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ΦΟΙΤΗΤΗΣ: ΑΥΓΕΡΟΣ ΝΙΚΟΛΑΟΣ
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POSTGRADUATE MASTER STUDIES IN : «TOXICOLOGY»

DIPLOMA THESIS: «*IN DEPTH ANALYSIS OF HISTOPATHOLOGICAL CHANGES OBSERVED IN RATS AFTER INDUCED CARDIOTOXICITY BY ANTHRACYCLINES ADMINISTRATION, AIMING TO IDENTIFY THRESHOLD VALUES*»

STUDENT: AVGEROS NIKOLAOS

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ΤΡΙΜΕΛΗΣ ΣΥΜΒΟΥΛΕΥΤΙΚΗ ΕΠΙΤΡΟΠΗ

- Κουρέτας Δημήτριος, Καθηγητής Φυσιολογίας Ζωικών Οργανισμών - Τοξικολογία
- Γεωργιάδης Νικόλαος, MSc, PhD, ERT
- Τσιτσιμπίκου Χριστίνα, MSc, PhD, ERT

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ABSTRACT

The identification of histopathological alterations in the heart resulting from prolonged exposure to various chemicals, such as insecticides, anthracyclines, and others, is deemed necessary due to the continuously increasing incidence of heart failure, decreased ejection fraction from the left ventricle, and elevated rates of fibrosis and inflammation in cardiac tissue.

This study is oriented towards conducting an extensive analysis of data derived from experimental studies that describe findings in laboratory rats exposed to anthracyclines. To this end, a thorough literature review was conducted from March 1, 2020, to October 13, 2023, using the English language in databases such as PubMed. A total of 384 studies were found, of which 130 were deemed suitable for data extraction, as they included the appropriate species, exclusive use of anthracyclines, and the appropriate experimental format, specifically in vivo studies. The results were analyzed in depth, recorded in detail, and revealed numerous categories of pathological alterations both macroscopically, in the heart tissue, and microscopically, at the cellular level.

The abundance of these data underscores the need for the introduction of a new hazard categorization regarding the characterization of chemical substances, which presents a challenge for the scientific community.

INTRODUCTION

Chemotherapy-induced cardiotoxicity is a significant concern for oncologists treating various types of cancer due to its impact on treatment options and patient survival rates. Different guidelines in the United States and Europe have distinct cutoff values for identifying cardiotoxicity caused by chemotherapeutic agents. In Europe, a left ventricular ejection fraction (LVEF) lower than 50% is considered abnormal, while in the USA, the threshold is 53%. Both guidelines stress the importance of a decline in LVEF compared to a patient's baseline values, prompting treatment with ACE inhibitors, ARBs, and beta-blockers. However, the adjustment of anticancer treatments for patients with declining cardiac function remains debated among specialists.(1)

In animal studies, where new anticancer drugs are evaluated and potential antidotes for cardiotoxicity are tested, accurately assessing the extent of cardiotoxicity is essential. Anthracyclines, a category of chemotherapy drugs derived from the Streptomyces bacterium, are widely used to treat various cancers, including leukemias, lymphomas, breast, stomach, uterine, ovarian, bladder, and lung cancers. Anthracyclines such as doxorubicin, daunorubicin, epirubicin, and idarubicin are known for their well-established cardiotoxic effects, leading to myocardial suppression in many patients. The first anthracycline discovered was daunorubicin, with a trade name “Daunomycin”. The most important is doxorubicin which is administered in considerable number of clinical cases of cancer. Their mechanism of action is to provoke cytotoxicity, especially in the cancerous, rapidly duplicating cells. Anthracyclines produce free radicals which can build up in cells and cause damage to other molecules, such as DNA, lipids, and proteins, leading to their malfunction. Furthermore, anthracyclines have the ability to inhibit topoisomerase I & II, which nuclear enzymes that play essential roles in DNA replication, transcription, chromosome segregation and recombination. All the above concerned can lead to irreversible, dose-dependent adverse effects on specific organs such as the heart of rats and humans which is known as cardiotoxicity.(2)

In animal models, anthracyclines are employed as a cost-effective method to induce dilated cardiomyopathy, unlike interventional research models involving infarction and myocardial ischemia. Various animal species and administration schemes have been utilized to study anthracycline-induced cardiotoxicity and test interventions to mitigate myocardial damage. Cardiac imaging is commonly employed and of outstanding importance to evaluate cardiotoxicity in animal studies.(1)

Besides anthracyclines, other pharmaceutical compounds such as anabolic steroids and everyday chemicals like metals and pesticides have been implicated in adverse cardiac pathology, leading to impaired cardiac function. Despite comprehensive regulatory networks in Europe taking under control the risks posed by chemical substances, cardiotoxicity lacks a autonomous hazard classification, and specific criteria for classifying substances as cardiotoxic are unavailable. This regulatory gap may lead to long-term cardiovascular complications burdening national health systems.(2)

There is a critical need to address the classification of cardiotoxicity in chemical substances. Currently, no animal testing methods for cardiotoxicity are available in the OECD Testing Guidelines, and cardiovascular measurements are not part of current evaluation programs for environmental chemicals. To address this, a weight-of-evidence approach incorporating anatomical, histopathological, echocardiographic, biochemical, and mechanistic data is necessary to reduce uncertainties in classifying cardiotoxicity.(2)

Scientific evidence, including anatomical, histopathological, echocardiographic, biochemical, and mechanistic data, should be utilized to classify cardiotoxicity accurately.

Anatomical and histopathological data, along with echocardiographic assessments of contractility, documentation of cardiac frequency, and biochemical markers, should be considered. Additionally, the identification of pathways and mode of actions, along with in silico data, can aid in understanding and classifying cardiotoxicity.(1)

Addressing the regulatory gap in describing cardiotoxicity as a separate hazard class is essential. A methodological approach to collect animal evidence should be developed to identify cardiotoxic chemicals and endorse legislative measures to protect human health from exposure. Targeted research is necessary to further investigate specific indices and biochemical markers for setting regulatory criteria and classifying cardiotoxicity induced by chemicals.(1)

In conclusion, efforts to classify cardiotoxicity in chemical substances are crucial for protecting human health from potential cardiovascular risks associated with chemical exposure. Addressing this gap in regulation and research methodologies will contribute to more effective management and mitigation of cardiotoxicity-induced complications in clinical practice and regulatory frameworks.(1)

THEORETICAL BACKROUND

Heart Anatomy

As far as the heart concerned, it is the most debated organ after the human brain. Its anatomy is complicated along with its functionality. It is located at the center of the human body, within the chest, positioned directly above the diaphragm in an area of the thorax known as the mediastinum, specifically the middle mediastinum. Its placement can vary depending on an individual's height and weight. The apex of the heart points forward, downward, and towards the left side of the body. Its inferior surface rests directly on the diaphragm. (3)

The fibrous pericardium envelops the heart and attaches to the great vessels. The serous pericardium is a closed sac comprising two layers—the visceral layer, also known as the epicardium, which forms the outer lining of the great vessels and the heart, and the parietal layer, which lines the inner surface of the fibrous pericardium. (3)

The heart is compartmentalized into four distinct chambers, each characterized by muscular walls of varying thickness. Among these chambers, the left atrium (LA) and right atrium (RA) stand as small, thin-walled compartments positioned just above the left ventricle (LV) and right ventricle (RV), respectively.(3)

Cardiac muscle represents an involuntary striated muscle type, characterized by mononucleated cells and exhibiting cross-striations formed by alternating segments of thick and thin protein filaments. These filaments are anchored by structures known as Z-lines. Compared to skeletal muscle, cardiac muscle is relatively shorter in length. Actin and myosin serve as the primary structural proteins within cardiac muscle fibers.(3)

The conduction system of the heart comprises specialized cells primarily responsible for transmitting impulses to various regions of myocardium. This system consists of three different cell types, each one of them possessing distinct morphological and functional characteristics: P-cells (Pale/Pacemaker-cells), transitional cells and Purkinje cells. Together, these cells play crucial role in ensuring the orderly regulation of the heart’s electrical activity.(3)

Heart Functionality

The cardiac myocyte stands as the most energetically active cell in the body, exhibiting a remarkable capacity for sustained contraction without fatigue. Throughout an average lifespan, it contracts over 3 billion times or more. Through its synchronized beating with approximately 3 billion neighboring cells within the human heart's primary pump, an astounding volume of over 7,000 liters of blood is propelled daily along a network of blood vessels stretching approximately 100,000 miles, all accomplished seamlessly without conscious effort.(5)

There is consensus regarding the spiral arrangement of left ventricular (LV) muscle fibers, which results in a twisting or wringing motion during LV systole. This motion involves the base of the LV rotating clockwise while the apex rotates counterclockwise, a phenomenon termed torsion, which is expressed as the rotational angle along the longitudinal axis of the LV. Despite this understanding, the specific muscular mechanisms responsible for this phenomenon have been inferred rather than conclusively determined. These mechanisms hold significant functional implications, although they have yet to be fully elucidated.(6)

Twisting plays a minor role in contributing to stroke volume, which contrasts with the prevailing understanding of its significance. Current functional knowledge suggests that septal twisting primarily accounts for 80% of right ventricular output. Furthermore, the loss

of left ventricular twisting stands out as the earliest discernible indication of heart failure, underscoring the critical role of twisting dynamics in cardiac function and pathology.(6)

Within the cardiac cell, bundles of protein strands known as myofibrils are housed. These myofibrils are encased by the sarcoplasmic reticulum, a specialized structure containing sac-like compartments called cisternae.(3)

Heart's Physiology

The main purposes of the cardiovascular system is to supply various nutrients, to distribute oxygen and to regulate internal balance of the body. It can be divided into two different circulation systems: 1) the pulmonary circulation, which is short and leads to the lungs and back and 2) the systemic circulation, in which the blood is circulated from the heart throughout the body.(7)

Cardiac pacemaker cells refer to SA node, AV node and bundle His/Purkinje fibers. These specific parts of the heart are essential for the heart rate to take place. AP is crucial for ensuring appropriate rate management. SA node assumes a pivotal role as the first pacemaker, since it retains pulse from 60 to 100 BPM whereas AV node ensures the pulse fluctuation between 40 and 60 BPM. This happens on the grounds of malfunction of the first pacemaker due to atrial fibrillation etc.(7)

To comprehensively elucidate the cardiac physiology, it is imperative to establish foundational terminologies. Diastole denotes the cardiac cycle phase characterized by ventricular relaxation and blood filling. Conversely, systole delineates the phase involving ventricular contraction and blood ejection. Preload signifies the maximum blood volume within the heart at diastolic conclusion, equivalent to end-diastolic volume (EDV). End Systolic Volume (ESV) denotes the residual blood volume in the ventricles post-systole.(7)

Heart's Pathophysiology

The most important organ throughout the body that regulates internal balance is the heart. Nevertheless, particular circumstances such as exposure to chemicals, anabolic drugs, anthracyclines etc may lead to heart dysfunction.

