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Review | Обзор

Cardiovascular Safety Assessment of Medicines in Preclinical *in vivo* Studies: A Review

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ABSTRACT

INTRODUCTION. The cardiovascular safety evaluation of medicines using *in vivo* models is a necessary preclinical step that is performed either in safety pharmacology studies or in toxicity studies. The design of safety pharmacology studies primarily involves assessing the potential of a test substance to prolong cardiac ventricular repolarization, without in-depth investigation of potential structural damage to the heart and blood vessels. Toxicity studies usually do not include electrophysiological testing. The regulatory standards of the Eurasian Economic Union (EAEU) and the International Council for Harmonisation (ICH) lack detailed guidance on the use of specific markers of cardiovascular dysfunction.

AIM. This study aimed to develop an integrated approach to assessing the cardiac and vascular toxicity of medicinal products in preclinical *in vivo* studies.

DISCUSSION. Cardiovascular function can be assessed in both small laboratory animals (rodents) and larger animals, such as rabbits, ferrets, dogs, minipigs, and primates. The toxic effects of a test medicinal product on the cardiovascular system of animals may be manifested as physiological, biochemical, and structural changes in the systems and organs. Therefore, the assessment of cardiovascular function should be based on a combination of instrumental, laboratory, and histological methods. First of all, physiological and laboratory studies are applicable. It is recommended to perform electrocardiography, heart rate and blood pressure measurements, and quantification of markers of cardiovascular dysfunction and structural cell damage. For more in-depth analysis, histological and immunohistochemical studies of cardiac and vascular tissues are recommended to assess changes at the tissue and cellular levels.

CONCLUSIONS. An effective strategy for detecting cardiovascular disorders is the use of an integrated approach that, on the one hand, facilitates a comprehensive assessment of the possible toxic effects of a medicinal product and, on the other hand, increases the translational potential of the data obtained at the preclinical stage of research.

Keywords: systematic review functional activity; cardiotoxicity; electrocardiography; blood pressure; cardiac markers; troponins; histological studies; preclinical studies; study design; laboratory animals

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Оценка безопасности лекарственных средств в отношении сердечно-сосудистой системы в доклинических исследованиях *in vivo*: обзор

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РЕЗЮМЕ

ВВЕДЕНИЕ. Оценка безопасности лекарственных средств в отношении сердечно-сосудистой системы с использованием моделей *in vivo* является необходимым этапом доклинических исследований, которая проводится либо в исследованиях фармакологической безопасности, либо в рамках токсикологических исследований. В дизайне исследований фармакологической безопасности в первую очередь подразумевается оценка потенциала исследуемого вещества замедлять реполяризацию желудочков сердца без углубленного изучения возможного структурного повреждения сердца и сосудов. При этом в токсикологических исследованиях, как правило, не оцениваются электрофизиологические параметры. В нормативной документации Евразийского экономического союза и Международного совета по гармонизации (ICH) отсутствуют подробные указания по использованию специфических маркеров нарушений функционирования сердечно-сосудистой системы.

ЦЕЛЬ. Разработка комплексного подхода по оценке кардио- и васкулотоксичности лекарственных препаратов в доклинических исследованиях *in vivo*.

ОБСУЖДЕНИЕ. Для оценки функционального состояния сердечно-сосудистой системы могут быть использованы как мелкие лабораторные животные (грызуны), так и более крупные, такие как кролики, хорьки, собаки, карликовые свиньи и приматы. Токсические эффекты изучаемых лекарственных препаратов в отношении сердца и сосудов у животных могут проявляться в виде изменений в физиологическом, биохимическом или структурном статусах систем или органов, поэтому оценка функционального состояния сердечно-сосудистой системы должна опираться на совокупность инструментальных, лабораторных и гистологических методов. В первую очередь применимы физиологические и лабораторные исследования. Рекомендовано проводить электрокардиографию, измерение частоты сердечных сокращений и артериального давления, а также количественно оценивать маркеры нарушения функций и структурного повреждения клеток. Для более глубокого анализа рекомендуется применять гистологические и иммуногистохимические исследования тканей сердца и сосудов, чтобы оценить изменения на тканевом и клеточном уровнях.

ВЫВОДЫ. Эффективным способом обнаружения нарушений сердечно-сосудистой системы является применение комплексного подхода, который, с одной стороны, позволяет всесторонне оценить возможные токсические проявления лекарственного средства, а с другой – увеличивает трансляционной потенциал данных, получаемых на доклиническом этапе исследований.

