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Cardiac Protection with Targeted Drug Delivery to Ischemic-Reperfused Myocardium

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1. Introduction

The concept of targeted drug delivery implies selective accumulation of the drug in the tissue affected by the pathological process after systemic administration of the drug and its carrier with minimal effect of the former on the intact organs and tissues (Lammers et al., 2010). The idea of targeted delivery has been first suggested by Paul Erhlich in 1906 when he introduced the concept of «magic bullet» which is directed against target cells only without any damage to healthy tissue (Erhlich, 1906). In the current medical practice, most of the drugs are administered orally or parenterally, resulting in natural biodistribution and systemic effect on the organism. This type of distribution is justified for the drugs which act on the systemic mechanisms of disease development and progression. At the same time, in case of focal pathological processes such as tumor growth, inflammation and ischemia it may be more clinically attractive to ensure the local rise in the drug concentration within the pathological area thus avoiding the putative side effects on neighboring tissues. This point can be illustrated by several examples. It is known that the administration of antitumor drug doxorubicin is associated with severe cardiomyopathy, suppression of myelo- and megakaryopoiesis, nausea, vomiting, and development of alopecia (Carvalho et al., 2009). Encapsulation of doxorubicin into liposomes resulted in dramatic reduction of these dose-limiting side effects (Leonard et al., 2009). The implementation of site-specific drug delivery may also benefit female patients receiving estrogens for treatment of osteoporosis. Along with desirable effect on the bone, estrogens may cause some unwanted effects, especially increased risk of uterine bleeding and development of endometrial cancer (Romer, 2006). One might suggest that bone-targeted delivery of estrogens will decrease the probability of these side effects. The application of nano-sized particles for drug transport may offer a new means of delivering drugs selectively into the affected tissue.

Typical nanoparticulate carrier for targeted drug delivery comprises several functional elements. The major part of the carrier is drug-loaded nanoparticle. An important step in nanocarrier fabrication is the functionalization of its surface which implies binding of the targeting ligands to the nanoparticle surface. Targeting ligand ensures specific interaction of the nanoparticle with the complementary molecules on the surface membrane of the target cell. In order to facilitate the process of functionalization and prevent rapid clearance of nanoparticles by the reticulo-endothelial system, the surface of the nanoparticle is often covered with biocompatible coating such as polyethylene glycol. The nanoparticles can be

additionally labeled with radioactive isotopes or fluorescent dyes which allows visualization of their accumulation in the damaged tissue. Successful targeted drug delivery with use of nanoparticulate carriers can bring solutions to several serious problems. In particular, it may result in reduced toxicity, increased solubility and stability of the drugs.

To date, the research on targeted drug delivery has been mostly concentrated on the development of tumor-targeted nanomedicines (Ali et al., 2011). It might be hypothesized that targeted drug delivery strategy approved in oncology can be applied in other clinical fields, now not for destruction but for the sake of salvage of reversibly injured cells, in particular of those destined otherwise to die within the area of myocardial ischemia-reperfusion. One reasonable approach to the problem may be targeted intramyocardial delivery of the agents (i.e., certain angiogenic growth factors, erythropoietin, ATP-sensitive potassium channel openers, etc.), either covalently and/or non-covalently bound to the nanoparticulate carriers. Thus, the purpose of this chapter is to provide an account of acquired knowledge on nanoparticle-based targeted drug delivery with particular emphasis on heart targeting. Besides, the original experimental data on the fabrication of silica nanoparticles which can be used as prototype carriers for cardioprotective drugs are presented. We also present the data on silica nanoparticle biodistribution, biodegradation, and acute toxicity. Finally, the preliminary evidence for enhanced infarct size limitation with use of silica nanoparticle-bound adenosine is provided.

2. Major types of nanocarriers for targeted drug delivery

The various types of nano-sized particles can be utilized for transport of drugs into the diseased tissues. Liposomes, drug-polymer conjugates, polymeric micelles, dendrimers, nanoshells, and nucleic acid-based carriers can all be used as nanocarriers for drugs (Wang et al., 2008). Among them, drug-polymer conjugates and liposomes are currently most commonly used for drug delivery in the clinical settings and collectively amount to at least 80% of all nanopharmaceuticals available on the market. The choice of the material for nanocarrier fabrication mainly depends on the chemical structure of the drug to be transported, characteristics of the target tissue and the route of nanocarrier administration into the organism (Ganta et al., 2008). It is generally accepted that the properties of ideal nanocarrier should include biocompatibility, biodegradability, and evasion of rapid uptake by the macrophages.

2.1 Liposomes

Liposomes are spherical membrane structures that consist of a phospholipid bilayer which can entrap aqueous solutions. In the past decade, considerable information has been accumulated on the therapeutic applications of liposomes (Fenske & Cullis, 2008). By now, there are 9 liposomal drugs approved for clinical use. Liposomal doxorubicin and daunomycin are successfully used for treatment of cancer while liposomal amphotericin remains to be a cornerstone for the treatment of some fungal infections.

2.2 Polymer-drug conjugates

Peptides and the drugs with low molecular weight are often characterized by the short half-life in the circulation thus requiring repeated administration and, furthermore, can exhibit

non-specific adverse effect on organs and tissues. Binding of the drugs with polymer nanocarriers may reduce these negative effects. Despite the fact that a great number of diverse polymers have been initially considered for drug delivery, the very few of them have been finally approved for clinical applications. Polyethylene glycol is the most universal polymer drug carrier. It has been introduced in the clinical practice in the early 90s and has rapidly become popular as drug vehicle because of its capability to increase the stability of drugs in the plasma, improve solubility of hydrophobic drugs and reduce their immunogenicity. Apart from polyethylene glycol, gamma-polyglutamic acid, N-(2-hydroxypropyl) methacrylamide and certain polysaccharides with linear structure are currently viewed as promising polymeric drug carriers (Sanchis et al., 2010). Tissue-recognition ligands engrafted on the polyethylene glycol chain conjugated with the drug may facilitate targeted delivery of the entire complex into the tissue of interest.

2.3 Dendrimers

Dendrimers represent three-dimensional highly branched polymeric macromolecules with the diameter varying from 2.5 to 10 nm. Dendrimers can be synthesized from both synthetic and natural monomers, e. g. amino acids, monosaccharides and nucleotides. High surface area, narrow range of polydispersity and abundance of functional groups on the outer shell of dendrimers make them an attractive tool for targeted drug delivery (for review, see Cheng et al., 2008). Two classes of dendrimers are most commonly used for biomedical applications – polyamidoamines and polypropyleneimines. Although polycationic macromolecules such as dendrimers were shown to be moderately toxic for living cells, the attachment of polyethylene glycol (PEGylation) to the dendrimer can overcome this limitation. The methods of drug binding to the surface of dendrimer include covalent conjugation and electrostatic adsorption. Besides, low molecular weight drugs can be placed into the cavities within the dendrimer molecules being temporarily immobilized there with hydrophobic forces, hydrogen and covalent bonds.