Heart failure syndrome(HFS) is defined as a systemic disease and equals to the incapability of the heart to circulate adequate oxygenated blood through organs, peripheral tissues and the body organs' metabolic needs both at resting condition and exercise. There are several categories of myocardial dysfunction such as: 1) systolic and/or diastolic, 2) acute or chronic, 3)compensated or uncompensated and 4)uni- or biventricular. As soon as heart failure occurs, neurohormonal reflexes are activated to restore the imbalance, case in point sympathetic adrenergic system, renin-angiotensin cascade and renal and peripheral alterations. (8)

Left heart failure(LHF), caused by left ventricular dysfunction, is a medical condition of great importance for physicians. It is defined by cardiomyocyte's disrupted structure and function leading to left ventricular pump dysfunction and the patient's clinical state is characterized by dyspnea, fatigue, exercise intolerance, including volume overload such as pulmonary crackles and peripheral edema. Despite the fact that the organism makes a concerted effort to ameliorate the symptoms by utilizing compensatory mechanisms, this could result in further myocardial deterioration and exacerbate the situation.(8)

In systolic LHF, the left ventricle fails to maintain a sufficient stroke volume on the grounds that systolic contractile function is diminished, which leads to hyperfunction of the right side of the heart. On the other hand, in diastolic LHF, the left ventricle fails to get filled sufficiently since the occurrence of incompliance of left ventricle, impaired relaxation and worsened end-diastolic pressure.(8)

As so long left ventricular ejection fraction inadequacy takes place, sympathetic nervous

system is activated and cardiac contractile frequency and strength are aroused. At first, the heart rate frequency is increased in order to boost total stroke volume per minute, without surpassing 140-150 beats per minute. Subsequently, neurohormonal system amplifies the intravascular volume, ventricular champers become augmented and cardiomyofibers' tension is enhanced. Unfortunately, no physiologic mechanism is capable of restoring the cardiac decompensation resulting in gradual modifications in left ventricle from reversible to irreversible cardiomyocyte remodeling. The heart regenerates its myocytes when it undergoes pathological stress. This remodeling is enhanced by the increasing loss of myocytes during heart failure leading to the progression of ventricular renovation and at last the dysfunction of the whole body.(Table .1)(8)

1) Activation of neurohormonal systems
2) Increasing preload to help to sustain cardiac performance
3) Myocardial cell regeneration and apoptosis
4) Myocardial hypertrophy and/or ventricular dilatation
5) Compensation of symptoms
6) Irreversible myocardial remodeling
7) Decompensation of clinic status
8) End-stage multi-organ dysfunction

Table. 1: Concise overview of the progression of stages occurring in cardiac failure following oxidative stress in the left ventricle of the heart, leading to irreversible damage.[8]

As far as the gradual modifications taking place during left ventricular failure concerned, could generate intricate alterations of cardiac myocytes, let alone non myocyte components. In the event that an individual promptly receives appropriate treatment to address the symptoms, the heart's anatomy returns to its normal state. If not, the reversible changes become irreversible, resulting into progressive loss of myofilaments, loss of contractile function, alterations in excitation-contraction coupling, fatal arrhythmias and desensitization of β-adrenergic signaling. This contributes the development of left ventricual hypertrophy with augmented left ventricular wall stiffness, with or without left ventricular thickening. A gradual deterioration in the connectivity of the collagen network leads to progressive dilation of the left ventricle, while maintaining the structural integrity of the heart. When the left ventricle takes the form of a sphere, it creates gradual wall stress and mechanical energy, which causes left ventricular dilation and the walls of it becomes thin. The dilation leads to of great importance mitral regurgitation due to the failure of the valve leaflets to properly meet. Last but not least, myocardial fibrosis is a serious histopathology situation in which severe arrhythmias and sudden death occurs as a result.(8)

Vascular congestion

Heart failure is a serious condition where the heart does not pump blood as efficiently as required. While this comes about, it lacks its ability to contract properly. Consequently, the heart fails to meet body's demands, causing blood to accumulate and back up, leading to vascular congestion, not only in the heart, but also the whole system. This inefficiency in pumping prevents sufficient oxygen-rich blood from diffusing oxygen throughout the body organs.(9)(10)

When considering the body's total circulating volume, the arterial system accounts for only about 30% of the blood volume. In states of congestion, the venous system offers a significant capacitance bed to accommodate the excess volume. Disorders of autonomic function, in which adrenergic modulation of the splanchnic circulation mobilizes blood

compartments in response to different postures, form the basis for tilt-table testing and blood pooling evaluations.(10)

In transitioning from the chapter on the anatomy of the heart and its fundamental pathological elements to the subsequent discussion on histopathological findings at the tissue and cellular levels, it is essential to bridge these topics by emphasizing the connection between structural anatomy and microscopic pathology. Understanding the heart's anatomical framework and the primary pathological conditions lays the groundwork for a deeper exploration of how these conditions manifest at a cellular level. The upcoming chapter will delve into histopathological evidence, illustrating how anatomical abnormalities correspond to specific cellular and tissue changes, thereby providing a comprehensive view of cardiac pathophysiology. This integrated approach is crucial for a holistic understanding of cardiac health and disease.

Cardiomyocyte regeneration

Cardiomyocytes have the ability to produce force in order to bring about blood circulation. When this force comes to be inadequate or the energy supply is insufficient, then coping mechanisms are activated in the heart resulting in rearrangement of myocardial structure in which condition heart failure takes place. Particular genome sections in the expression pattern of “mature” and “immature” are stimulated in order to provoke cardiomyocyte dedifferentiation and therefore to sustain altered metabolic and/or hypoxic stress by breaking down the energy-intensive contractile machinery and reducing the cell's energy consumption. The most important parts of cardiomyocytes’ dedifferentiation: 1) contractile apparatus restruction, 2) cytoskeleton’s rearrangement and 3) energy metabolism alterations.(11)

Oxygen deficiency provoke coping mechanisms in cardiac cells that alters the structure of myocardium and involves responses of inflammatory infiltration. Not only the ischemic parts of the heart’s ventricles are affected, but also non ischemic. Small fractures result in cardiomyocytes’ hypertrophy to normalize wall stress, while large infarcts cause reformation of the entire heart. Therefore champer size, shape and function are modified, so heart’s function can partially be restored. At last, one underlying mechanism is myocardial cell death and cardiomyocyte slippage. In this case, cardiomyocytes tend to lose the contact in-between and they subsequently get dynamically disorganized. As the cardiomyocytes are disarranged and the heart function is diminished, heart failure develops into dilative cardiomyopathy. Causality behind this condition might be generic predisposition or environmental factors.(11)

Inflammatory cell infiltration & Fibrosis

Both myocardial ischemia, and myocardial infarction (MI) are two of the most frequent causes of cardiac injury which results in the reinforcement and activation of immune responses and cell death procedure. Inflammatory cell infiltration is a dynamic response which includes the infiltration of cardiomyocytes with neutrophils, monocytes, macrophages, dendritic cells and lymphocytes in order to heal the infarcted area and clear it from the dead cells. Toll-like receptor(TLR)-mediated pathways are activated and both chemokines and cytokines are upregulated in the infarcted part. TLRs are found on endothelial cells and on cardiac myocytes which recognize endogenous danger signals. Chemokines promote the chemotactic recruitment of inflammatory cells into the infarcted area. CC chemokine ligand 2 (CCL2) attracts monocytes, macrophages, T cells and NK cells. The receptor of it CCR2 is expressed by monocytes and macrophages. (12-13)

The first step of the immune response is neutrophils infiltration when the infraction

is initiated. Afterwards, monocytes and macrophages begin to infiltrate and release inflammatory mediators, reactive oxygen species and proteolytic enzymes. Settlement of cellular processes such as phagocytosis, proteolysis, angiogenesis, infarct healing and reformation of the left ventricle begin.(12) On the grounds that this process does not remain under wraps, matrix degradation and cardiac cell's apoptosis will occur, which will lead to dilative reformation, progressive dysfunction of the heart and scar formation.(13)

Fibrosis is a response of reparative mechanisms of the cells and it is a source of organ dysfunction. It is mostly caused under conditions of stress or mechanical stress due to chemosensitive and mechanosensitive receptors. Furthermore it is driven by the unexpected and abrupt death of numerous cells, which simultaneously signals the activation of myofibroblasts. Fibrogenic signaling cascade is activated through three main pathways: 1)fibrogenic growth factors (transforming growth factor-β, platelet-derived growth factors), 2) cytokines (Tumor Necrosis Factor[TNF]-α, interleukin[IL]-1, -6, -10, -4) and 3)neurohormones, having as a result matricellular macromolecules deposition in the reformatted myocardium.(14)

In order to clarify the fibrotic lesion, there are three types. Firstly, interstitial fibrosis delineates the enlargement of endomysial and perimysial space due to the fact that extracellular matrix protein are concentrated on the grounds of massive cardiomyocyte loss. On the other hand, perivascular fibrosis commonly refers to expanded microvascular adventitia. These first two categories demonstrate a prolonged fibrogenic stimuli activity, they are mostly seen in primary processes and are linked to diastolic dysfunction and decline left ventricular compliance. Lastly, replacement fibrosis is a condition in which collagen accumulates and replaces the necrotic cardiac cells leaving a collagen-based scar. This happens because of the adult heart's inability to regenerate its own cells and it is commonly linked to systolic dysfunction.