Ключевые слова: функциональная активность; кардиотоксичность; электрокардиография; артериальное давление; кардиомаркеры; тропонины; гистологические исследования; доклинические исследования; дизайн исследования; лабораторные животные

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INTRODUCTION

Identifying potential toxicity in experimental animals, i.e. assessing the impact on the functional state of systems and organs is a necessary stage in preclinical trials of drugs under development. Guidelines S7A[1], S7B[2] of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), as well as Guideline No. 18[3] of the Board of the Eurasian Economic Commission of October 27, 2020 oblige preclinical researchers to analyze the potential adverse pharmacodynamic effects of drugs on physiological functions. Prior to clinical trials, a core battery is performed to assess the impact of a developed drug on the central nervous, respiratory, and cardiovascular system; this emphasizes acute effects without a thorough pharmacological profiling. Thus, the design of pharmacological safety studies primarily involves assessing the ability of the test substance to slow down ventricular repolarization without an in-depth analysis of possible structural damage to the heart and blood vessels; for example, laboratory or histological examinations are used. Meanwhile, toxicology studies do not measure blood pressure, heart rate, or electrocardiographic parameters.

Toxic effects of test drugs in animals can manifest themselves as changed physiological, biochemical, or structural status of systems or organs [1]. Therefore, a cardiovascular assessment must combine instrumental, laboratory, and histological methods.

Early tests assessing cardiovascular effects of the test substance are performed *in vitro*; this can help the researchers understand its effect on the action potential duration and ionic currents in the heart, in particular on hERG ion channels (potassium channels encoded by *hERG* gene, human *Ether-à-go-go*-related gene) [2]. Blocking cardiac hERG channels can have hazardous effects: impaired ventricular repolarization and ventricular tachyarrhythmia [3]. These studies are essential for assessing the potential for QT prolongation and understanding cellular mechanisms that underline myocardial repolarization. There remains a need for *in vivo* studies to determine the hemodynamic and electrophysiological effects of medicines, since alternative approaches are currently unavailable.

Both small laboratory animals (rodents) and larger ones, such as dogs, minipigs and primates, can be used to assess cardiovascular functions [4, 5]. Using mice and rats as an *in vivo* model is likely the best option for early pharmacological testing, since these laboratory animals are a preferred choice for toxicology studies and can provide initial data on the test compound. However, ionic mechanisms of repolarization in rats and mice differ from those of larger animals, including humans; thus guinea pigs, rabbits, ferrets, dogs, minipigs, and primates are used for more in-depth studies[4].

A comprehensive strategy for *in vivo* safety assessment of the developed drugs that goes beyond conventional approaches to toxicological and pharmacological examinations will increase the probability of identifying and studying adverse effects essential for the safe pharmacotherapy.

The aim of the study is to develop an integrated approach to assessing the cardiac and vascular toxicity of medicinal products in preclinical *in vivo* studies.

This study systematized and analyzed methods used to assess toxic effects of drugs on the cardiovascular performance of test animals. Scientific publications on the prerequisites, methods, advantages/disadvantages, and prospects of assessing cardiac and vascular toxicity in preclinical studies were summarized. Literature search included PubMed, eLIBRARY.RU databases and Google Scholar search engine. The review covered full-text publications available as of August 20, 2024.

[1] ICH S7A Safety pharmacology studies for human pharmaceuticals. CPMP/ICH/539/00. ICH; 2001.
[2] ICH S7B Non-clinical evaluation of the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals. CPMP/ICH/423/02. ICH; 2005.
[3] Guideline No. 18 of the Board of the Eurasian Economic Commission dated October 27, 2020 "On the Guidelines for the Pharmacological Safety Study of Drugs for Medical Use."
[4] Ibid.

Articles published within the past five years were prioritized. Search terms for Russian and English literature included "functional activity", "cardiac toxicity", "blood pressure", "cardiac markers", "troponins", "histology", "preclinical studies", and "laboratory animals".

MAIN PART

Instrumental Methods

Cardiovascular safety assessment of drugs (core battery) includes blood pressure (BP) and heart rate (HR) measurements, as well as an electrocardiogram (ECG)[5] [6]. Echocardiography measuring cardiac output and ventricular contractility is considered a supplementary test [6, 7]. It is recommended to use awake animals[6] (not anesthetized) for *in vivo* studies. One of the main points to consider when using animals not anesthetized in advance is the prevention of discomfort and pain. To achieve this, a long-term pre-training is recommended for the animals to get used to these procedures. In this regard, it is allowable and even advisable to anesthetize animals in order to record the parameters. This will reduce intersubject variability of parameters caused by distress and motor activity of animals while reducing the time spent on manipulations. To become aware of the potential influence of general anesthetics on the test parameters, it is necessary to record their initial values prior to the intervention and include a control group in the experiment.

Electrocardiography. ECGs obtained from awake laboratory animals often show a fast heart rate and electric noise caused by the moving animals. All these variables make it difficult to obtain an accurate and consistent ECG, which is inherently complicated in laboratory animals due to unclear signals at recording [8]. Anesthesia/sedation balances out these variables and offers some advantages compared to awake animals. Baseline test parameters should be recorded to identify potential individual traits of an animal. When selecting an anesthetic dosage sufficiently effective with a minimal effect on the test parameters, reference intervals, researchers' own data, or literature data are preferred. Moreover, ICH S7A guideline recommends using negative and positive control groups during the examinations to help determine the model system sensitivity and distinguish any pathology caused by the test substance from individual animal characteristics and artifacts [9].