2.4 Polymeric nanoparticles

Biodegradable polymeric nanoparticles were extensively studied as drug carriers. This type of nanocarriers is usually synthesized by means of self-assembly of copolymers consisting of two or more blocks with different hydrophobicity. In the aqueous environment, these copolymers spontaneously form micellar structures with hydrophilic shell and hydrophobic core (Torchilin, 2007). Hydrophobic core may serve as an ideal container for water-insoluble drugs while the hydrophilic shell can be additionally modified for attachment of water-soluble drugs. Polymeric nanoparticles can in principle transport not only low molecular weight hydrophilic and hydrophobic drugs but also the macromolecules such as proteins and nucleic acids (Perez et al., 2001). The kinetics of the release of nanoparticle-bound drug depends on the duration of nanoparticles residence in the organism and characteristics of their microenvironment. Polylactide, polyglycolide and poly(ε–caprolactone) as well as their diblock copolymers with polyethylene glycol at different molar ratios are most commonly used for fabrication of biodegradable nanoparticles. In general, biodegradable polymeric nanoparticles maintain therapeutic drug concentration in the tissue of interest for a longer period of time than other nanocarriers. This feature makes them suitable platforms for transport of highly toxic, water insoluble and unstable drugs.

2.5 Metallic nanoshells

Metallic nanoshells typically consist of dielectric core and thin metallic shell which increases their biocompatibility and optical absorption. The resonance frequency at which the nanoshells demonstrate maximal absorption of energy and its minimal dissipation strongly depends on the ratio between the diameter of the core and the thickness of the metallic layer on the surface of the particle. Gold nanoshells heated with use of near-infrared light were successfully used for photothermal destruction of tumors *in vivo* (Hirsch et al., 2003). Similarly, thermosensitive polymer hydrogels and optically active nanoshells may be used for targeted delivery of drugs. For instance, Arias et al. (2006) developed nanoshells consisting of magnetic core (carbonyl iron) and biodegradable coating (polybutylcyanacrylate) designed for controlled release of 5–fluorouracil in the tumor tissue.

2.6 Nucleic acid-based nanoparticles

RNA and DNA can also play a role of macromolecular carriers for delivery of the drugs. For fabrication of nucleic acid nanoparticles, modified chains of RNA or DNA with non-linear shape are utilized. One of the studies on this topic provided the description of 100 nm multifunctional complex on the basis of DNA designed for consecutive targeted delivery of the drug, visualization of the pathologic focus and gene therapy (Li et al., 2004). The effects of RNA nanoparticles (25-40 nm) containing inactive RNA within the core, RNA aptamers as targeting ligands and small interfering RNA for inhibition of tumor cells were investigated in another recent study (Khaled et al., 2005).

3. Passive and active targeting

Two main strategies currently utilized for site-specific drug delivery are passive and active tissue targeting. In passive targeting, nanoparticles of specific size and charge are nonspecifically accumulated within the affected tissue because of the special characteristics of its vascular bed. Good example of passive targeting is selective accumulation of PEGylated nanoparticles within the tumor tissue after their intravenous administration. This phenomenon is explained by the increased permeability of the tumor microvessels which, in turn, is due to defective endothelial lining and local fenestrations of basement membrane. These observations were first made by Matsumura & Maeda (1986) in the murine solid tumors and are usually referred to as enhanced permeability and retention phenomenon.

Due to the enhanced permeability and retention phenomenon, systemic administration of polymer-drug conjugates results in 10-100-fold higher concentration of the drug in the tumor tissue than after administration of aqueous solution of the drug. Enhanced permeability and retention effect has been observed not only in tumors but also in chronic inflammation and infection. Furthermore, it is well established that nanoparticles without polymer coating are rapidly eliminated from the circulation because of the uptake by the reticulo-endothelial system elements. This fact provides a rationale for passive targeting of liver and spleen in chronic inflammatory diseases associated with prolonged persistence of pathogens within the macrophages (e.g.candidiasis , listeriosis, leishmaniasis, brucellosis, etc.). Stimuli-responsive nanoparticles have recently emerged as another feasible approach to passive targeting (Ganta et al., 2008).

It has been shown that nanoparticles diameter and charge can have a significant impact on their ability to accumulate in the damaged tissue. For instance, van Vlerken et al. (2007) suppose that the most intensive and prolonged accumulation within the tumor is typical for positively charged nanoparticles with diameter of less than 200 nm.

Active targeting employs nanoparticles with specific targeting ligands which selectively bind to biomarkers on target cells. These biomarkers include integrins $\alpha_V\beta_5$ and $\alpha_V\beta_3$ which are expressed on the tumor cells and endothelial cells of the tumor vessels. It is documented that integrins $\alpha_V\beta_5$ and $\alpha_V\beta_3$ specifically interact with arginine-glysine-aspartic acid (RGD) tripeptide sequence. It follows, therefore, that the attachment of RGD peptide to the surface of antitumor drug-loaded nanoparticles may result in their targeted delivery to the tumor tissue. Other variants of targeting ligands are considered in the following section.

4. Tissue-recognition ligands

Monoclonal antibodies and their fragments, aptamers, peptides and low molecular weight compounds may function as tissue-recognition ligands. Considerable efforts have been directed towards developing targeted drug delivery systems with use of monoclonal antibodies. Synthetic monoclonal antibodies are most commonly used for this purpose. For example, the conjugation of fluorescent polystyrene nanoparticles with monoclonal antibodies against platelet endothelial cell adhesion molecule-1 resulted in efficient internalization of the entire complex into the endothelial cells (Garnacho et al., 2008). It was shown in this study that the fate of the nanoparticle (presence of endocytosis, intracellular trafficking, and fusion of vesicles with lysosomes) was dependent on the type of the monoclonal antibody epitope against platelet endothelial cell adhesion molecule-1. On the basis of these data, one might suggest that the drugs can be delivered not only in the affected cells but also in the specific intracellular compartments.

The use of native monoclonal antibodies for tissue targeting is limited by their considerable immunogenicity. In this connection, the development of chimeric and humanized monoclonal antibodies is of importance. The use of monoclonal antibodies as targeting ligands has several other disadvantages such as high cost of monoclonal antibodies fabrication and significant variability of their specificity from batch to batch. Antigen recognition fragments such as Fab fragments, single-chain variable fragments, minibodies, diabodies, and nanobodies may become an alternative to antibodies. Nanobodies are the smallest antibody-derived structures which are produced from the variable domain of the heavy chain of single-domain immunoglobulins and retain the ability to recognize specific antigen. Nanobodies seem to be optimal tissue recognition ligands since they have small size and low immunogenicity. Qiu et al. (2007) produced low-molecular weight antibody mimetics by virtue of fusion between two complementarily-determining regions of the prototype antibodies. Obtained mimetics with molecular weight of 3 kDa were characterized by the better distribution pattern than corresponding antibodies, suggesting that these mimetics may be used as highly specific targeting ligands.

Aptamers are small nucleic acid molecules which may function as specific receptors for low-molecular weight organic substances. The selection of aptamers specific for certain molecular target is a laborious and complicated procedure consisting of several stages. One of the major stages is the enrichment of combinatorial oligonucleotide libraries including up

to 10^{15} random sequences resulting in the identification of aptamers specifically interacting with target molecule. The advantages of aptamers include low molecular weight (around 15 kDa) and low immunogenicity leading to improved pattern of tissue distribution (Zhou & Rossi, 2011). It is worth noting that the technology of aptamer synthesis can be easily scaled to industrial production, as opposed to synthesis of monoclonal antibodies. It has been shown that RNA aptamers for recognition of vascular endothelial growth factor can cause regression of tumor microvessels and demonstrate high stability in plasma of the monkey (Ruckman et al., 1998). Anti-vascular endothelial growth factor aptamer pegaptanib has been approved by the American Food and Drug Administration in 2004 for clinical use in the patients with age-related macular degeneration. This fact underscores rapid advancement of aptamers from the bench to clinical applications.