Anthracyclines

Anthracyclines play a significant role in the cancer treatment process. They are used in several cancer drug schemes such as breast cancer, lymphoma, childhood cancer, stomach cancers, etc. The most common anthracyclines (Figure.1) used are doxorubicin, daunorubicin, epirubicin and idarubicin, with the first two of them be the first in patient treatment. Doxorubicin was the first to be discovered from *Streptomyces peucetius* as an antibiotic. Epirubicin is a stereoisomer of doxorubicin and remains in the system ten times more than doxorubicin. Idarubicin derives from daunorubicin and it has the ability to pass through the cell more efficiently than its precursor. (15)

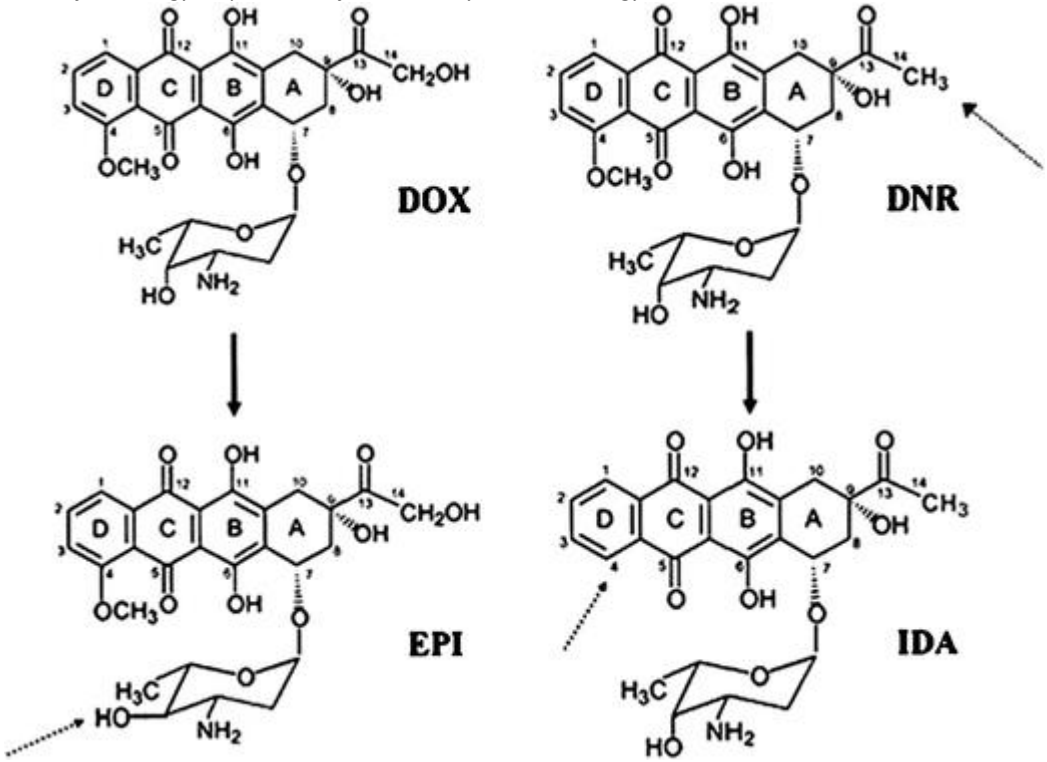


Figure.1: Demonstration of chemical structure of the anthracyclines Doxorubicin (DOX), Daunorubicin (DNR), Epirubicin (EPI) and Idarubicin (IDA), the four anthracyclines mostly used to induce cardiotoxicity in the studies reviewed in the present report

Anthracyclines tend to produce reactive oxygen species (ROS) and interfere with DNA, more specifically with topoisomerase 2, causing damage to the genome. Doxorubicin interfere between DNA and topoisomerase 2 when DNA starts to replicate resulting in double-stranded DNA breaks. Simultaneously, the Top2-doxorubicin-DNA complex provokes a cascade of events especially in cardiomyocytes which results in impaired calcium handling, mitochondrial dysfunction, activation of altered p53 tumor suppressor pathway and augmented apoptosis.(15)

Weight of evidence

The European Chemicals Agency (ECHA) ensures the safe use of chemicals in the EU by managing chemical regulations like REACH and CLP. In hazard classification, ECHA assesses and classifies substances to identify risks to health and the environment.

According to ECHA, the weight of evidence is the process of considering all available data, their quality, severity of the detected effects and relevance to make a balanced and comprehensive hazard assessment decision. This approach integrates various lines of evidence rather than relying on a single source of information. It is of utmost importance that all of evidence are explained through a scientific point of view and are sufficient to support the arguments. All of the information to be taken into account must be constructed in an organized way.(16)

A justification can be more supported with the following ways:

1) Published literature
2) Read-across from chemical analogues
3) QSAR predictions
4) Data from existing studies
5) In vitro studies
6) Epidemiological data/human experience (16)

MATERIALS & METHODS

A thorough research using the electronic database PubMed was carried out, in order to retrieve all original research studies from 1st of March, 2020 until 15th of October, 2023. This study aimed to identify articles containing histopathological data pertaining to rat species and investigate the cardiotoxic effects of anthracyclines and doxorubicin on cardiac tissue. PubMed Advanced Search Builder was used to search throughout All Fields and the utilized filters in separate searches were as follows:

1) “Cardiotoxicity” AND “rats” AND “anthracycline” AND “histopathology”

2) “Cardiotoxicity” AND “rats” AND “anthracycline” AND “lesion”

3) “Cardiotoxicity” AND “rats” AND “anthracycline” AND “staining”

4) “Cardiotoxicity” AND “rats” AND “doxorobucin” AND “histopathology”

5) “Cardiotoxicity” AND “rats” AND “doxorobucin” AND “lesion”

6) “Cardiotoxicity” AND “rats” AND “doxorobucin” AND “staining”

The list of studies obtained was additionally evaluated for the relevance of the subject and the eligibility by screening the titles/abstracts of the full paper. Only the original research studies were used. No other particular filter was used. The cumulative count of articles retrieved from each of the aforementioned individual searches amounted to 384 articles, with 84 articles from a different study being added to the list of studies. From this pool of articles, 130 were deemed suitable for inclusion in this study. 208 articles were excluded due to being duplicates, 11 articles were not available, 20 articles due to inappropriate species tested, 9 articles due to no anthracycline used, 84 articles due to no histopathological data and at last 6 articles due to irrelevant organ tested. (Figure.2)

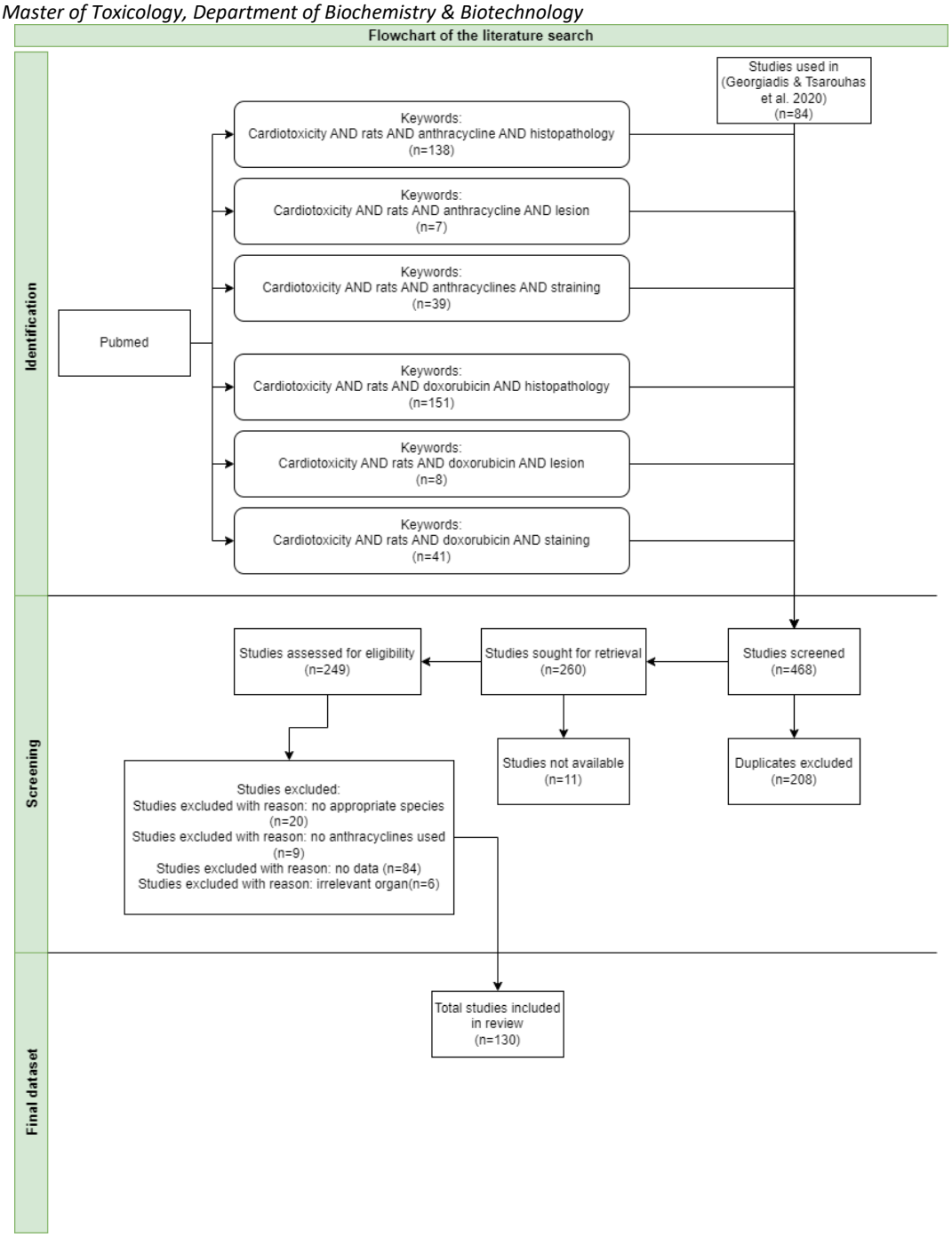


Figure. 2 : Prisma flow chart (literature search) about histopathological data for the present study design.

Each of the accepted articles was subsequently utilized to gather data on the in vivo experiments conducted within each study, facilitating the creation of a comprehensive summary table comprising six columns. The initial column delineates the authors of the respective articles, the second column delineates the number, variety, and species of rats involved, while the third column specifies the type of anthracycline utilized. In the fourth column, the duration over which the anthracycline was administered is detailed, and the fifth column encapsulates the total findings. Finally, the last column delineates supplementary observations gleaned from the studies.