Dogs, primates, and minipigs are considered the most suitable laboratory animals for electrophysiological studies. Guinea pigs, rabbits, and ferrets are also acceptable. Mice and rats are often used in toxicology and pharmacodynamic studies, as well as in combined studies where some pharmacological safety endpoints are included in the design. Noteworthy are specific ECG characteristics of small rodents, including an inferior Q wave and ST segment or the presence of a J wave (identified as the ST segment slope) [10].

Despite the labor-intensive recording and analysis, ECG is deemed the most important indicator and the main biomarker assessing cardiovascular safety at preclinical studies. Regulatory guidelines[7] emphasize the need to evaluate QT interval (the time from the start of the QRS complex to the end of the T wave), an indicator of cardiac conduction. With a delayed ventricular repolarization and the increased QT interval in humans, the risk of ventricular tachyarrhythmias increases. Therefore, the study of the potential proarrhythmic effects associated with prolongation of the QT interval[8] is of great importance. Alongside with the QT interval, the most frequently determined parameters are the length of the PR intervals, duration and morphology of the P wave, QRS complex, T and U waves that help to understand the anatomical site that can be changed under the influence of the test drug [11].

[5] ICH S7A Safety pharmacology studies for human pharmaceuticals. CPMP/ICH/539/00. ICH; 2001.
[6] Guideline No. 18 of the Board of the Eurasian Economic Commission dated October 27, 2020 "On the Guidelines for the Pharmacological Safety Study of Drugs for Medical Use."
[7] ICH S7B Non-clinical evaluation of the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals. CPMP/ICH/423/02. ICH; 2005.
[8] Ibid.

Given the importance of this method, it is noteworthy that even small discrepancies in the placement of ECG markers increase the variability of the analyzed values and can affect the interpretation of the ECG data; subsequently, it can modify sensitivity of the statistical analysis crucial for detecting small but significant changes.

Another non-invasive ECG recording method is a 24-hour ECG monitoring (Holter monitoring), a continuous ECG recording that can be performed both at rest and during physical activity. Due to the size of the equipment, this electrophysiological method is suitable for large animals such as dogs and pigs. Monitoring in primates can be technically challenging due to their high mobility. 24-hour ECG recording in cats is also uninformative due to the large amount of noises [12–14]. Before applying the electrodes, the animal's skin must be prepared by shaving the fur and degreasing the surface. Disposable self-adhesive electrodes are then attached to the chest skin, ensuring a secure connection with the recording device. Vests or elastic bandages are also used for the animal's comfort; they help to hide the wires from the animal and ensure a better adherence of the electrodes to the skin. Holter monitoring is used in veterinary medicine to diagnose rhythm and conduction disorders, cardiomyopathy, and to evaluate the effectiveness of antiarrhythmic therapy. This method has not yet become widespread in preclinical studies due to the high equipment cost.

Heart rate (HR) is yet another electrophysiological indicator of cardiac activity used for the primary differentiation between normal heart rhythms and various abnormalities. HR in laboratory animals is often recorded together with ECG. HR can be determined directly at any time point or in the intervals between successive QRS complexes, that is, between the two main depolarization waves (R–R intervals); these data can then be converted into HR values [15].

Blood pressure (BP). While ECG recording and analysis present some challenges, BP measurement requires relatively simple data recording and interpretation strategies. The most common method of BP measurement is sphygmomanometry, where species-specific sphygmomanometers with a cuff (with auscultation) are applied around a peripheral artery in the animal's limb or tail. Proper cuff size and position are crucial for accurate results. Using a cuff that is too narrow or too loose tends to result in overvaluated BP, while using a cuff that is too wide or too tight leads to underestimated BP [16].

BP values recorded in laboratory animals may vary depending on the site where the cuff is applied. Systolic BP increases in a distal direction as a result of pressure wave being reflected from arterioles due to a stronger muscular wall and higher resistance, while diastolic BP decreases insignificantly, with mean BP being virtually stable [11]. If BP is measured in large animals, it is possible to evaluate systolic and diastolic BP to calculate the mean BP, whereas in rodents, only systolic BP is measured using a tail cuff for caudal artery [17].

Invasive BP measurement has been developed, such as the use of a peripheral arterial catheter. However, direct BP measurement involves a difficult placement and carries risks associated with a catheter placed in a peripheral artery, including ischemic injury, bleeding, arterial embolization, and infection. Notably, this method is primarily suitable for large animals [18].

Echocardiography is another non-invasive method for assessing cardiac activity, which provides data on the heart morphology and its structural changes (e.g. hypertrophy and dilated cardiomyopathy), as well as function capabilities (left ventricular contractility, systolic discharge, etc.) [7, 19]. When performing echocardiography in rodents and small animals, neonatal phased array transducers can be used; for larger species, such as dogs, primates, and miniature pigs, a pediatric transducer is preferred.