In the last years, with the advent of combinatorial peptide libraries, the peptide targeting ligands are becoming more and more widespread. The peptides can bind to target molecule with very high specificity and affinity. For instance, cyclic peptide cilengitide, an integrin-targeting RGD peptide, is currently being tested in the phase I/II clinical trial for therapy of recurrent and/or metastatic squamous cell cancer of the head and neck (Vermorken et al., 2011).

Low-molecular weight compounds may also serve as efficient tissue-recognition ligands because of their low cost and negligible immunogenicity. One of the most intensively studied molecules within this group is folic acid. Folate is especially helpful for tumor targeting since tumor cells are over-expressing folate receptor on their surface. Folic acid has been used for tumor-targeted delivery of cytostatics immobilized on different types of nanoparticulate carriers (e. g., polymeric nanoparticles, liposomes, dendrimers, etc.).

Various methods of binding targeting ligand with the nanoparticle surface have been suggested, which can be generally divided into covalent and non-covalent techniques. Covalent binding has been suggested more than 25 years ago for immobilization of proteins on the surface of liposomes. Protein conjugation with the nanoparticle is usually mediated through the bifunctional polyethylene glycol which contains hydrophobic anchor for fixation in the liposomal coating and molecular spacer with amino or sulfhydryl moieties for drug binding. Specific interaction between protein A and Fc-fragments of monoclonal antibodies as well as the biotinylation of the nanoparticles and their subsequent conjugation with ligand-streptavidin complex can both be used for non-covalent binding of nanoparticle with targeting ligand.

5. Heart targeting with nanoparticulate carriers

Much of the work on targeted drug delivery has focused on cancer treatment. The reasons for this include high morbidity and mortality due to cancer as well as focal tumor growth associated with severe alterations in tumor molecular phenotype, thus making possible the design of highly selective targeting ligands. However, it can be hypothesized that the concepts of passive and active targeted drug delivery can be applied efficiently to any type of focal pathological process including ischemia-reperfusion and inflammation. Ischemic heart disease is generally thought to be a major cause of morbidity and mortality worldwide. It follows, therefore, that prevention and/or alleviation of myocardial ischemia-

reperfusion injury remains to be among the most important goals of the medical care. One solution to the problem might be direct delivery of cardioprotective drugs into the ischemic myocardium.

At present, there is some, albeit limited, published evidence that the ischemic heart can be actively and passively targeted with drug-loaded liposomes. In particular, anti-myosin monoclonal antibody-doped liposomes containing ATP were administered to the isolated rat heart prior to global ischemia-reperfusion (Verma et al., 2006), which was associated with better postischemic contractile recovery. Intracoronary infusion of non-targeted ATP-loaded liposomes before regional ischemia-reperfusion resulted in significant reduction in infarct size as compared to controls in rabbit model (Verma et al., 2005). One possible limitation of these studies is that drug-loaded nanocarriers were administered prior to ischemia instead of administering at the end of ischemia or at the early stage of reperfusion. It seems justified that myocardial nanoparticle accumulation is most intensive at the time of reperfusion. Therefore, one can also expect to get the best result as to the extent of myocardial protection when infusing nanoparticle-loaded drug at this time. There are several lines of evidence in support of this notion. First, myocardial cell injury associated with exposure/expression of injury markers required for active targeting becomes more pronounced after reperfusion. Second, myocardial reperfusion initiates the inflammatory response associated with increased microvascular permeability, the latter being a major prerequisite of passive targeting. Third, reperfusion is accompanied by reactive hyperemia which may contribute to better delivery of nanocarriers to the area of interest. Fourth, early stage of reperfusion itself strongly contributes to the formation of irreversible myocardial injury (Yellon & Hausenloy, 2007). Hence, the release of nanoparticle payload at this narrow time interval may prevent or block some of the mechanisms of lethal reperfusion injury. Thus, in order to optimize the pharmacokinetic profile of the heart-targeted drug delivery system, it should be administered at the final stage of ischemia or at the very beginning of reperfusion. In conclusion, the timing of nanoparticle-loaded cardioprotective drug administration is crucial for successful heart targeting.

In the study of Scott et al. (2009), the vascular endothelial growth factor-loaded liposomes functionalized with monoclonal antibodies against P-selectin were administered intravenously to the rats after permanent coronary artery ligation. The authors showed increased density of capillaries within the infarct area 4 weeks after infarction and better post-infarct left ventricular function. Despite positive findings, it is difficult to extrapolate the results of this study to the current clinical practice since it was performed with use of permanent ischemia model without reperfusion. At the same time, current gold standard for treatment of acute coronary syndrome is prompt myocardial revascularization. Besides, the benefits of applied treatment regimen might be explained rather by the improved neovascularizarion of peri-infarct area, and not by the direct prevention of cardiac myocyte death within the ischemic area.

Important results were obtained in a landmark study by Takahama et al. (2009) who used liposomal adenosine for treatment of myocardial ischemia-reperfusion in rats. Adenosine-loaded liposomes were infused intravenously for 10 minutes starting 5 minutes prior to myocardial reperfusion. Selective accumulation of liposomes within the ischemic-reperfused area was verified with electron microscopy, optical fluorescence, and radionuclide imaging. Besides, liposomal adenosine administration was associated with more significant infarct size limitation and less severe arterial hypotension as compared to free adenosine treatment.

The research of our group working at the Institute of Experimental Medicine, V.A. Almazov Federal Heart, Blood and Endocrinology Centre is focused on the development of both active and passive techniques of heart targeting. We hypothesized that local accumulation of drug-loaded nanoparticles within the ischemic area of the heart may be achieved by selective binding of the annexin V to the nanoparticle surface (Fig. 1).

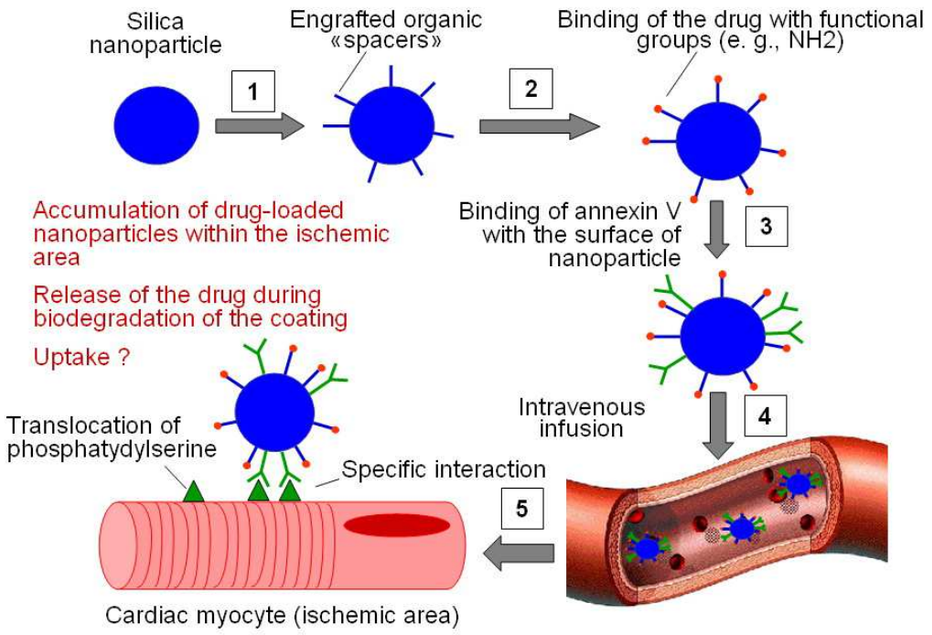

Fig. 1. Proposed algorithm of active targeted drug delivery into the ischemic cardiac muscle