Publication	No. Rats/Sex/Species Used	Anthracycline administered	Anthracycline total dose	Duration	Summary of findings	Secondary findings & Annotiations
Patintingan et al (2023)	24/Sprague-Dawley rats	Doxorubicin	4 mg/kg, ip	Once per week for 4 weeks	**Altered cardiac morphology** (reduced cardiac muscle cell size, excessive inflammatory cell infiltration, myocardial necrosis, bleeding, extensive myocarditis)	
Pereira et al (2023)	39/male/Wistar rats	Doxorubicin	7.5 mg/kg	Three times per week for 2 weeks	**Altered cardiac morphology** (myocardial lesions, Type III collagen deposition, cardiac mast cells increased)	
Samir et al (2023)	60/male/Swiss albino rats	Doxorubicin	15 mg/kg, ip	One week	**Altered cardiac morphology** (perivascular inflammatory cell infiltration with edema surrounding the dilated and congested blood vessels, focal hemorrhages, inflammatory cell infiltration within the myocardial bundles)	
Wang, Y. et al (2023)	32/male/wild-type Sprague-Dawley rats	Doxorubicin	2.5 mg/kg, ip	Three times per week for 3 weeks	**Altered cardiac morphology** (cardiac fibrosis, collagen deposition on cardiomyocytes, swollen mitochondrial cristae and vacuolization of mitochondria)	

Satyam et al (2023)	30/female/Wistar rats	Doxorubicin	20 mg/kg, ip	Single injection	**Altered cardiac morphology** (significant cardiomyocyte degeneration, intermuscular edema, mild inflammatory cell infiltration, myofibrillar loss)	
Wang, T. et al (2023)	Rats	Doxorubicin	cumulative dose: 15 mg/kg	(no data)	**Altered cardiac morphology** (inflammatory cell infiltration, vacuoles, watery lesions in cardiac cells, excessive autophagy in cardiomyocytes)	
Prathumsap et al (2022)	40/male/Wistar rats	Doxorubicin	3 mg/kg/day, ip	On 4th, 8th, 15th, 22nd and 29th day	**Altered cardiac morphology** (cardiomyocyte apoptosis, cardiomyocyte autophagy and mitophagy [grade 3], cardiac inflammation [grade 3], cardiomyocyte pyroptosis [grade 3], massively swollen mitochondria)	
Amin et al (2023)	24/male/Wister rats	Doxorubicin	cumulative dose: 21 mg/kg, ip	Twice per week for 3 weeks	**Altered cardiac characteristics** (elevation in heart weight and function)	
Majhi et at (2022)	40/male/Wister rats	Doxorubicin	2.5 mg/kg, i.p. cumulative dose: 15 mg/kg	Three times per week for 2 weeks	**Altered cardiac morphology** (cardiac necrosis with edema, leukocyte infiltration, intramuscular haemorrhage)	

Furcea et al (2022)	(no data)	Doxorubicin	(no data)	(no data)	**Altered cardiac morphology** (foci of necrosis, dissolution of intracytoplasmic myofibrils, cardiomyocytes with intracytoplasmic vacuolization)	
Naderi et al (2023)	42/male/Wistar rats	Doxorubicin	2.5 mg/kg, ip	Three times per week for 2 weeks	**Altered cardiac morphology** (extensive vacuolization, cardiomyocytes degeneration)	
Alyasiry et al (2022)	28/male/Sprague Dawley rats	Doxorubicin	2.5 mg/kg, ip	Three times per week for 2 weeks	**Altered cardiac morphology** (cellular swelling, perinuclear vacuolation, cytoplasm vacuolization)	
Refaie et at (2022)	50/male/Wistar albino rats	Doxorubicin	15 mg/kg, ip	Single injection	**Altered cardiac morphology** (extensive necrosis, loss of muscular striations with vascular congestion)	

Adıyaman et al (2022)	28/male/Sprague Dawley rats	Doxorubicin	10 mg/kg, ip	Single injection	**Altered cardiac morphology** (severe disarrangment and hypertrophy of cardiomyocytes)	
Shekari et al (2022)	32/male/Wistar rats	Doxorubicin	2.5 mg/kg, ip	Three times per week for 2 weeks	**Altered cardiac morphology** (myocardial hypertrophy, mononuclear inflammation, myofibrillar loss, perinuclear and cytoplasmic vacuolization)	3 rats severe, 2 moderate, 2 mild
Zhou et al (2022)	60/Sprague-Dawley rats	Doxorubicin	15 mg/kg, ip	Single injection	**Altered cardiac morphology** (disorganised myocardium [broken,wavy, loose], cell morphology blurred, fibrosis, inflammatory cell infiltration, collagen accumulation, myoplasmic dissolution, dissarangement of myofibres)	
Sandamali et al (2022)	70/Wistar albino rats	Doxorubicin	18 mg/kg, ip	Single injection	**Altered cardiac morphology** (severe necrosis in peripheral and subendocardium of the myocardium [grade 4], congestion of blood vessels, intracellular vacuoles, interstitial oedema,	

					haemorrhages, inflammatory infiltrations, and wavy myocardial fibers)	
Wang, S. et al (2022)	48/male/Sprague-Dawley rats	Doxorubicin	2.5 mg/kg, ip	Once per week for: 1st group: 4 weeks, 2nd group: 6 weeks, 3rd group 8 weeks, 4th group: 10 weeks respectively	1st, 2nd, and 3rd group: early myocardial fibrosis, 4th group: vacuolarly degenerated cardiomyocytes, myocardial disarrangement, myocardial cell necrosis, eosinophilic cardiomyocyte necrosis, disarrayed myocardial structure	
Eisvand et al (2022)	42/male/Wistar rats	Doxorubicin	2 mg/kg, ip	On alternate days for 12 days	**Altered cardiac morphology** (collagen deposition, degeneration of myocardial fibers, myocardiocytes cytoplasmic vacuolization)	
Chen et al (2021)	18/male/Sprague Dawley rats	Doxorubicin	3 mg/kg, ip	Once every 3 days (5 total)	**Altered cardiac morphology** (disarrangement of cardiomyocytes, vacuolation with damaged organelle)	

Fouad et al (2021)	24/male/Wistar albino rats	Doxorubicin	20 mg/kg, ip	Single injection	**Altered cardiac morphology** (congestion of myocardial blood vessels, focal Zenker's necrosis of cardiac myocytes, intermyocardial edema, inflammatory cells infiltration)	
Tan et al (2021)	90/male/Sprague-Dawley rats	Doxorubicin	2 mg/kg, ip	Once per week for 6 weeks	**Altered cardiac morphology** (inflammation, desarcomerization, interstitial fibrosis[moderate to severe]inflammation, tissue disorganization)	
Wan, Y. et al (2021)	48/male/Sprague-Dawley rats	Doxorubicin	2.5 mg/kg, ip	Once per week for 6 weeks	**Altered cardiac morphology** (muscle fibre disarrangement, myofilament loss and enlargement, aberrantly shaped mitochondria, disarrayed myocardial structure, obvious cardiomyocyte vacuolization, increased extracellular fibrosis)	

Wan, Y. et al (2021)	45/male/Sprague-Dawley rats	Doxorubicin	4 mg/kg, ip	Four injection per week for 6 weeks	**Altered cardiac morphology** (myocardial edema, cardiac strain, cardiomyocytes fibrosis, cell apoptosis, cardiomyocyte vacuolization and myofibril loss)	
Abdelatty et al (2021)	56/male/Albino rats	Doxorubicin	2 mg/kg, ip	On 1st, 4th, 8th, 15th and 30th day of 90 days	**Altered cardiac morphology** (sarcoplasmic vacuolation,coagulative necrosis of cardiac myocytes, perivascular and subendocardial coagulative necrosis,perivascular edema, dilatation of mitochondrial cristae, severe degree of myolysis, fibrosis, atrophy)	
Refaie et al (2021)	40/male/Wistar albino rats	Doxorubicin	15 mg/kg, ip	Single injection	**Altered cardiac morphology** (degenerated wavy muscle fibers with absent striations, fibers with pyknotic nuclei or absent nuclei, congested dilated blood capillaries,	Grade 3 characteristics (severe disruption of cardiac muscle architecture, severe loos of muscular striations, severe vascular

					blood extravasation with inflammatory cell infiltration, hemosiderin pigment between fibers)	congestion and hemosederin, severe inflammatory cell infiltration, severe nuclear pyknosis)
Shi et al (2021)	40/male/Sprague Dawley rats	Pirarubicin	3 mg/kg, iv	Once per week for 8 weeks	**Altered cardiac morphology** (cardiomyocyte disarrangement, enlarged intercellular space, focal vacuolization, steatosis)	
Wang, C. et al (2021)	40/male/Sprague Dawley rats	Doxorubicin	2 mg/kg, ip	On alternate days for 14 days	**Altered cardiac morphology** (disordered myocardial fiber, severely dissolved myofibrils, inflammatory cells, fibrosis, collagen deposition around small blood vessel)	
Younis et al (2021)	30/male/Sprague-Dawley rats	Doxorubicin	5 mg/kg, ip, cumulative dose: 15 mg/kg	One injection every 5 days(3 total)	**Altered cardiac morphology** (severe cardiomyocyte injury with extensive cytoplasmic vacuolization, significant cardiomyocyte injury with extensive cytoplasmic vacuolization and myofibrillar degeneration)	

Rahmanifard et al (2021)	42/male/Sprague–Dawley rats	Doxorubicin	2.5 mg/kg, ip	One injection every 3 days	**Altered cardiac morphology** (myocytes, microvessels and the number of cardiomyocyte nuclei reduced)	
Alhazzani et al (2021)	24/Sprague–Dawley rats	Doxorubicin	15 mg/kg, ip	Once every other day for 14 days.	**Altered cardiac morphology** (cardiomyocyte vacuolar degeneration, perivascular fibrosis,severe fibrotic scarring, interstitial tissues, mild chronic inflammation with perivascular fibrosis)	
Lai et al (2022)	24/male/Sprague Dawley rats	Doxorubicin	5 mg/kg, ip	Two weekly injections	**Altered cardiac morphology** (myocytes disarrangement, myofibrillar degeneration, extensive inflammatory cell infiltration, cardiomyocyte interstitial fibrosis and perivascular fibrosis, cardiac fibrosis)	
Park et al (2021)	40/male/Sprague–Dawley rats	Doxorubicin	1 mg/kg, iv, Groups(1-5): cumulative dose: 4, 8, 12, 16, 24 mg/kg, respectively	Twice per week for 2, 4, 6, 8, 12 weeks respectively	**Altered cardiac morphology** (vacuolar changes [mild to moderate], interstitial fibrosis [mild, severe 5th group], inflammation[mild to severe], and edema[mild])	Group 1= 2 deaths, Group 4 & 5: 1 death