Laboratory Methods

Cardiac-specific markers determined in serum or plasma are a group of indicators that can be assessed when studying cardiovascular effect of a drug [20]. Notably, laboratory methods assessing the functional state of the

cardiovascular system in preclinical studies or sensitivity studies to endogenous/exogenous substances are optional. They can be used as supplementary studies (ICH S7A).

Ideally, a cardiac biomarker should be highly specific; its level should rise and fall rapidly after the injury and correlate with its severity; it should depend on the function of other organs and be identifiable by standard and affordable methods [21]. In the context of preclinical studies, cardiac markers should be both identifiable in laboratory animals and feasible for use in clinical practice to ensure high translational value of the data. However, currently none of the markers can meet all these criteria.

All cardiac toxicity markers used today have certain limitations; the most common are low organ specificity and their protein nature making them organospecific, which, in turn, makes the research costly. Other constraints in the use of specific toxicity markers include their short lifespan, difficulty to predict blood sampling time, and limited translational potential [22].

Biomarkers used to detect cardiac damage are either those that identify structural (direct) damage and necrosis of cardiac myocytes (e.g. troponins, heart-type fatty acid-binding protein) or those indicating heart function abnormalities (e.g. natriuretic peptides).

Structural damage and cardiac myocyte necrosis markers. Troponin is a regulatory globular protein involved in muscle contraction that consists of three subunits. Troponin is found in skeletal and cardiac muscles, however, it is absent from smooth muscles [23].

US Food and Drug Administration (FDA) has approved the use of cardiac-specific troponins (cTn) T and I (cTnI and cTnT) in preclinical studies to assess potential myocardial damage in rats, dogs, and primates [24]. cTnI and cTnT proteins are found only in the cardiac muscle, determining their high organ specificity; they can be released into the bloodstream after myocardial damage (cardiac myocyte necrosis), while their increased blood level correlates with the damage extent [25]. The troponin level in response to damage increases in a generally similar manner in different laboratory animals: in the first 2 to 3 h after damage, correlating with histopathological changes in the myocardium [26–29]. Depending on the model of the cardiovascular pathology, an increased concentration of troponins in test animals can persist for up to 10 days. This ensures a fairly long period of their diagnostic significance (diagnostic window), including the excretion periods of creatine kinase and lactate dehydrogenase.

Despite the described potential, cTn as a marker of drug cardiac toxicity has some limitations. For instance, the detection methods have been developed and refined primarily for humans, but not for laboratory animals [30]. Furthermore, cTn is rapidly cleared from the blood in some laboratory animals, such as mice and rats, requiring adjustments to blood sampling points [31].

Alongside with troponins, other cytoplasmic proteins that indicate ischemia and necrosis of cardiomyocytes emerge in the blood after myocardial injury, such as myoglobin (Mb) and heart-type fatty-acid-binding protein (H-FABP). Mb and H-FABP are early and sensitive markers of myocardial damage in humans, entering the blood stream within the first hours after injury. However, both indicators alone are not sufficiently specific as myocardial injury markers. They reach peak blood levels after 3 to 5 h and usually return to normal by the end of 24 h. Moreover, they are also present in other tissues. Thus, it appears more appropriate to measure Mb and H-FABP as part of a multimarker panel [32, 33].

An entire range of markers (enzymes) are used to detect cardiomyocyte damage in humans and animals: aspartic transaminase (AST), lactate dehydrogenase (LDH), and creatine kinase (MB isoform, CK–MB). However, these enzymes are localized not only in myocardial cells, but also in other body tissues. For instance, AST is found in all body cells, mainly in the heart and liver and to a lesser extent in the kidneys and muscles [34]. LDH is also found in virtually all body cells and is most active in skeletal muscle, cardiac muscle, kidneys, liver, and red blood cells [35]. There are five different isoforms of this enzyme, differing in molecular structure and location. Isoforms 1 and 2 are the basic ones for the myocardium;

however, they are also present in red blood cells and the renal cortex. The MB isoform of creatine kinase is found almost entirely in the cardiac muscle; however, this isoenzyme is also typical for skeletal muscle [36].

The lack of tissue specificity is of high importance for the use of these enzymes as biomarkers in animals, since stress and restricted physical activity during manipulation can cause mild to moderate release of muscle components. Moreover, cardiotoxic drugs are often myotoxic as well; this complicates the identification of target organs. However, despite the limitations, these enzymes can be used as auxiliary markers in preclinical testing.

Heart function change markers. Atrial and brain natriuretic peptides (ANP and BNP) may be used as potential biomarkers for identifying cardiotoxic compounds. Their precursors, proANP and proBNP, respectively, are synthesized in cardiomyocytes of the atria and ventricles and are released in response to their distension caused by increased pressure or neurohormonal stimulation. During secretion, proANP and proBNP molecules are split into active ANP and BNP and N-terminal fragments (NT-proANP and NT-proBNP, respectively). Their secretion amounts are closely correlated, while the half-life of NT-proANP and NT-proBNP (~2.5 and 15.5 min, respectively) is longer than that of ANP and BNP (~0.5 and 6 min, respectively) [37].