Annexin V is a naturally expressed protein that binds to phosphatidylserine in a calcium-dependent fashion. Phosphatidylserine is normally localized on the inner surface of the plasmalemma, but it is translocated on the exterior cell surface when sublethal cell injury occurs (Vance & Steenbergen, 2005). Annexin V is widely used as one of the marker proteins for cell injury and apoptotic cell death *in vitro*. Besides, the intravenous administration of annexin V conjugated with fluorescent probe to the dogs subjected to 1 hour regional ischemia and 1 hour reperfusion resulted in bright fluorescence of ischemic area indicative of site-specific conjugate accumulation (Ohnishi et al., 2006). Specific interaction of the nanoparticle-anchored annexin V with phosphatidylserine would potentially result in the binding of nanoparticles to the cardiomyocyte membrane with their subsequent accumulation in the area of interest.

Apart from active heart targeting with use of annexin V as a tissue-recognition ligand, we also feel that ischemic-reperfused myocardium may offer an advantage of passive targeting. The basic idea is that the nanoparticulate carriers, if administered in proper time (see above), can be retained within the ischemic-reperfused area of the heart owing to local inflammatory response and severely increased microvascular permeability. In this regard, the functional characteristics of myocardial microvessels are more or less similar to those of the tumor. Therefore, it seems not unlikely that drug-loaded nanoparticles can tend to accumulate within the ischemic-reperfused area of the heart just because of the raised permeability of myocardial capillaries.

5.1 Fabrication, characterization, and surface modification of silica nanoparticles

Among inorganic drug carriers, nanodisperse silica, titania, zirconia, and iron oxide as well as fullerenes and carbon nanotubes have been considered to be the most promising. In our studies, the prototype carriers for antiischemic drugs are suggested to be nanodispersed silica particles. It has been shown before that silica nanomaterials are characterized by fairly good biocompatibility and biodegradability (Slowing *et al.*, 2008). Chemical modification of silica nanoparticle surface can be performed with use of two different synthetic approaches, namely, immobilization and chemical assembly. In immobilization strategy, the modifier is attached to the carrier surface with use of a single-stage reaction. Chemical assembly represents another approach toward synthesis of engrafted surface compounds that has recently emerged as a promising alternative to immobilization strategy. Chemical assembly is based on multistage synthesis of engrafted chemical compounds on the surface of solid phase particles which play a role of matrices. The major advantage of chemical assembly in comparison to immobilization is the larger diversity of functional groups that can be applied to the matrix surface.

Taking into account the above considerations, we suppose that nanodisperse silica of the Aerosil type may be a perspective drug carrier for biomedical applications. In our studies, the standard pyrogenic highly dispersed silica (Aerosil A380 mark obtained from Vekton, Russia) was used throughout experiments. Surface area of silica nanoparticles was determined with use of Brunauer-Emmett-Teller (BET) method and averaged from 170 to 380 m^2/g (Galagudza et al., 2010). The mean particle diameter varied from 6 to 13 nm. The technique of silica nanoparticle functionalization included three sequential steps: modification of silica nanoparticles with (3-aminopropyl)triethoxysilane, hydrolysis of unreacted alkoxysilane groups, and binding of fluorescein. Chemosorption of (3-aminopropyl)triethoxysilane was performed from the gaseous phase with use of dried nitrogen as a carrier gas. The synthesis was done in the vertical quartz flow reactor at 220°C during 2 hours. Hydrolysis of the unreacted alkoxysilane groups was achieved with water vapor at 150°C during 1 hour. The covalent binding of fluorescein to the aminated silica was done using carbodiimide technique during 1 hour. The surface reactions were controlled with infrared spectroscopy. The amount of fluorescein bound to the surface of silica nanoparticles was determined spectrophotometrically at λ=490 nm. Fluorescein content within the samples of modified silica nanoparticles varied from 0.01 to 0.02 mmol/g. Another fluorescent dye that can be used for labeling of silica nanoparticles is considered to be indocyanine green. This fluorophore has an absorption band in the near-infrared region (λ=780 nm). We have developed the technique of indocyanine green immobilization on the surface of aminated silica. Then, fluorescently labeled silica nanoparticles were used for biodistribution studies.

The above-described techniques of matrix synthesis of engrafted surface compounds can be easily applied for Aerosil modification. Combination of the molecular layering method with chemical assembly of engrafted organic compounds is especially promising for development of targeted nanopharmaceuticals. With use of this approach, thin layers of inorganic substances possessing magnetic properties can be laid on the Aerosil surface with subsequent modification of the latter by the organic compounds containing active centers crucial for immobilization of drug molecules. We believe that the use of chemically modified silica as a prototype carrier for targeted drug delivery holds great promise because of

several advantages, such as biocompatibility and biodegradation, low cost, and detailed account on the techniques of surface immobilization of drugs, fluorophores, and targeting ligands.

5.2 Acute toxicity, biodistribution and biodegradation of silica nanoparticles

To investigate the acute toxicity of silica nanoparticles experimentally, we studied the acute hemodynamic effects of nanoparticle formulations in the rat model. For this purpose, the suspensions of silica and fluorescein-doped silica nanoparticles in 0,9% sodium hydrochloride solution were prepared and administered intravenously 3 times with 20-minute intervals between infusions. Control animals received vehicle. Measurements of heart rate, mean arterial pressure and pulse arterial pressure were done throughout the experiments. In all groups of animals, there were no significant alterations in heart rate over time. The first infusion of any tested nanoparticle formulation had no effect on hemodynamic parameters. Furthermore, there were no statistically significant differences in mean arterial pressure between the baseline time point and the end of the experiment. However, the second and the third infusions of silica nanoparticles evoked transient increases of mean arterial pressure. There were significant increases in pulse arterial pressure in the animals that received silica nanoparticles at the end of the observation. Administration of fluorescein-labeled silica nanoparticles resulted in increase of pulse arterial pressure too, while pulse arterial pressure in controls was unaffected throughout the entire experiment. Thus, intravenous infusion of silica nanoparticle formulations caused mild changes in systemic hemodynamic parameters which appears to be indicative of appropriate biocompatibility of these nanomaterials.