Wu et al (2021)	48/male/specific pathogen-free Sprague-Dawley rats	Doxorubicin	3 mg/kg, iv	On days 9, 16, 23 and 30	**Altered cardiac morphology** (disordered myocardial fiber arrangement, localized inflammatory infiltration in myocardial tissue, vacuolar degeneration)	
Barış et al (2021)	38/male/Sprague–Dawley rats	Doxorubicin	Cumulative dose: 18 mg/kg, ip	On days 2, 4, 6, 8, 10, 12 and 14	**Altered cardiac morphology** (infiltrative cell quantification, cardiomyocyte degeneration with karyorrhexis, pyknosis, karyolysis, myofibril loss , edema)	
Soliman et al (2021)	28/male/Swiss albino rats	Doxorubicin	4 mg/kg, ip	Daily for 1 week	**Altered cardiac morphology** (scanty collagen deposition in between the cardiomyocytes, focal disruption of myofibers, karyolytic or pyknotic nuclei, areas of focal sarcoplasmic vacuolation, cardiomyocytes with irregularly indented nuclei, abnormally condensed chromatin, focal lysis of myofibrils, loss of normal alignment of sarcomeres, vacuolation of mitochondria with irregular cristae, focal sarcoplasmic reticulum vacuolation)	

Zhang et al (2021)	40/male/Sprague-Dawley rats	Doxorubicin	2.5 mg/kg, iv	Once per week for 4 weeks	**Altered cardiac morphology** (irregular morphology, disarrangment, increased inflammatory cell infiltration, mitochondria swelled, cristae breakage)	
Hekmat et al (2021)	35/male/Sprague-Dawley rats	Doxorubicin	3.75 mg/kg, ip, cumulative dose: 15 mg/kg	On days 14, 21, 28, and 35	**Altered cardiac morphology** (vascular congestion [grade 4], infiltration of inflammatory cells [grade 3], disrupted cardiac muscle architecture [grade 3], loss of muscular striations, hydropic degeneration, focal apoptosis, coagulative necrosis)	
Ahmed et al (2021)	24/female/Wistar rats	Doxorubicin	25 mg/kg, ip	Single injection	**Altered cardiac morphology** (intermuscular edema, myofibrillar loss, infiltration with inflammatory cells, vacuolization and cardiomyocytes degeneration)	
Eid et al (2021)	24/male/Wistar rats	Adriamycin	10 mg/kg, ip	Single injection	**Altered cardiac morphology** (capillary dilation and congestion, atrophy and degeneration of cardiac fibers, interfibrillar spaces increased)	

da Cunha Menezes Souza et al (2021)	140/male/Wistar rats	Doxorubicin	4mg/kg, ip	1st group: single dose, 2nd group: one dose per week for 4 weeks	**Altered cardiac morphology** (myocyte vacuolization, inflammation, necrosis and atrophy, increased volume and retraction of nuclei)	
Sadek et al (2021)	32/male/Albino rats	Doxorubicin	10 mg/kg, ip	On days 3, 9, 15, and 21 for 4 weeks	**Altered cardiac morphology** (degeneration of the myocardial tissue, myofibrillar loss, eosinophilia of myofibers)	
Durdagi et al (2021)	32/male/Wistar Albino rats	Doxorubicin	45 mg/kg, iv	Single injection	**Altered cardiac morphology** (myofibrillary disorganization, interstitial edema, interstitial hemorrhage, nuclear degeneration, infiltration, fibrosis)	
Samra et al (2021)	48/male/Wistar rats	Doxorubicin	3.5 mg/kg, ip	Twice per week for 21 days	**Altered cardiac morphology** (cardiomyocytes disarrangement & degeneration, fibrotic lesions & perivascular fibrosis)	
Saleh et al (2020)	40/female/Wistar albino rats	Doxorubicin	200 mg/kg, ip	Single injection	**Altered cardiac morphology** (expanded vacuolization of cardiomyocyte sarcoplasm associated with loss of striations and individual necrosis)	

Olorundare et al (2020)	49/male/Wistar Albino rats	Doxorubicin	15 mg/kg, ip	Single injection	**Altered cardiac morphology** (mildly congested cardiomyocytes and antemortem coronary microthrombi)	
Hung et al (2020)	male/Wistar rats	Adriamycin	3 mg/kg, ip	Three times per week for 2 weeks	**Altered cardiac morphology** (moderate collagen accumulation, altered heart foci, apoptosis of cardiomyocytes)	
Ahmed, L. A. et al (2021)	48/male/Wistar rats	Doxorubicin	15 mg/kg, ip	Single injection	**Altered cardiac morphology** (necrosis with mononuclear inflammatory cells, nuclear pyknosis, intercellular edema, congested blood vessels, cardiac lesion [grade 3])	
Karabulut et al (2020)	40/male/Wistar rats	Doxorubicin	15 mg/kg, ip	Single injection	**Altered cardiac morphology** (disorganized in myocardial fiber, expansion in cells, necrosis)	

Radeva-Ilieva et al (2020)	36/male/Wistar rats	Doxorubicin	10 mg/kg, ip	Two injections on 1st day and 3rd day	**Altered cardiac morphology** (disrupted myofibril architecture, myocyte disorganization, cytoplasmic fading, and pyknotic nucleus formation, collagen fibers)	
Birari et al (2020)	42/male/Wistar rats	Doxorubicin	6 mg/kg, ip	On alternate days for 10 days	**Altered cardiac morphology** (cellular damage, inflammation, architecture perturbation)	
Xu et al (2020)	48/Sprague Dawley rats	Doxorubicin	2 mg/kg, ip	One injection per week: 1st group: 1 week, 2nd group: 2 weeks, 3rd group: 6 weeks	**Altered cardiac morphology** 1st group: irregularly arranged fibers, irregular and rough endothelium, collagen around vessels, 2nd group: irregular fibers, gaps, rougher endothelium, collagen around vessels, 3rd group: not perfused with red cells vascular cavities, rougher endothelium	1st group: 1 rat died, 2nd group: 4 rats dies, 3rd group: 2 rats died before completion of study (hair loss, abdominal distention, ascites and pericardial effusion)
Li et al (2020)	24/male/Wistar rats	Doxorubicin	2.5 mg/kg, ip	On alternate days for 14 days	**Altered cardiac morphology** (severe tissue damages, inflammatory cells infiltration, distracted tissue architecture)	

Chaudhary et al (2020)	72/Wistar albino rats	Doxorubicin	5 mg/kg, ip	Once per week for 3 weeks	**Altered cardiac morphology & complications** (ischemia & myocardial necrosis)	
Nordgren et al (2020)	20/male/Sprague Dawley rats	Doxorubicin	2 mg/kg, sc	Six weekly injections	**Cellular alterations** (swollen mitochondria)	
Sun et al (2020)	33/male/Wistar rats	Doxorubicin	2.5 mg/kg, ip	Once per week for 6 weeks	**Altered cardiac morphology** (collagen I & III accumulation on left ventricles, cardiomyocyte apoptosis, myocardial fibrosis)	
Chu et al (2020)	24/male/Sprague Dawley rats	Doxorubicin	10 mg/kg, ip	Daily for three consecutive days	**Altered cardiac morphology** (necrotic cardiomyocytes, inflammatory cells, enlarged myocardial space)	
Babaei et al (2020)	30/male/Wistar rats	Doxorubicin	1st group: 2 mg/kg ip, 2nd group: 2.5 mg/kg, ip	On alternate days for 12 days	**Altered cardiac morphology** Both groups: (swollen mitochondria with cristae)	Two deaths in 2nd group
Bin Jardan et al (2020)	24/male/Wistar rats	Doxorubicin	15 mg/kg, ip	Single injection	**Altered cardiac morphology** (perivascular cuffing, interstitial fibrosis, myocytolysis, myonecrosis, infiltration of cells)	
Chen et al (2020)	36/male/Sprague–Dawley rats	Doxorubicin	5 mg/kg, iv	On days 7, 14, 21 and 28	**Altered cardiac morphology** (apoptosis, nuclear lysis, dissasemble myocardial fiber, vacuolization, necrosis, inflammatory infiltration)	Increased eye secretion, erected back hair and 1 death noticed.

Sun et al (2020)	24/male/Sprague Dawley rats	Doxorubicin	2.5 mg/kg, iv	One injection per week for 6 weeks	**Altered cardiac morphology** (myocardial fibrosis, cardiac hypertrophy)	
Wen et al (2020)	male/Sprague Dawley rats	Doxorubicin	2.5 mg/kg, ip	Daily for 7 days	**Altered cardiac morphology** (focal necrosis, myocardial fibrosis, interstitial edema, infiltration of inflammatory cells)	
Adıyaman et al (2020)	28/male/Sprague Dawley rats	Doxorubicin	10 mg/kg, ip	Single injection	**Altered cardiac morphology** (severe myocyte misalignment, hypertrophy, fibrosis, MM & MH severely increased)	
Aziz et al (2020)	56/male/Albino rats	Doxorubicin	20 mg/kg, ip	Single injection	**Altered cardiac morphology** (cytoplasmic vacuolization of cardiomyocytes, inflammatory cells infiltration, Zenker's necrosis of sporadic myocytes)	
Hassan et al (2020)	60/Wistar rats	Doxorubicin	2.5 mg/kg, ip	On alternate days for 2 weeks	**Altered cardiac morphology** (cytoplasmic vacuoles, myocardial inflammation, fibrosis)	

