommend a matrix composed of fibrin, fibronectin, gelatine and vascular EC growth factor (VEGF). Here cells retained their ability to proliferate, remained viable and were relatively less thrombogenic [60]. Future studies should further analyse the effect of matrix characteristics on endothelial differentiation.

Long-term maintenance of highly purified primary porcine endothelial cells is not only useful for toxicity assays of LPEI, but are also required for xenotransplantation and stem cell research. In this study pEC were isolated and maintained up to passage 22. Until passage 19 more than 90% of the cells were CD31 positive. Cells showed cobblestone morphology with little fibroblast-like cells. At passage 20 pECs started to decrease. This may have been due to freezing of cells at passage 19 or caused by significant cell transformation on serial passage. Also coating with gelatine could have had an influence on cell differentiation. Additionally cells were passaged rather irregular due to holidays and weekends.

Fetal calf serum is a commonly used nutritional supplement in cell culture media. The composition of various proteins enables cell growth and stimulates proliferation of cells. Yang et al. (2009) demonstrated that 20% FCS treated primary endothelial cells show higher proliferative potential than those treated with 10% FCS. This is consistent with our findings. In this study 20% FCS conditions resulted in higher cell number than in 10% and 5% conditions. Interestingly, lower levels of LPEI were not adverse for CD31 positivity. This data may be beneficial for controlling passage time by varying concentrations of FCS. Low passage number decreases the possibility of cell differentiation and lower concentrations of FCS extend split ranges and reduce proliferation rate. Complete withdrawal of FCS in medium however led to detachment of cells from culture plate surface and finally cells died off.

In the very brief comparison of freshly prepared antibody solution and solution prepared 2 days before differences in CD31 positivity were not significant. For a scientific evaluation this experiment should be repeated at least 2 times. Additionally the result is only valid for 2 days old solution. Conclusions on older solutions are not permitted. However, it can be assumed that similar results can be expected for slightly older preparations.

Toxicity of cationic particles is an important issue. Here cytotoxicity of LPEI at different molecular weight and different concentration was examined. It has to be noted that probably mistakes in application led to imprecise results, yet a tendency can be deduced. As shown in fig. 11, fig. 13, fig. 15 cell viability decreases dramatically with higher doses. At the same time it can be observed that LPEIs with higher MW induce higher cytotoxicity than their low-MW analogues. It seems obvious to use low molecular weight LPEI, however, polycation vectors possessing high MW often afford stronger DNA condensation capacity and higher transfection efficiencies.

The characterization of porcine endothelial cells in this study is limited to microscopic examination of morphology and CD31 staining. But endothelial cells also possess the ability to uptake acetylated low density lipoproteins(LDL). Additionally they express the endothelial specific marker von Willebrand factor(vWF). These properties can be analysed using flow cytometry and are urgently required to achieve optimal characterization.

6. CONCLUSION

The objective of the study was to investigate optimal culture conditions for long-term maintenance of highly purified primary porcine endothelial cells. The results provide clear evidence of the influence of gelatine-coating, different levels of FCS and cell density on proliferation rate, purity and cell viability. Unexpectedly, increasing fibroblast growth was observed when pECs reached complete confluency. It is therefore recommended to split cells at lower cell density. Removal of supernatant during harvesting process showed minor impact. Hence this procedure is considered unnecessary. Gelatine-coating of plate surfaces enables constant conditions for the cells. This study revealed higher proliferation rate with gelatine-coating, yet long-term effects or alternative coating were not investigated. Therefore the effect of gelatine coating in cell culture needs to be examined more precisely. The use of 20% FCS is deemed too high. 10% FCS-treated cells showed slightly lower proliferation rate at consistent high CD31 positivity. Even 5% FCS can be recommended. If these points are followed in the future, it is possible to obtain non-transformed, pure pEC with a long replicative lifespan.

7. REFERENCES

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