Biodistribution of silica nanoparticles was studied with use of optical fluorescence. Fluorescein and indocyanine green were used for fluorescent labeling of silica nanoparticles. The animals received intravenous infusion of fluorescently labeled nanoparticles suspended in normal saline at a volume of 1 ml for 10 minutes. 20 minutes after the end of infusion, the animals were sacrificed by an overdose of anesthesia, and the following organs were removed for subsequent measurements: heart, brain, liver, spleen, lung, and kidney. Control animals were treated with the vehicle. The registration of fluorescence was performed using FLUM3 setup for fluorescent imaging of small animals and biopsy specimens consisting of the mercury lamp illuminator and TV camera 285. The fluorescence of fluorescein- and indocyanine green-labeled nanoparticles was elicited by the use of band-pass interference filters of 435 and 780 nm, respectively. The images obtained in the experiments with fluorescein-labeled nanoparticles were split into RBG channels. Channel G has been chosen as target channel. The relative change in the level of fluorescence (i. e., percent change from control, ΔG, %) has been calculated as follows: $\Delta G=(G2 - G1)/G1$, where G1 is the level of (auto)fluorescence in controls, G2 – the level of fluorescence in animals treated with fluorescein-labeled silica nanoparticles. In the experiments with indocyanine green-labeled nanoparticles, black-and-white images were obtained, and the relative change in the level of fluorescence (i. e., percent change from control, ΔF, %) was calculated as described above. The biodistribution data are shown in the Table 1. It should be noted that exposure of organ samples to UV light (λ=435 nm) for detection of fluorescein-doped nanoparticles was associated with significant deal of auto fluorescence which may interfere with the desired signal. Despite that, we observed predominant accumulation of fluorescein-labeled

nanoparticles within the lung, spleen and liver, that is, in the organs of reticulo-endothelial system. The use of indocyanine green was helpful in avoiding the high level of auto fluorescence because this fluorophore is excited at λ=780 nm. Generally, we found the highest accumulation of indocyanine green-labeled nanoparticles within the liver and kidney. Therefore, we recommend the use of infrared fluorophores for fluorescent labeling of silica nanoparticles and investigation of their biodistribution.

Organ	λ=780 nm			λ=435 nm		
	Control, F1	Indocyanine green-labeled nanoparticles, F2	ΔF, %	Control, G1	Fluorescein-labeled nanoparticles, G2	Δ G,%
Heart	12±0.5	22±0.4	81±3.9	90±13.2	106±7.0	18±9.3
Brain	26±0.5	35±0.5	77±7.0	115±7.1	119±13.4	5±2.8
Liver	14±0.6	1213±193.0	8311±1232.0	95±31.5	113±24.8	22±14.6
Spleen	16±9.9	20±4.2	62±27.0	81±10.2	99±7.1	23±6.8
Lung	29±5.7	127±24.8	357±123.0	88±6.0	113±2.1	30±11.4
Kidney	16±5.7	164±21.2	1018±373.0	76±0.4	82±6.2	9±6.7

Table 1. Distribution of indocyanine green- and fluorescein-labelled silica nanoparticles at the organ level of Wistar rats after 30 min of dose administration

Silica nanoparticle biodegradation was studied both *in vitro* and *in vivo*. When designing the experiments, we speculated that silica nanoparticles are undergoing biodegradation due to gradual erosion of their surface resultant in the formation of water-soluble salts of silicic acid which are excreted from the organism by the kidney (Fig. 2).

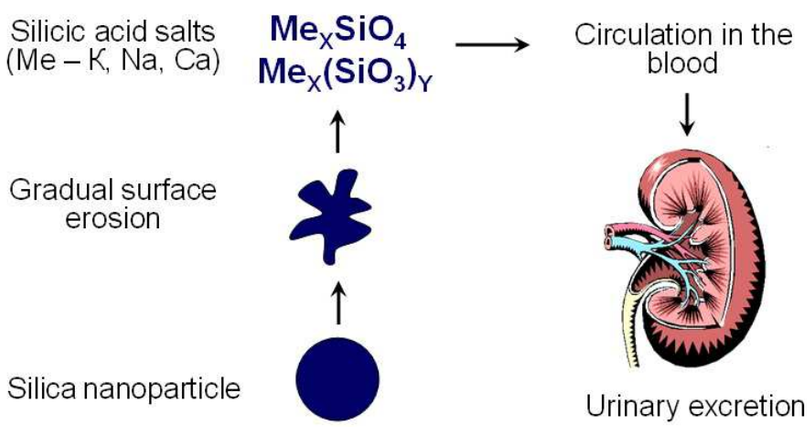

Fig. 2. Hypothetical mechanism of silica nanoparticle biodegradation

The suspension of Aerosil A380 in Krebs-Henseleit buffer having electrolyte composition similar to that of blood plasma with final concentration of silica nanoparticles 2 mg/ml and pH=7,4 was used for investigation of biodegradation *in vitro*. The experiments were performed in the settings of continuous stirring in the polymer 100 ml glass. The temperature of the media was maintained at 37°C by means of water jacketing. The samples

were continuously gassed with carbogen (95% O_2 and 5% CO_2). Total incubation time was equal to 15 hours. The samples were taken each 5 hours and analyzed for silicate content spectrophotometrically after reaction with molybdenum blue. The concentration of silicate in samples increased logarithmically with time. With use of mathematical analysis, we derived the value which corresponds to 95% biodegradation of silica and equals to 41 day. These data fit well to the results of Finnie et al. (2009) who studied biodegradation of sol-gel mesoporous silica microparticles. It has been shown in this study that silica microparticles are rapidly degraded in physiological buffer. Besides, the authors demonstrated that the addition of plasma proteins to the buffer retarded biodegradation by 20-30%. It follows, therefore, that *in vivo* biodegradation might be slower than in the *in vitro* settings.

In vivo biodegradation of silica nanoparticles was studied in the male Wistar rats weighting 200-250 g. Silica nanoparticles were infused intravenously at a dose of 2 mg/ml and volume of 1 ml followed by sampling of the liver at 1 h, 10, 20, and 30 days after infusion. The controls were animals received intravenous infusion of 1 ml of vehicle. Liver samples were dried at 90°C during 24 h to obtain constant weight. The liver was chosen for sampling and analyses on the basis of biodistribution experiments which showed maximal accumulation of silica in this organ. Quantitative analysis of silicon content within the samples was performed with atomic absorption spectroscopy. The mineralizat obtained after drying was analyzed on the atomic absorption spectrometer with electrothermic atomization and Zeman correction. Silicon content within the mineralizat was recalculated for the dry sample weight and expressed in μg/g. Silicon content in the liver of the rats according to the results of atomic absorption spectroscopy is shown on the Fig. 3.