Ana Flávia M Botelho et al (2020)	20/Wistar rats	Doxorubicin	12.5 mg/kg, ip	Single injection	**Altered cardiac morphology** (moderate congestion, edema & fibrin between cardiomyocytes, emboli, perivascular edema, fiber fragmentation, loss of striations)	
Radonjic et al (2020)	36/female/Albino Wistar rats	Doxorubicin	15 mg/kg, ip	Single injection	**Altered cardiac morphology** (moderate interstitial fibrosis, degenerately modified cardiac muscle fibres, vacuolar degeneration, myocytolysis, perivascular collagen accumulation)	
Akdemir et al (2021)	24/Wistar type rats	Doxorubicin	2.5 mg/kg, ip	Six injections for 2 weeks	**Altered cardiac morphology** (deteriorated structure, Zenker's necrosis, hyperaemia, mild haemorrhage)	
Efentakis et al (2020)	48/male/Wistar rats	Doxorubicin	3 mg/kg, ip	Six injections for 2 weeks	**Altered cardiac morphology** (cardiomyocyte hypertrophy, myocardial fibrosis)	
Podyacheva et al (2022)	75/male/Wistar rats	Doxorubicin	Cumulative doses: 1st group: 25mg/kg, 2nd group: 20.4 mg/kg, 3rd group: 15 mg/kg, 4th group: 15mg/kg,	1st group: 10 times for 1.5 week daily, 2nd group: 6 times for 1.5 week on alternate days, 3rd group: 6 times for	**Altered cardiac morphology** (capillary plethora, dilated vessels, loss of cardiomyocyte myofibrils, necrosis of epitheliocytes, vacuolization of cytoplasm,	1st group: all rats dead, 2nd group: 30% of rats dead, 3rd group: no deaths

			5th group: 10 mg/kg, 6th group: 5 mg/kg	1.5 week on alternate days, 4th group: 6 times for 2.5 weeks in 2 days, 5th group: 6 times for 2.5 weeks in 2 days, 6th group: 6 times for 2.5 weeks in 2 days	loss of nuclei, bands necrosis, minor heamorrhage)	
Ghaleb et al (2021)	21/male/rats	Doxorubicin	2.5mg/kg, ip	Three times per week for 2 weeks	**Altered cardiac morphology** (cardiomyocytes apoptosis, inflammation, cellular swelling [grade 4] damage necrotic myocardial cells, cellular swelling, increased cytoplasmic eosinophilic, karryolysis)	
Meeran et al (2021)	72/male/Albino Wistar rats	Doxorubicin	2.5 mg/kg, ip	Once per week for 5 weeks	**Altered cardiac morphology** (cardiac muscle fibers degradation, inflammation, fibrosis, fibrillar collagen deposition)	
Cheng et al (2020)	40/male/Sprague-Dawley rats	Doxorubicin	2.5 mg/kg, ip	Once per weer for 6 weeks	**Altered cardiac morphology** (collagen accumulation, cardiomyocyte apoptosis)	

Zhang et al (2023)	30/male/Sprague-Dawley rats	Doxorubicin	5 mg/kg, ip	On days 7, 14 and 21 for 28 days	**Altered cardiac morphology & cellular complications** (vascular congestion, necrosis cells, pyknotic nuclei, degeneration of myocardial fiber, vacuolization of sarcoplasm)	
Syahputra et al (2023)	Rats	Doxorubicin	15 mg/kg, ip	(no data)	**Altered cardiac morphology** (cardiomyocyte apoptosis, inflammation, mitochondrial damage)	
Fan et al (2023)	99/male/Sprague Dawley rats	Doxorubicin	Cumulative doses: 1st group: 12 mg/kg, 2nd group: 15 mg/kg 3rd group: 18 mg/kg Modes of administration: ip and iv	Once per week for 6 weeks	**Altered cardiac morphology** All groups: myocardial collagen deposition, myocardial fifibrosis, partial inflammatory cell infiltration 2nd group: focal fibrotic hyperplasia, 3rd group: diffuse fibrotic hyperplasia	During 4-6 week: activity decreased, eyelid secretion increased, food & water intake decreased, depilation, respiration accelerated, diarrhea. In the 2nd group only 8 rats survived and 9 rats in the others
Ma et al (2023)	80/male/Sprague–Dawley rats	Doxorubicin	2 mg/kg, ip	Once every 4 days for 30 days	Altered cardiac morphology (cellular necrosis and vacuolization, myocardial fibers disarrangement, myocardial fiber lysis, vacuolar degeneration)	Gradually developed poor diet, decreased activity, dull hair, yellowing, occasional diarrhea, and significant weight loss
Wu et al (2023)	54/male/Sprague-Dawley rats	Doxorubicin	3 mg/kg, ip	Once per week for 6 weeks	**Altered cardiac morphology** (myocardial apoptosis,	

					autophagy, myofibril loss, myocardial necrosis)	
Shabaan et al (2023)	28/Wistar rats	Doxorubicin	12.5 mg/kg, ip	Single injection	**Altered cardiac morphology** (branched disorganized cardiac muscle fibers with wide interstitial spaces, dilated congested blood capillaries, interstitial hemorrhage, inflammatory cell infiltration, darkly stained pyknotic nuclei, enlarged mitochondria, swollen matrix with loss of cristae)	
Shi et al (2021)	40/male/Sprague-Dawley rats	Pirarubicin	3 mg/kg, iv	Once per week for 8 weeks	**Altered cardiac morphology** (disarrangment of cardiomyocytes, enlarged intercellular space, focal vacuolization and steatosis)	
Wang et al (2020)	48/male/Sprague-Dawley rats	Pirarubicin	3 mg/kg, iv	Once per week for 6 weeks	**Altered cardiac morphology** (loss of striation of myocardial fibers, hypertrophic myocardial fibers, pyknotic & deformed nuclei, interstitial edema)	

Li et al (2020)	Wistar rats	Doxorubicin	(no data)	6 injections within 2 weeks	**Altered cardiac morphology** (cardiomyocytes' areas reduced, collagen deposition induced, cardiomyocyte apoptosis rate increased, myocardial fibrosis)	
Elblehi et al (2021)	40/male/Wistar albino rats	Doxorubicin	2.5 mg/kg, ip	6 injections within 2 weeks	**Altered cardiac morphology** (interfibrilar infiltration, focal areas of hemorrhage, collagen deposition, vacuolization of sarcoplasm, Zenker's necrosis & degeneration, mononuclear inflammatory cells infiltrations, fibroblasts proliferation, hyperemic interstitial blood vessels, myocytolysis)	2 rats died, scruffy appearance, significant body weight loss, heart weight raised
Olorundare et al (2020)	56/male/Wistar albino rats	Doxorubicin	2.5 mg/kg, ip	On alternate days for 14 days	**Altered cardiac morphology** (myocyte congestion with scanty pyknotic and predominant hyperchromatic, meganuclei with interstitial fibrosis, myocardial hypertrophy, scattered cardiac myocyte necrosis)	

Chen et al (2023)	24/male/Sprague-Dawley rats	Doxorubicin	2.5 mg/kg, ip	Twice per week for 4 weeks	**Altered cardiac morphology** (vacuolation, myocardial fiber disarrangement, myocardial cell degeneration and necrosis, inflammatory cell infiltration)	
Sharifiaghdam et al (2023)	40/male/Wistar rats	Doxorubicin	2 mg/kg, ip	On alternate days for 12 days	**Altered cardiac morphology** (bigger intercellular distances and spaces, myocardial apoptosis)	
Malik et al (2022)	Rats	Doxorubicin	15 mg/kg, ip	Single injection	**Altered cardiac morphology** (mildly distorted cardiac architecture with edema, dilated blood vessels, mild lymphocytic infiltration)	
Rajangam et al (2022)	24/Wistar Albino rats	Doxorubicin	10 mg/kg, ip	Single injection	**Altered cardiac morphology** (necrotic cardiac tissue damage, proliferated granulation tissues)	

Nagoor Meeran et al (2023)	Rats	Doxorubicin	12.5 mg/kg, ip	Single injection	**Altered cardiac morphology** (muscle fiber degradation with inflammatory cells [grade 2], disrupted myofibrils, separation of cardiac muscle fibers, necrosis, edema, inflammatory cells infiltration)	
Avagimyan et al (2023)	80/male/Wistar rats	Doxorubicin	2.5 mg/kg, ip	Three times per week for 2 weeks	**Altered cardiac morphology** (left ventricle myocardium: edema, inflammatory infiltration, myocardium wave-like deformation and fragmentation, multiple fuchsinofilic substrates, severe venous hyperemia [grade 3])	
Bradic et al (2023)	24/male/Wistar albino rats	Doxorubicin	15 mg/kg, ip	Single injection	**Altered cardiac morphology** (degenerative changes, focal necrosis, cofluent and massive necrosis of myocardium, fiber fragmentation, loss of nuclei, hypereosinophilia of the cytoplasm)	
Cicek et al (2023)	28/male/Wistar Albino rats	Doxorubicin	15 mg/kg, ip	Single injection	**Cardiac muscle complications** (mononuclear cell infiltration, hemorrhage)	

Dantas et al (2022)	80/male/Wistar rats	Doxorubicin	5 mg/kg, ip	Once per week for 4 weeks	**Altered cardiac morphology** (LV cardiomyocyte hypertrophy)	
Hosseini et al (2022)	40/male/Albino Wistar rats	Doxorubicin	2.5 mg/kg, ip	On alternate days for 2 weeks	**Altered cardiac morphology** (extracellular edema, moderate congestion, small foci of hemorrhage)	
Rankovic et al (2021)	48/Wistar albino rats	Doxorubicin	15mg/kg, ip	Single injection	**Altered cardiac morphology** (degenerative changes, unicellular or focal necrosis of cardiomyocytes, muscle fiber damage, hyperaemia, edema)	
Sandamali et al (2021)	70/male/female/Wistar albino rats	Doxorubicin	18 mg/kg, ip	Single injection	**Altered cardiac morphology** (extensive early changes of necrosis in both peripheral and subendocardium of the myocardial tissue, intracellular vacuoles, wavy myocardial fibers, inflammatory infiltration, haemorrhages, interstitial edema, congestion of blood vessel)	