NT-proBNP is the biomarker most commonly used in preclinical toxicology studies. NT-proBNP is a sensitive and specific biomarker for diagnosing heart failure in humans and animals. BNP is also elevated in case of dilated and hypertrophic cardiomyopathy, diastolic dysfunction, and systemic hypertension. The potential value of serum natriuretic peptides as biomarkers for differentiating cardiac hypertrophy mechanisms in preclinical drug safety assessments has been demonstrated in rats [37]. Increased NT-proANP and NT-proBNP were observed with drug-induced cardiac hypertrophy, but not with exercise-induced hypertrophy in rats.

Notably, natriuretic peptide levels also increase significantly in a number of non-cardiac conditions associated with increased circulating blood volume and blood pressure. If the fluid is retained in the body, the left ventricle works harder and its walls stretch, resulting in significantly increased BNP and NT-proBNP blood concentrations. Such changes most often occur in heart failure, but can also be observed in pulmonary embolism, liver cirrhosis, and kidney diseases [38].

Analyzed references showed that these markers were detected within 5 to 24 h after simulation of injury in most laboratory animals. When simulating cardiovascular disease in laboratory animals, a combined detection of both natriuretic peptides and specific markers of structural heart damage is recommended for general characterization of both structural and functional effects on the heart *(Table 1)*.

A procedure for detecting cardiac markers in the blood of laboratory animals in response to acute damage, with approximate time ranges of marker increase and decrease *(Figure 1)*, involved the analysis of a large number of references and provided average values [20, 24–44]. This procedure was based on data obtained from studies using rats, dogs, and primates. For other species, it can serve as a reference.

Troponins have the longest diagnostic window and can be detected in laboratory animals within the first 24 h or later. Natriuretic peptides are also found in the blood for several days. H-FABP, CK–MB, and Mb are early markers of cardiac pathology in both humans and many laboratory animals, so their peak activity occurs within the first 1 to 6 h of exposure to a cardiotoxic substance. AST and LDH concentrations in many laboratory animals peak within 5 to 12 h. Generally, the rate of occurrence and increased cardiac marker blood concentrations are significantly influenced by the specific study design, the selected pathology modeling method (for pharmacodynamic studies), and the drug class, dosage, and administration frequency (for drug-induced damage). The best way is to collect blood samples at specific time intervals, such as 2, 4, 6, 8, 12, 16, 24 h, etc. This will enable the dynamic assessment of changes in the concentration of the test marker without ignoring its increase or decrease. With repeated administration

Table 1. Biomarkers used to detect and identify cardiac damage in preclinical studies (adapted from [20, 24–38])

Parameter	cTn	ANP	BNP	H-FABP	CK–MB	Myoglobin	AST	LDH1/LDH2
Tissue specificity	Absolute	Absolute	Absolute	*Moderate*	Moderate	Low	Low	Moderate
Presence in circulating blood under normal conditions	No	No	No	*Yes*	Yes	Yes	Yes	Yes
Stability in blood	High	High	High	*Low*	Moderate	Low	High	High
Frequency of use in preclinical studies	High	High or moderate	High or moderate	*Low*	Moderate	Low	High	Moderate
Preferred test systems for preclinical studies	Rats, dogs, primates	Rats, rabbits, cats, dogs, minipigs, primates	Rats, cats, dogs, minipigs, primates	*Mice, rats*	Mice, rats, cats, dogs, minipigs, primates	Mice, rats	Mice, rats, rabbits, ferrets, cats, dogs, minipigs, primates	Mice, rats, rabbits, ferrets, cats, dogs, minipigs, primates

The table was prepared by the authors

Note. cTn, cardiac troponin; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; H-FABP, heart-type fatty-acid-binding protein; CK-MB, creatine kinase–myocardial band; AST, aspartate aminotransferase; LDH1/LDH2, lactate dehydrogenase isoenzymes 1 and 2.

of cardiotoxic substances, the cardiac marker assessment period should be extended.

Vascular damage markers. Assessing potential vascular damage is an important part of safety studies when developing a drug. The blood and lymphatic system facilitates metabolic processes, such as transport of various substances, thus the vessels are at a high risk when exposed to drugs. Identifying drug-induced vascular injury (DIVI) is an important but very complex task, since specific circulating biomarkers of early vascular damage in humans have not been identified as yet [45].

The Predictive Safety Testing Consortium (PSTC) created a Vascular Injury Working Group (VIWG) to develop and qualify translational DIVI biomarkers [46] that has identified some of the most promising biomarkers.