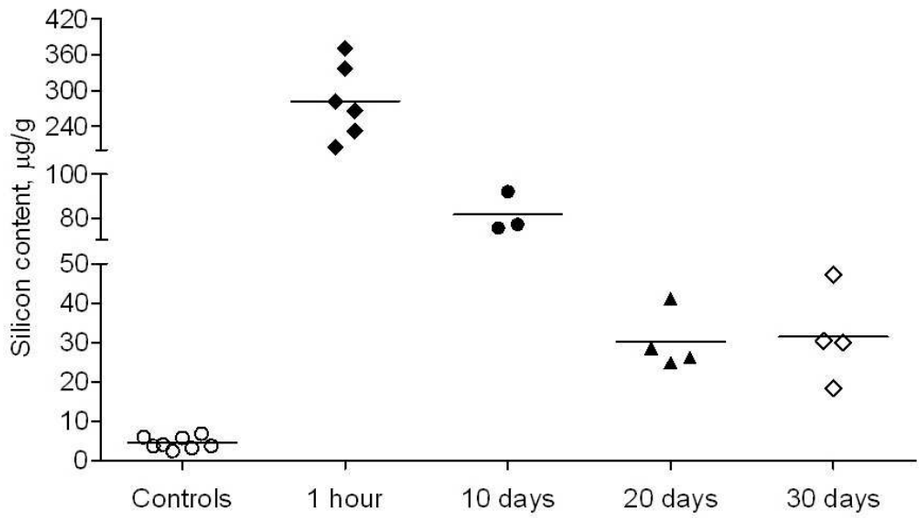

Fig. 3. Biodegradation of silica nanoparticles *in vivo*: silicon content in the liver at different durations after infusion of nanoparticles

Silicon content in the liver of healthy rats averaged 4.4±1.55 μg/g. At 1 hour after silica nanoparticles infusion silicon content was increased up to 282.3±62.65 μg/g. 10 and 20 days after silica nanoparticle infusion silicon content in the liver was significantly lower (81.5±9.25 and 30.2±7.48 μg/g, respectively). This dynamics of silica dissolution is consistent with the data obtained in the *in vitro* model. However, at 30 days after silica nanoparticle

administration we failed to see any additional decrease in silicon tissue content (31.5±11.87 μg/g). This fact might be accounted for by the higher stability of the intracellular pool of silica nanoparticles, produced as a result of their phagocytosis by the Kupffer cells and, to a lesser extent, silica nanoparticle internalization into hepatocytes and other liver cells. It remains unclear, however, whether this increased silicon content in the liver cells affects liver function or not.

5.3 Passive heart targeting with silica nanoparticles

For investigation of passive heart-targeted drug delivery with silica nanoparticles, we first studied biodistribution of unmodified nanoparticles in the rats with regional myocardial ischemia-reperfusion. For this purpose, the animals were randomly allocated into one of three groups: 1) controls (these animals received intravenous infusion of saline), 2) silica nanoparticle-treated animals (2 mg/ml, 1 ml intravenously) with sham surgical procedure; 3) silica nanoparticle-treated animals with myocardial ischemia-reperfusion. Regional myocardial ischemia was induced by 30-minute left coronary artery ligation followed by 60 minutes of reperfusion. Silica nanoparticles were infused for 10 minutes starting 5 minutes prior to reperfusion (Fig. 4). The left ventricle of the heart and liver were sampled at the end of the experiments, rinsed and dried at 90°C during 24 h to obtain constant weight. Silicon content in the organ samples was done with atomic absorption spectroscopy as described above.

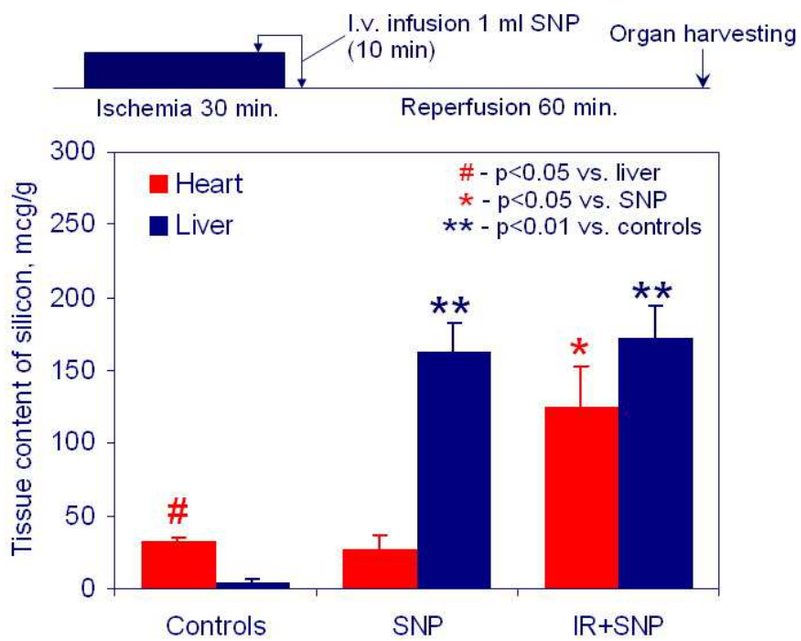

Fig. 4. Biodistribution of non-modified silica nanoparticles in rat model of myocardial ischemia-reperfusion. SNP – silica nanoparticles, IR – ischemia-reperfusion

In controls, silicon content was found to be significantly higher in myocardial tissue then in the liver (32±2.8 vs. 4±2.3 μg/g, respectively, $p<0.05$) (Fig. 4). Of note, physiological role of this fact is currently unknown. Silica nanoparticle administration to sham-operated rats

resulted in dramatic rise in liver silicon content as compared to controls (163±19.5 vs. 4±2.3 μg/g, respectively, $p<0.01$), while myocardial silicon concentration remained unaffected (27±9.7 vs. 32±2.8, respectively, $p>0.05$). Administration of silica nanoparticles to the animals with cardiac ischemia-reperfusion was associated with significant increase in myocardial silicon content in comparison to sham-operated animals (125±28.2 vs. 32±2.8 μg/g, respectively, $p<0.05$). Thus, the first evidence that non-modified silica nanoparticles can accumulate within the anatomical area at risk after regional myocardial ischemia-reperfusion was provided in this study.

The next series of experiments was performed to study the hemodynamic effects of nanoparticle-bound and free adenosine. Adenosine adsorption on the surface of silica nanoparticles was achieved by mixing the suspension of silica nanoparticles in 0.9% sodium chloride and adenosine solution in the same vehicle. The samples thus prepared were subjected to 10-minute sonication and left at +4°C overnight. Silica nanoparticle-bound adenosine was administered to the rats intravenously for 10 minutes at a dose of 300 μg/kg×min. Control animals received 10-minute intravenous infusion of adenosine at a dose of 300 μg/kg×min. Mean blood pressure was registered before the beginning of drug infusion, at 5th and 10th minutes of infusion, and at 5th and 10th minutes of recovery. The data on blood pressure values are shown in Table 2. The extent of blood pressure decrease was significantly less marked when adenosine was immobilized on the surface of nanoparticles (Fig. 5).