Sandamali et al (2020)	50/male/female/Wistar albino rats	Doxorubicin	18 mg/kg, ip	Single injection	**Altered cardiac morphology & nuclear changes** (myocytes with hypereosinophilic cytoplasm, pyknosis, karrheorhexis, and karyolysis, necrosis, haemorrhages, inflammatory infiltrations, interstitial edema, congestion of blood vessel, wavy myocardial fibers, intracellular vacuoles)	
Saad et al (2020)	24/male/Wistar rats	Doxorubicin	15 mg/kg, ip	Single injection	**Altered cardiac morphology** (pathological lesions [grade 3], edema, loss of cellular boundaries, myocardial fibers disorganization, cytoplasmic vacuolization, and infiltration of the lymphocytes)	33% of rats died, minor body weight loss, significantly lower heart weight, rats with scruffy fur with pink tinge, red exudates around eyes and nose, soft watery feces and necrosis
Zhang et al (2018)	30/male/Sprague-Dawley rats	Doxorubicin	1 mg/kg, ip	Daily for 2 weeks	**Altered cardiac morphology** (enhancement and loose arrangement of the myocardial fibers, loss of myocytes, vacuolar degeneration)	At the beginning of week 4, the rats became lethargic, had decreased food and water intake, slow movement, loss of hair luster, decreased activity, and symptoms of eyeball hyperemia and diarrhea. One rat died on

						each of days 35, 47, and 70 of the experiment in the ADR group, and the survival rate was 70%.
Tian et al (2017)	70/male/Sprague Dawley rats	Doxorubicin	3.0 mg/kg, ip	Once per week for 6 weeks	**Altered cardiac morphology** (myofibrilar disorganization, marked interstitial edema and fibrosis, focal cytoplasmic vacuolation, interstitial fibrosis, perivascular fibrosis)	
Andreadou et al (2014)	90/male/Wistar rats	Doxorubicin	3 mg/kg, ip	Three times per week for 2 weeks	**Altered cardiac morphology** (focal edema, vacuolization and degeneration of myocardial fibers)	19% mortality by the end of DXR injection protocol
Vasić et al (2018)	68/male/Wistar out-bred rats	Doxorubicin	2.5 mg/kg, ip	On alternate days for 2 weeks (6 total doses)	**Altered cardiac morphology** (vacuolar degeneration of myocardial cells, interstitial mononuclear infiltration of cardiac tissue and myofibrillar contraction band necrosis, perivascular and interstitial fibrosis)	
Arozal et al (2010)	20/male/Sprague–Dawley rats	Daunorubicin	3 mg/kg/day (18 mg/kg total dose)	On alternate days for 12 days	**Altered cardiac morphology** (edema, haemorrhage, congestion)	

Argun et al (2015)	40/male/Wistar albino rats	Doxorubicin	4 mg/kg, ip	Twice per week for 2 weeks	**Altered cardiac morphology** (disarrangement of muscle fibers, myofibril loss, intracytoplasmic vacuole formation, congestive and hemorrhagic areas)	
Tatlidede et al (2009)	32/Wistar albino rats	Doxorubicin	cumulative dose: 20 mg/kg	On alternate days for 2 weeks (6 total doses)	**Altered cardiac morphology** (enhanced fibrotic activity, collagen accumulation, congestion & vacuolization in the connective tissue of muscle fibers)	
Teng et al (2010)	46/male/Sprague-Dawley rats	Doxorubicin	2 mg/kg, ip	Once per week for 8 weeks	**Altered cardiac morphology** (swelling of mitochondria and nuclei [grade 3], focal subendocardial fibrosis, disorganization of cardiomyocytes)	9 rats died throughout experiment
Kondru et al (2017)	24/male/Wistar rats	Doxorubicin	2 mg/kg, ip	Once per week for 5 weeks	**Altered cardiac morphology** (cardiac fibrosis & perivascular fibrosis [grade 3], higher transmural fibrosis)	
Moriyama et al (2016)	66/male/Sprague-Dawley rats	Doxorubicin	2 mg/kg ,iv	Once per week for 6 weeks	**Altered cardiac morphology** (slight intracytoplasmic vacuolation)	5/6 rats with grade 1 and 1/6 rats with grade 2

Shen et al (2016)	150/Sprague-Dawley rats	Doxorubicin	Cumulative dose : 12 mg/kg 1st group: 1 mg/kg, ip 2nd group: 2 mg/kg, ip	1st group: twice a week 2nd group: once a week	**Altered cardiac morphology** (apoptosis, cardiomyocyte vacuolization [grade 3], degeneration and necrosis, interstitial edema, inflammatory cell infiltration)	"body hair was ruffled and alopecia occurred, physical activity was reduced, and the rats ingested less food. (11/50) of the rats died in the Dox 1 group, while 48% (24/50) of rats died in the Dox 2 group 22% (11/50) of the rats died in the Dox 1 group, while 48% (24/50) of rats died in the Dox 2 group (acute heart failure, sudden cardiac death)"
Shoukry et al (2017)	32/male/Wister rats	Doxorubicin	2.5 mg/kg, ip	Three times per week for 2 weeks	**Altered cardiac morphology** (widely spaced deep acidophilic fibers, multiple disrupted fibers, dark peripheral nuclei, congested blood vessels, dense collagen fibers)	

Bertinchant et al (2003)	35/male/Wistar rats	Doxorubicin	1.5 mg/kg, iv	Weekly for up to 8 weeks	**Altered cardiac morphology** (perivascular fibrosis, Interstitial fibrosis,myocyte vacuolisation and degeneration)	6 mg/kg: 1 rat :perivascular fibrosis(very slight), interstitial fibrosis (moderate), myocyte vacuolisation and degeneration, 7.5 mg/kg: perivacular fibrosis (2/6 rats very slight, 1/6 rat slight, 3/6 rats moderate), interstitial fibrosis (2/6 rats very slight), myocyte vacuolisation and degeneration (2/6 rats very slight), 12 mg/kg: perivascular fibrosis (6/11 rats very slight, 4/11 rats slight, 1/11 rats moderate), interstitial fibrosis (5/11 rats very slight, 2/11 rats slight, 1/11 rats moderate), myocyte vacuolisation & degeneration (3/11 very slight, 1/11 rats slight)
Gao et al (2016)	90/male/Wistar albino rats	Doxorubicin	2 mg/kg, ip	Every 3 days for 30 days	**Altered cardiac morphology** (infiltration of inflammatory cells, focal myolysis, karyopyknosis, vacuolar degeneration)	12/30 rats died in the Dox group

Lu et al (2015)	48/male/Sprague-Dawley rats	Doxorubicin	1 mg/kg on the 2nd and 4th days, 2 mg/kg on the 6th and 8th days, 3 mg/kg on the 10th and 12th days, and 4 mg/kg on the 14th and 16th days, ip (cumulative dose: 20mg/kg)	10 weeks	**Altered cardiac morphology** (myocardial fiber disruption and disarray, hyperplastic cardiomyocytes infiltration with inflammatory cells, fibrosis)	
Carresi et al (2018)	40/male/Wistar rats	Doxorubicin	2.5 mg/kg, ip	Three times per week for 2 weeks	**Altered cardiac morphology** (myocardial degeneration, significant myocyte apoptosis, reactive myocyte hypertrophy, loss of myofibrillar structure)	
Ma et al (2017)	170 rats	Doxorubicin	2.5 mg/kg, ip	6 doses over a period of 2 weeks	**Altered cardiac morphology** (cardiac collagen deposition, inflammatory cells invasion, loss of myofibrils and disorganization)	23 rats died, scruffy, light yellowish fur, diarrhea and red exudates around the eyes with grossly enlarged abdomen and ascites
Zhang et al (2017)	26/male/Sprague-Dawley rats	Doxorubicin	4 mg/2 ml/kg, ip	Twice per week for 2 weeks	**Altered cardiac morphology** (disorganization of myofibrillar morphology, myofibrillar loss and cytoplasmic vacuolization, apoptosis)	
Sun et al (2017)	Experiment 1: 32/male/Sprague–Dawley rats, Experiment 2: 16/male/Sprague–	Doxorubicin	Experiment 1: 20 mg/kg, ip, Experiment 2: 5 mg/kg,	Single injection	**Altered cardiac morphology** (cardiac damage [grade 3], myocardial fibrosis, myocardial degeneration and necrosis, inflammatory	

	Dawley rats, Experiment 3: 60/male/Sprague–Dawley rats		iv, Experiment 3: 5 mg/kg, iv		cell infiltration, occasional vacuolization)	
Hiona et al (2010)	24/female/Sprague Dawley rats	Doxorubicin	Cumulative dose: 25 mg/kg, ip	Once per week for 6 weeks	**Altered cardiac morphology** (scattered foci of myofibrillar degeneration)	
Tang et al (2013)	24/male/Sprague-Dawley rats	Doxorubicin	2.5 mg/kg, ip	On alternate days (6 total doses)	**Altered cardiac morphology** (myocardial hypertrophy, myocardial edema, fiber breakage)	
Al-Taee et al (2019)	48/male/Albino Wistar rats	Doxorubicin	12.5 mg/kg, ip	Single injection	**Altered cardiac morphology** (extensive muscle fiber degradation with inflammatory cells, extensive myofibrillar loss and mitochondrial degeneration)	
Sahyon et al (2019)	20/male/Sprague Dawley rats	Doxorubicin	2.5 mg/kg, ip	On alternate days for 2 weeks	**Altered cardiac morphology** (myocardial degeneration, vacuolation of the myocardial fibres, extensive myolysis, interstitial fibroblastic cell proliferation and numerous apoptotic cells [grade 3])	
Sheibani et al (2020)	40/male/Wistar rats	Doxorubicin	2.5 mg/kg, ip	Three times per week for 2 weeks	**Altered cardiac morphology** (cardiac tissue disarrangement, lesion [grade 1-2], mononuclear	

					cell infiltration, edema without hemorrhage and necrosis)	
Abdelrahman et al (2019)	24/male/Wistar rats	Doxorubicin	17.5 mg/kg, ip	Single injection	**Altered cardiac morphology** (significant increase of collagen deposition, fibrosis, tubuli damage, inflammatory cell infiltration)	
Jang et al (2019)	45/male/Wistar rats	Doxorubicin	20 mg/kg, sc	Two injections for 2 days	**Altered cardiac morphology** (interstitial edema, hemorrhage, loss of myofibrils with fiber disorganization [grade 3], myofibril injury with cytoplasmic vacuolization)	
Zhang et al (2020)	32/male/Wistar rats	Doxorubicin	4 mg/kg, iv	Once per week for 4 weeks	**Altered cardiac morphology & complications** (significant increase in intracellular space, cytoplasmic vacuolation and myocardial cell disorders)	