DIVI is typically characterized by damaged endothelium and vascular smooth muscle cells, as well as inflammation. Therefore, vascular toxicity can be detected by 1) assessing specific biomarkers associated with the release of endothelial cell adhesion molecules and/or their activation markers (angiopoietin-2, endothelin-1, E-selectin, thrombospondin-1, and VEGF-α), 2) by determining non-specific acute inflammation proteins (Timp-1, lipocalin-2, KC/GRO (Cxcl1), α-1 acid glycoprotein 1, and total nitric oxide) [38].

A complete blood count (CBC) and serum electrolyte assessment (potassium, sodium, and chloride) is also recommended. CBC allows diagnosing most diseases associated with the cardiovascular system, including myocardial infarction, stroke, inflammatory processes, and many other pathologies. Electrolyte balance is an important indicator of overall health, including kidney and heart function [38].

Morphological Methods

Morphological test methods are applicable if the drug can mediate degeneration and ne-

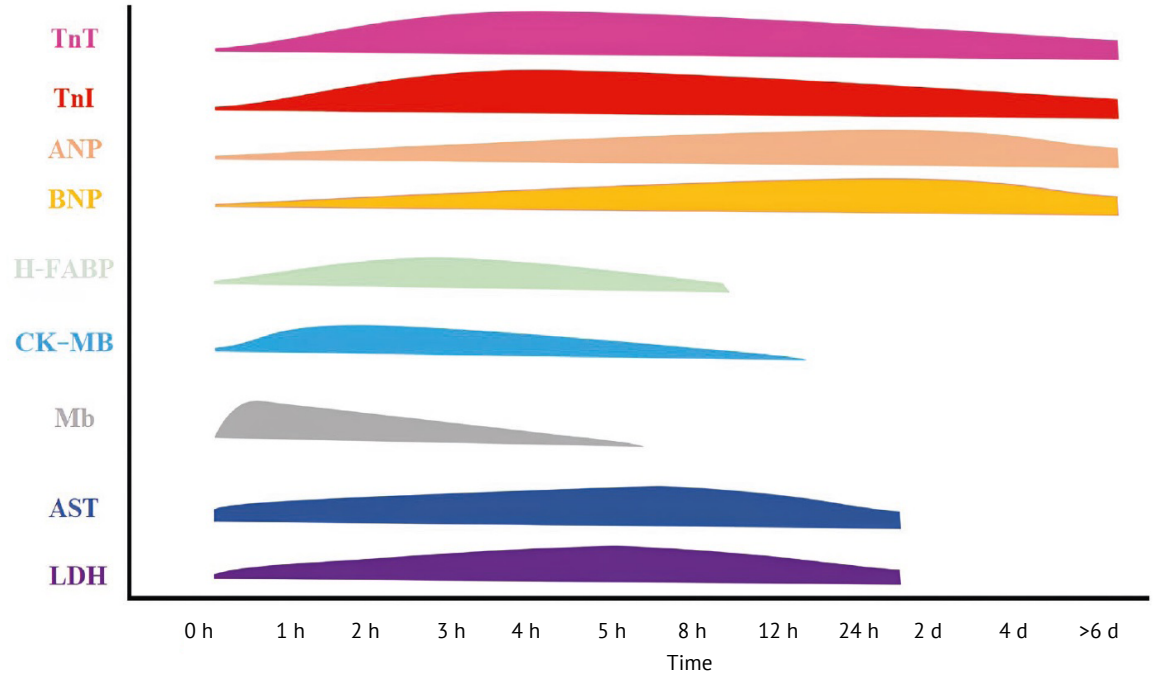

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Figure 1. Summary of changes in the levels of cardiac markers in the blood of laboratory animals in response to acute injury [20, 24–44]. TnT, troponin T; TnI, troponin I; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; H-FABP, heart-type fatty-acid-binding protein; CK–MB, creatine kinase – myocardial band; Mb, myoglobin; AST, aspartate aminotransferase; LDH, lactate dehydrogenase

crosis of cardiac myocytes. If the cardiotoxic compound affects only conduction and contractility, no major morphological changes in the myocardium are typically detected [47].

The most common approaches for assessing cardiac toxicity is to record the heart weight, assess morphology with a standard histological examination (hematoxylin and eosin staining) or in combination with histochemical methods, as well as immunohistochemistry [47].

The administration of certain drugs can change heart weight due to hypertrophy or atrophy of cardiac myocytes [47, 48]. Changing heart weight is sometimes the only indicator of hypertrophy or atrophy; however, these data may be hard to interpret due to biological variability in the test parameter and the lack of precise species-specific reference intervals for organ weight coefficients, which necessitates the establishment of intermediate reference intervals for organ weights and their coefficients [49–54].

A standard histological examination can identify and classify morphological changes caused by the drug, as well as provide data on the correlation between these changes and the dose / administration time [48, 55].

In order to best assess cardiac changes in toxicological studies, sampling should be standardized [56, 57]. For example, in rats and mice, we recommended to dissect the heart longitudinally, penetrating both ventricles, to ensure that all necessary structures are included in the section [58].