MBP, mmHg	Baseline	5 min infusion	10 min infusion	5 min recovery	10 min recovery
Free ADO	125±12	82±7	85±9	128±5	119±13
ADO+SNP	131±8	108±14*	113±11*	134±12	130±10

Table 2. Attenuation of adenosine-induced hypotension after its immobilization on the surface of silica nanoparticles. MBP – mean blood pressure, ADO – adenosine, SNP – silica nanoparticles. * - $p<0.05$ vs. free adenosine

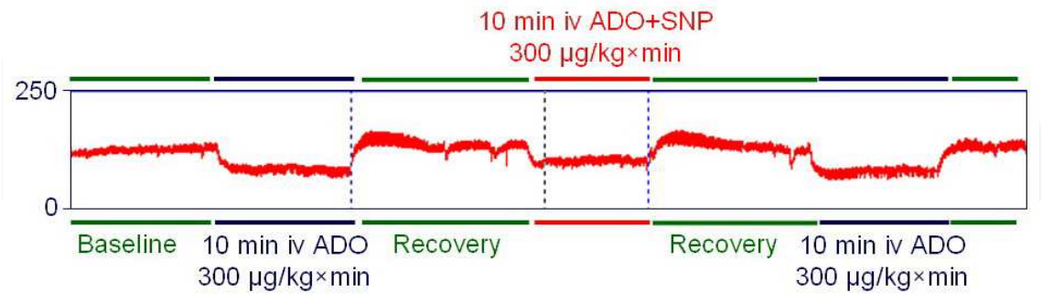

Fig. 5. Representative blood pressure recording showing different blood pressure response to free and silica nanoparticle-bound adenosine. Initial free adenosine infusion was followed by recovery period and infusion of silica-nanoparticle bound adenosine. After recovery, one more episode of adenosine infusion was performed. ADO – adenosine, SNP – silica nanoparticles

The final series of experiments was focused on investigation of the effect of free adenosine and nanoparticle-bound adenosine on infarct size. Five minutes prior to the end of 30-minte

ischemia, animals received either 10-minute adenosine infusion at a dose of 300 µg/kg×min or infusion of adenosine adsorbed on silica nanoparticles in the equivalent dose. Control animals were treated with vehicle. Myocardial area at risk and infarct size were determined after 90 minutes of reperfusion with use of Evans blue and triphenyltetrazolium chloride staining, respectively. There were no differences in risk area between groups (Fig. 6).

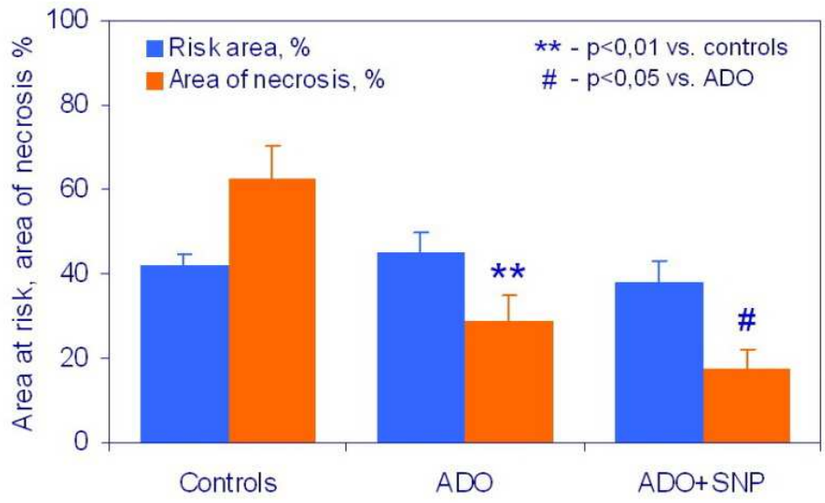

Fig. 6. Increased infarct-limiting effect of adenosine after its immobilization on silica nanoparticles. ADO – adenosine, SNP – silica nanoparticles

Adenosine infusion caused significant reduction in infarct size as compared to controls (29±6,2 vs. 63±7,3%, respectively, $p<0.01$). Administration of adenosine adsorbed on silica nanoparticles resulted in additional significant decrease in infarct size in comparison to free adenosine (18±4.2 vs. 29±6,2, respectively, $p<0.05$).

In conclusion, adsorption of adenosine on silica nanoparticles results in its passive delivery to the ischemic area of the heart which, in turn, leads to more pronounced infarct size limitation and attenuation of hemodynamic side effect typical for the drug used.

5.4 Candidate drugs for heart-targeted delivery

Several groups of drugs with different mechanisms of action might potentially be targeted to the heart with nano-sized carriers (Table 3). One of the most efficient means of myocardial protection known to date is ischemic preconditioning, a phenomenon which describes dramatically increased heart tolerance to prolonged ischemia occurring after one or several brief episodes of ischemia-reperfusion (Galagudza et al., 2008). It is well established that preconditioning can elicit several cardioprotective effects including infarct-limitation, reduction of ischemic and reperfusion arrhythmias, prevention of endothelial dysfunction and attenuation of stunning. The mechanisms of preconditioning include generation of chemical triggers during brief bouts of ischemia, their interaction with corresponding sarcolemmal G-protein-coupled receptors with subsequent activation of multiple intracellular kinases, and, eventually, engagement of putative mitochondrial effectors responsible for initiation of energy-sparing processes. It has been shown that such preconditioning triggers as adenosine, bradykinin, and opioid peptides are all able to mimic

the preconditioning response when administered exogenously. However, their clinical use at doses required for preconditioning-like response is hampered by the high risk of dangerous side effects. For example, intravenous administration of adenosine is associated with arterial hypotension and bradycardia (Takahama et al., 2009), while administration of bradykinin might lead to hypotension and bronchial spasm (Homma & Irvin, 1999). It follows, therefore, that targeted delivery of preconditioning mimetics or synthetic agonists of G-protein-coupled receptors might contribute to improved safety and efficacy profile of these compounds, especially taking into account the encouraging results with heart-targeted delivery of adenosine (Takahama et al., 2009).

Mechanism	**Drug/ligand**
Activation of G-protein-coupled receptors (preconditioning)	Bradykinin, opioids, adenosine and synthetic ligands of corresponding receptors
Activation of tyrosine kinase receptors (angiogenesis, cardiac myocyte proliferation?)	Insulin, insulin-like growth factor-1, transforming growth factor-β1, erythropoietin, vascular endothelial growth factor, etc.
Inhibition of mitochondrial permeability transition pore opening (attenuation of reperfusion injury, antiapoptotic effect)	Cyclosporine
Activation of peroxisome proliferator-activated receptors gamma (reduction of inflammation, improved endothelial function)	Rosiglitazone, pioglitazone
Opening of ATP-sensitive potassium channels (improved mitochondrial function)	Nicorandil
Nitric oxide signaling pathway (preconditioning, vasodilation)	Nitric oxide donors, atorvastatin, atrial natriuretic peptide, etc.

Table 3. Candidate drugs for ischemic heart targeting

Another group of compounds demonstrating cardioprotective effects are growth factors which interact with tyrosine kinase receptors. Such molecules as insulin, insulin-like growth factor-1, transforming growth factor-β1, erythropoietin, vascular endothelial growth factor, and fibroblast growth factor may not only induce cytoprotective effect when administered either prior or just after index ischemia, but also possess late cardioprotective effects due to stimulation of angiogenesis and attenuation of inflammation (Boucher et al., 2008; Ueda et al., 2010). Again, heart-targeted delivery of growth factors may result in better outcome because of local increase in the concentration and reduction of such side effects as hypotension and hypersensitivity reactions.