Sharma et al (2020)	50/male/Sprague-Dawley rats	Doxorubicin	2 mg/kg, ip	Once per week for 4 weeks	**Altered cardiac morphology** (spread interstitial fibrosis, hypertrophic or atrophic myocardial fibers, degeneration of cardiomyocytes, inflammatory cell infiltrations)	
Baniahmad et al (2020)	36/male/Wistar rats	Doxorubicin	2.5 mg/kg, ip	Three times per week for 2 weeks	**Altered cardiac morphology** (rupture of cardiac muscle fibers, hemorrhage, myocyte wavy degeneration, necrosis, inter-fibrillar congestion)	
Todorova et al (2020)	57/female/Fisher344 rats	Doxorubicin	2 mg/kg, ip	Twice per week for 3 weeks	**Altered cardiac morphology** (excessive myofibrillar degeneration, swelling of sarcoplasmic reticulum, myocyte vacuolization, granulation of cytoplasm, hypereosinophilia)	3 rats died

Razmaraii, N. et al (2016)	18/male/Wistar rats	Doxorubicin	2 mg/kg, ip	On alternate days for 12 days	**Altered cardiac morphology** (cytoplasmic vacuolization, interstitial edema, hyaline degeneration and Zenker's necrosis, focal to extensive hemorrhages, inflammatory cells infiltration, injured vascular structures, necrotic changes in the nuclei of cardiomyocytes and mild cardiac fibrosis)	
Swamy, A. V. et al (2012)	24/male/female/Albino rats	Doxorubicin	2.5 mg/kg, ip	Three times per week for 2 weeks	**Altered cardiac morphology** (loss of myofibrils and vacuolization of the cytoplasm)	

Table. 2 **:** Detailed analysis of all data taken from the literature such as sex, species and number of rats, type of anthracycline received, dose and route by which rats received it, frequency and duration with which the rats received the anthracycline, the histopathological data obtained after necropsy of the rats which are reported at tissue and cellular level, the gradation of the damage caused to the tissues, where it was available as data throughout the literature and additional findings noted during each study conducted.[17-146]

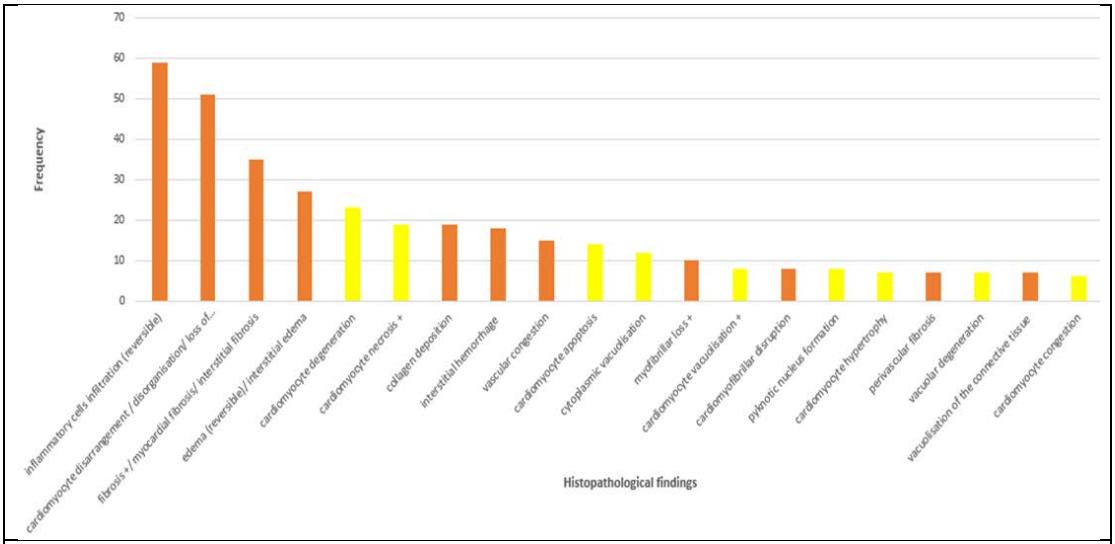
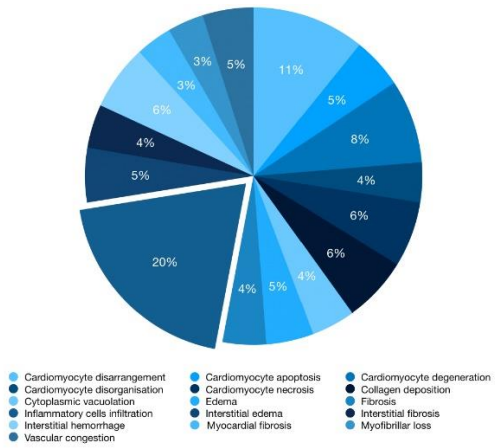


Figure. 3 : Histopathological effects that are reported more frequently (>1%). Orange code: Effects related to tissue level. Yellow code: Effects related to cellular level. +: The most important effects related to severity.



Most important findings	Frequency
Cardiomyocyte disarrangement	31
Cardiomyocyte apoptosis	14
Cardiomyocyte degeneration	23
Cardiomyocyte disorganisation	11
Cardiomyocyte necrosis	18
Collagen deposition	18
Cytoplasmic vacuolation	12
Edema	13
Fibrosis	12
Inflammatory cells infiltration	56
Interstitial edema	15
Interstitial fibrosis	12
Interstitial hemorrhage	18
Myocardial fibrosis	10
Myofibrillar loss	10
Vascular congestion	14

Table. 3 :Illustration of the main histopathological findings and their frequency

RESULTS

The main criterion, which was investigated and reviewed in the context of a weight-of-evidence approach, includes the findings from histopathological analysis of the heart tissue from animals exposed to well-established cardiotoxic chemicals. Histopathological data, when assessed properly, can provide reliable information. More specifically, significant functional changes in the heart muscle noted at necropsy and/or at microscopic examination, but also morphological, reversible or not, changes which provide evidence of marked heart dysfunction and cell death incapable of regeneration, could be of relevance. Preliminary data in the literature are encouraging.

The histopathological findings are illustrated in table 2, table 3 and figure 3. The findings have been categorized to the ones observed in tissue level and the ones observed in cellular level. When it refers to tissue level the major findings are reversible inflammatory cells in-filtration (10,21% of the examined rats), cardiomyocyte disarrangements and disorganizations and even loss of muscular striations (8,82% of the examined rats), fibrosis, myocardial and interstitial fibrosis (6,06% of the examined rats), reversible edema and interstitial edema (4,67% of the examined rats), collagen deposition which constitutes a part of fibrosis installation (3,29%), interstitial hemorrhage non specific to lead to functional decline (3,11% of the examined rats), vascular congestion (2,6% of the examined rats), myofibrillar loss (1,73% of the examined rats), cardiomyofibrillar disruption (1,38% of the examined rats), perivascular fibrosis (1,21% of the examined rats) and vacuolization of the connective tissue non indicative for functional decline and depicts cellular debris mainly (1,21% of the examined rats).

The most frequent findings related to cellular level were cardiomyocyte degeneration (3,98% of the examined rats), cardiomyocyte necrosis (3,29% of the examined rats), cardiomyocyte apoptosis (2,42% of the examined rats), cytoplasmic vacuolisation (1,38% of the examined rats), pyknotic nucleus formation (1,38% of the examined rats), cardiomyocyte hypertrophy (1,21% of the examined rats), vacuolar degeneration (1,21% of the examined rats) and cardiomyocyte congestion (1,04% of the examined rats).

DISCUSSION & CONCLUSIONS

More than 500 distinct histopathological observations have been documented. Figure 3 summarizes those reported with a frequency greater than 1%. Histopathological effects are identified at both the cellular and tissue levels. Additionally, some of these effects are considered more critical than others in relation to the functional deterioration of the cardiac muscle.

Myocardial fibrosis appears to be a pivotal event in anthracycline-induced cardiotoxicity in both animals and humans. Interstitial and replacement fibrosis were evident in patients undergoing cancer treatment, as demonstrated in a recent study evaluating biopsy and autopsy specimens (147) . Myocardial tissue disorganization and disarrangement, likely due to myocardial cell necrosis, constitute another critical event. Maintaining myocardial architecture is crucial, as its disruption is associated with functional impairment of the left ventricle in patients with cancer-related cardiotoxicity. Cardiomyocyte necrosis and replacement fibrosis are interrelated processes, but it is believed that another significant factor involves alterations in the heart cytoskeleton, which fundamentally affects the mechanical properties of the heart (148).

At the cellular level, cardiomyocyte necrosis and apoptosis are defining features of cancer-related cardiotoxicity, accompanied by cell vacuolization, as observed in histopathological examinations (149). These irreversible cellular alterations are linked to the established effects of anthracycline cardiotoxicity and underscore the importance of early detection of cardiotoxicity in clinical practice.

Thus, the histopathological findings of anthracycline cardiotoxicity, in relation to myocardial functional decline, can be categorized into critical findings highly predictive of subsequent functional loss and findings not definitively associated with it. Myocardial necrosis and vacuolization in histopathological assessments should be considered significant, particularly when frequently observed. Myocardial disorganization and fibrosis, both replacement and interstitial, should be regarded as key events closely related to functional decline, especially when the myocardial cytoskeleton is also compromised.

Recognizing the existence of the aforementioned histopathological pool of data, it is important to note that the current research aims to facilitate the utilization of this data in the hazard assessment of cardiotoxicity. Furthermore, the experience of risk assessment for newly emerging hazard classes, such as endocrine disruptors, has demonstrated that assessors, particularly for regulatory purposes, often rely on existing data to uphold animal welfare standards.

In conclusion, more targeted research is required from both scientists and regulators to further enhance the weight-of-evidence approach and accurately characterize the hazard of cardiotoxicity caused by chemicals relevant to humans as a regulatorily recognized toxicological category. Specifically, the updated roadmap (150) proposed in this manuscript for identifying regulatory criteria from animal studies for inclusion in regulations consists of the following steps:

1)Conducting a meta-analysis of each line of scientific evidence (e.g., histopathological, biochemical, echocardiographic indices, etc.) recognized in animal species following exposure to well-established human cardiotoxicants (e.g., anthracyclines) to identify threshold values or ranges of normal and/or altered values due to exposure

2)Validating the aforementioned evidence in animals exposed to other suspected cardiotoxic substances (e.g., anabolic-androgenic steroids and pesticides)

3)Establishing mechanisms of action based on information from known or suspected

cardiotoxicants and associating them with the parameters introduced as scientific evidence in the development of classification criteria

4)Introducing novel indices and in silico methods.

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