The pivotal morphofunctional structures in the morphological study of a drug cardiovascular effect are the papillary muscles, subendocardial region, valves, and coronary arteries [56]. For large animals, it is recommended to assess both atrial and ventricular walls, the interventricular septum, and the atrioventricular and aortic valves. In addition, the sinoatrial and atrioventricular nodes can be included to assess heart conduction system [56, 57].

Morphological criteria for myocardial changes include cardiac myocyte degenera-

tion/necrosis, cardiac myocyte vacuolization, hemorrhages, deposition of pigments and proteins, focal mineralization, fibrosis, and inflammation sites (*Figure 2 to Figure 6*, see Journal's website[9]) [47, 48, 55, 56, 59–62]. When assessing morphological changes, it is extremely important to distinguish them from spontaneous and background changes characteristic of animals [55, 56].

An important step is not to simply record microscopic findings, but to note the severity of the lesions and their distribution (focal, multifocal, diffuse); location (right ventricle, left ventricle, interventricular septum, etc.), as well as any additional pathology modifiers (e.g. inflammation type) [63]. Furthermore, the lesion severity can be assessed by measuring the degeneration/necrosis/fibrosis site. To assess the lesion size, 6 to 8 tissue specimens of an animal are deemed optimal [64].

Triphenyltetrazolium chloride (TTC) is a widely used and readily available indicator for visualizing ischemic damage and assessing heart damage in animals, including infarction. TTC-based analysis in cardiac toxicity studies offers advantages over standard histology thanks to identification of structurally intact but functionally compromised cardiac myocytes [65]. Colorless TTC is enzymatically reduced in living tissues by various dehydrogenases to the red-colored compound 1,3,5-triphenylformazan; as a result, intact myocardial tissue is stained dark red, while necrotic areas of the myocardium where TTC remains unchanged appear paler (*Figure 7* on the Journal's website[10]).

Immunohistochemistry can be used to identify markers that are found in the tissue of interest and lost during cell destruction (such as troponins cTnI and cTnT). Membrane CD markers reflecting the course of the inflammatory process are often used, as well as markers that accumulate in damaged tissue, such as complement and fibrin [66].

Vascular damage (*Figure 8* on the Journal's website[11]) is also a frequent finding in drug safety assessments [66]. Detected vascular changes are often hard to interpret due to a spontaneous vascular damage in laboratory animals (polyarteritis nodosa, spontaneous polyarteritis in beagles, etc.) [66, 67].

Despite the various DIVI mechanisms, the cellular response is generally similar in all cases. Regardless of the sequence of events and their pathophysiology, constant findings for all major DIVI types include activation and damage of endothelial cells; damage to the muscular wall; and inflammation characterized by vascular leakage and inflammatory cell infiltrates [66]. Thus, a standard histology can be successfully used to determine the vascular damage, but it cannot help deduce its mechanisms.

To successfully identify DIVI, VIWG has developed organ sampling guidelines. A standard organ sampling for routine evaluation includes the aorta, heart, administration site, kidneys, liver, skeletal muscle, mesentery, and testes. It is recommended to sample the mesentery by swabbing; the aorta and skeletal muscle must be sampled longitudinally and cross-sectionally, whereas for the heart, multiple sections are recommended to increase the probability of detecting coronary artery lesions [66]. This list can be expanded when needed.

Microscopic findings typical for DIVI include medial necrosis, inflammatory infiltration of the tunica media and adventitia, and hemorrhages [59].

DIVI specific immunohistochemical markers have not yet been identified. However, some markers are used to assess vascular damage and, when combined with other data, can help diagnose DIVI. These include CD31, a transmembrane glycoprotein that is a marker of endothelial cells involved in the migration of leukocytes to the inflammation site, and Von Willebrand factor that shows significant extracellular immunopositive staining as vascular damage progresses and indicates the destruction of endothelial cells [68].