One more key player in the mechanisms of ischemia-reperfusion myocardial injury is mitochondrial permeability transition pore (mPTP). mPTP represents multiprotein complex localized in the inner mitochondrial membrane which functions as voltage-dependent nonselective channel. The probability of mPTP opening is very low during in both normal and ischemic environment. However, at reperfusion mPTP becomes opened thus making

inner mitochondrial membrane permeable for water, ions, and molecules with molecular weight up to 1.5 kDa. Major stimuli for mPTP opening are increased calcium concentration, mitochondrial matrix pH>7.0, increased concentration of reactive oxygen species, and mitochondrial membrane depolarization (Di Lisa et al., 2011). mPTP opening results in immediate complete depolarization of mitochondrial membrane and, therefore, loss of electrochemical gradient. In this situation, ATP synthase starts to degrade ATP in a futile attempt to regenerate membrane potential. Besides, massive swelling of mitochondrial matrix may contribute to mechanical rupture of the organelles with resultant release of several pro-apoptotic molecules normally retained in the intermembrane space. It follows that mPTP opening at the early moments of reperfusion can lead to development of lethal reperfusion injury by means of both necrosis and apoptosis. mPTP inhibitors were shown to be cardioprotective in experimental and clinical settings. Intravenous administration of mPTP inhibitor cyclosporine to the patients with acute ST-elevation myocardial infarction just prior to percutaneous coronary intervention resulted in significant reduction in infarct size as compared to controls (Piot et al., 2008). Cyclosporine is commonly used for immune suppression, and its prolonged use is associated with several serious side effects including renal failure, hepatotoxicity, infection, and increased risk of tumor development. Thus, it is of prime importance to develop the technique of targeted delivery of cyclosporine to the ischemic heart which may help to attenuate its systemic toxicity.

Peroxisome proliferator-activated receptors (PPAR) are nuclear receptors that function as transcription factors for numerous genes. In terms of myocardial ischemia-reperfusion injury, activation of PPARγ with either endogenous ligands or thiazolidinedione derivatives has been shown to be cardioprotective (Di Paola & Cuzzocrea, 2007). Potential mechanisms of PPARγ-mediated cardioprotection include inhibition of the activation of nuclear transcription factor κB, stimulation of endothelial NO-synthase function, and increased expression of heme oxygenase-1. Decreased function of nuclear transcription factor κB in the ischemic-reperfused heart is supposed to be beneficial since this transcription factor positively regulates a battery of proinflammatory genes, all of which contribute to secondary injury in the infarcted area.

ATP-sensitive potassium channels have been believed to be major end effectors of ischemic preconditioning in the late 90s. There are two main populations of ATP-sensitive potassium channels, that is, sarcolemmal and mitochondrial. Currently, the cardioprotective role of sarcolemmal ATP-sensitive potassium channels has been unequivocally proved while there is some debate as to the role of mitochondrial channels (Hanley & Daut, 2005). Several ATP-sensitive potassium channel openers were shown to exert preconditioning-like protective effect in the experimental models. However, the only drug from this group that is approved for use in humans is nicorandil. The results of IONA trial demonstrated reduced incidence of acute coronary syndrome in the patients with stable angina receiving nicorandil. It seems, therefore, that targeted delivery of nicorandil to the heart might further strengthen its cardioprotective effect.

Finally, pharmacological modulation if nitric oxide (NO)-dependent pathway in the heart is also of interest. It has been shown that endogenous NO participation in the mechanisms of cardiac protection if unlikely but exogenous supplementation of the heart with NO can mimic the protective effect of preconditioning (Dawn & Bolli, 2002). NO-dependent

mechanisms were also proposed for infarct-limiting effect of atorvastatin (Atar et al., 2006) and atrial natriuretic peptide (Okawa et al., 2003).

In conclusion, the list of cardioprotective drugs which might be delivered to the heart with nanocarriers is incomplete. By now, hundreds of drugs were shown to be cardioprotective in the preclinical models. However, very few of them were proved to be effective in the patients with coronary artery disease. The reasons for this failure are multiple and analyzed elsewhere, but at least one is the fact that many drugs have significant side effects which hinder the process of clinical translation. Recent evidence indicates that some side effects can be substantially reduced when the drug is navigated to the tissue of interest by the nanocarrier. Targeted delivery concept holds promise for development of new efficient and safe therapies for ischemic heart disease.

6. Perspectives and future work

Although the concept of targeted drug delivery to the ischemic heart seems to be generally coherent, it is evident that large questions lie ahead. Systemic administration of multifunctional drug carriers results in their selective accumulation in the target tissues with normal blood flow. It might be problematic to achieve the minimal effective dose of the nanopharmaceutical within the tissue subjected to critical ischemia. This problem may be solved by the increased residence time of nanocarriers within the adjacent to ischemic tissues providing diffusion of the drug into the poorly vascularized area. Alternative solution may involve increased loading of the nanoparticles with payload and/or targeting ligands. On the other hand, intensive functionalization of nanoparticles may result in better recognition by the immune system and faster clearance by the reticulo-endothelial system.

Insufficient evidence is currently available on the clinical effectiveness of nanoparticulate drug carriers. Additional studies are required to elucidate the pharmacokinetics of nanocarriers as well as their biodistribution and putative toxicity. All newly developed nanosystems for targeted delivery possess unique properties requiring individual testing. The refinement of the techniques of nanocarrier fabrication is aimed at ensuring of better uptake of nanoparticles by the target cells, formation of higher gradient of drug between injured and intact tissue, and more complete avoidance of drug toxicity. The accomplishment of these goals will be a major step in development of an ideal tool for targeted delivery of the drug into the tissue of interest.

7. Conclusion

Concepts of passive and active targeting can be applied to the development of targeted drug delivery to the ischemic myocardial tissue. Targeted delivery of various cardioprotective agents to the ischemic cardiac muscle with use of nanoparticles seems to be a challenging approach to treatment of coronary artery disease.

Silica nanoparticles are non-toxic materials that might be potentially used as carriers for heart-targeted drug delivery. Silica nanoparticles can be functionalized with use of the techniques of immobilization and chemical assembly. Immobilization of adenosine on the surface of silica nanoparticles resulted in enhancement of free adenosine-mediated infarct size limitation in the animal model. Furthermore, hypotensive effect of adenosine was

attenuated after its adsorption on silica nanoparticles. Additional studies will be required to demonstrate silica nanoparticle biocompatibility in the chronic experiments.

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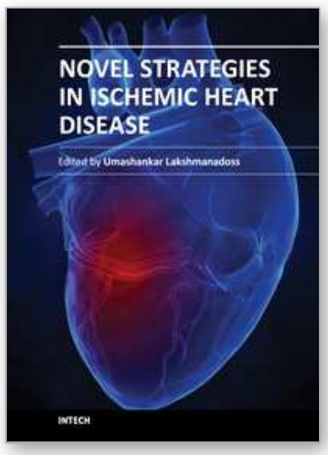

Novel Strategies in Ischemic Heart Disease

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The first edition of this book will provide a comprehensive overview of ischemic heart disease, including epidemiology, risk factors, pathogenesis, clinical presentation, diagnostic tests, differential diagnosis, treatment, complications and prognosis. Also discussed are current treatment options, protocols and diagnostic procedures, as well as the latest advances in the field. The book will serve as a cutting-edge point of reference for the basic or clinical researcher, and any clinician involved in the diagnosis and management of ischemic heart disease. This book is essentially designed to fill the vital gap existing between these practices, to provide a textbook that is substantial and readable, compact and reasonably comprehensive, and to provide an excellent blend of "basics to bedside and beyond" in the field of ischemic heart disease. The book also covers the future novel treatment strategies, focusing on the basic scientific and clinical aspects of the diagnosis and management of ischemic heart disease.

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