[9] https://doi.org/10.30895/2312-7821-2025-475-fig
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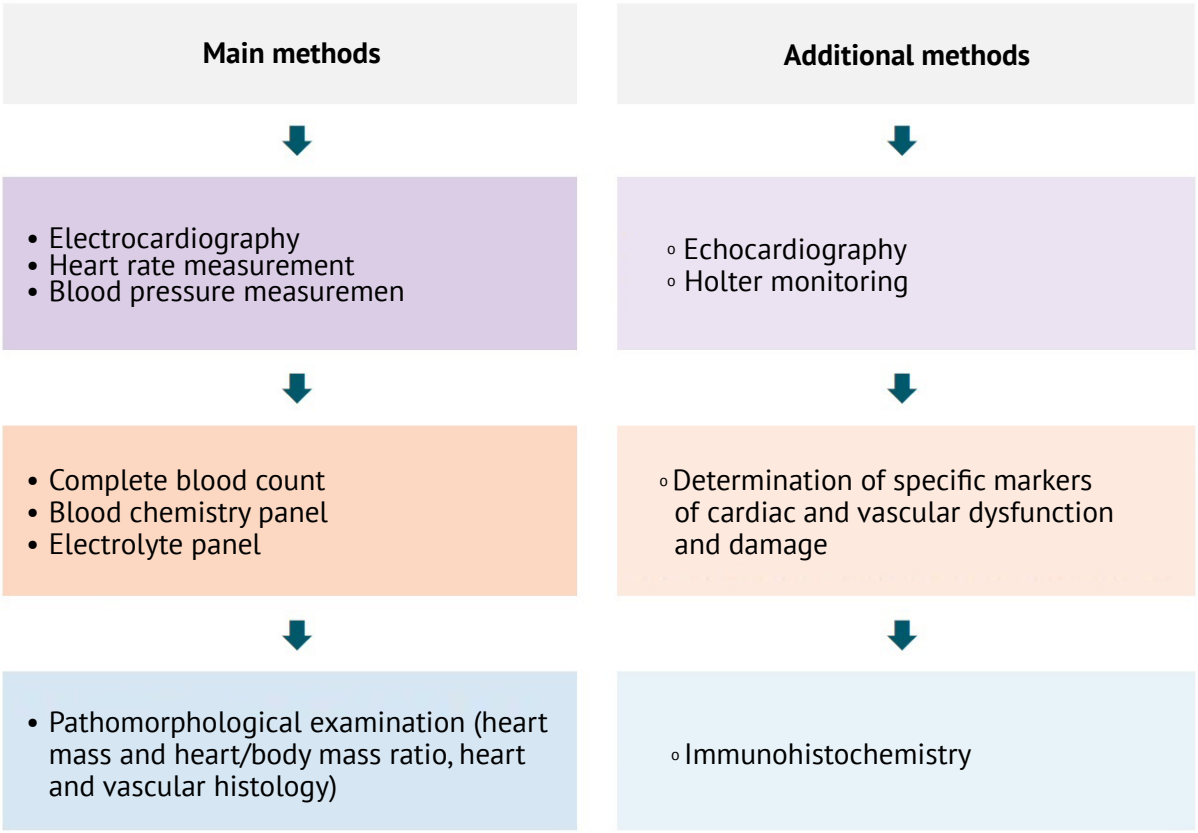
[11] https://doi.org/10.30895/2312-7821-2025-475-fig



The figure was prepared by the authors

Figure 9. Schematic representation of comprehensive cardiovascular function assessment in laboratory animals

Caveolin-1 (CAV-1) and smooth muscle antibodies (SMAs) are immunohistochemical markers of vascular smooth muscle cell damage, since they are present in normal cells but not in damaged cells [68, 69]. ZO-1, claudin, and connexin 43 are often used as disruption markers of intercellular contacts in vessel wall cells. Immunoreactivity of these proteins decreases or disappears at vascular damage sites [69]. In addition, immunohistochemistry can detect deposits of substances in the vessel wall that accumulate in case of a damaged vessel wall or surrounding tissues, such as complement C3, fibrin/fibrinogen, and immune complexes [69–71].

All the above immunohistochemical markers can confirm vascular damage and identify morphological traits. However, they are not exactly DIVI-specific and require a comprehensive assessment. Notably, this method only allows for a semi-quantitative assessment of immunohistochemical staining intensity, thus limiting its use.

Assessing the functional state of the cardiovascular system in preclinical studies is a crucial component of the pharmacology safety evaluation. Due to its structural and functional complexity, as well as significance of the system in question, it is impossible to develop a comprehensive and versatile assessment plan. However, it is possible to identify key (critical) significant patterns (blocks) that, in our opinion, are noteworthy. *Figure 9* shows the general list of methods for assessing cardiovascular functions at a preclinical stage.

CONCLUSIONS

Assessing the drug effects on the cardiovascular system is a key aspect of preclinical trials that requires a comprehensive and detailed approach. When assessing cardiotoxicity, instrumental methods are recommended

at the beginning; the most informative are electrocardiography, heart rate and blood pressure measurements. These are included in the minimum list of mandatory tests for assessing pharmacological safety (ICH S7A, ICH S7B). Diagnostic laboratory tests are recommended in addition to instrumental methods. One specific marker, such as troponin I or T, would be the best solution, as well as several additional markers of choice, such as LDH, AST, or CK–MB. A complete blood count is also recommended to assess the electrolyte profile. At the same time, the period when the concentrations of the test parameters increase should be taken into account.

For a more in-depth analysis, histological and immunohistochemical analysis of the cardiovascular system is recommended to assess changes at the tissue and cellular levels.

Thus, the suggested approach can help assess manifestations of the potential drug toxicity at the biochemical, structural, or physiological level, making it possible to completely and correctly assess the potential cardiac and vascular toxicity of new drugs, while increasing the translational potential of data obtained in preclinical trials.

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Additional information. Figures 2–8 are posted on the website of *Safety and Risk of Pharmacotherapy*. https://doi.org/10.30895/2312-7821-2025-475-fig

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