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REVIEW

The Problem of Determination of Cause of Laboratory Animal's Death: A Critical Review of Definitions of "Fatal" and "Incidental" Lesions

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Abstract—The determination of the cause of a laboratory animal's death in gerontological experiments has become extraordinarily urgent in connection with the appearance of ideas on the programmed death of organisms. Unfortunately, the past approach to diagnosis of fatal and incidental changes based only on data of autopsy and histopathology (according to the human pathology model) is not correct for laboratory rodents. Nevertheless, the exact determination of death causes is principally possible in the future under conditions of adequate experimental design (including a large set of clinical, physiological, biochemical, and morphological examinations). However, it seems that even in this case causes of some experimental animal's death will remain unclear.

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It is obvious that studies on the phenomenon of phenoptosis (i.e. the death of an organism according to a program included in its genome [1-3]) can hardly be productive without a strict approach to analyzing causes of spontaneous deaths. Unfortunately, the solution of the problem of determination of the cause of an animal's death under conditions of a chronic gerontological experiment is still far from sufficient for correct analysis of mechanisms of the organism's programmed death. Therefore, results of observations on effects of geroprotectors are doubtful and perhaps even useless.

In this review, problems of death cause diagnosis in laboratory practice are critically considered in comparison with the traditional approach in medicine, and a possible line is proposed for resolving this very difficult problem.

CERTIFICATION OF DEATH CAUSE IN HUMAN PATHOLOGY

From the time of appearance of classical works by Th. Bonet (1679) and J.-B. Morgagni (1761), certification of death cause is a major problem of pathological anatomy or, as it is more correctly called, of clinical pathology. Any anatomopathological (and medicolegal) study results in the formulation of a diagnosis with a clear subordination of detected changes, on one hand, due to the main disease, and its complications causing the death mediated through a certain pathophysiological mechanism (a direct cause of the death) and, on the other hand, due to other vitally insignificant concomitant pathologies [4]. Pathologists were forced to observe this subordination and improve the clinical and morphological analyses not because of scientific interest, but mainly because of the great social and legal significance of the exact formulation of death cause. From the very beginning, pathological anatomy was fundamentally based on clinical data; upon appearance of a large set of biochemical and functional examination approaches, data without fail are taken into account for designing a pathophysiological picture of the given case of a patient's death, and often the pathomorphological and clinical information is essentially complete. Finally, a special field of medicine has been delineated, so-called "thanatology" (i.e. "science about death"), which is dedicated to analyzing causes and mechanisms of dying basing on pathomorphological and pathophysiological data. Because of its rather peculiar position in medical knowledge system (it requires high

Abbreviations: IARC, International Agency for Research on Cancer; ICD-10, International Classification of Disease, 10th revision; SPF, specific pathogen free.

professional erudition in such far fields as resuscitation and pathological anatomy), this field of medicine is not yet a major scientific trend. This situation can be explained in part by the absence in many cases of difficulties for medical clinical pathologists and forensic pathologists in determination of diseases resulting in the death, certainly, on not taking into consideration pronounced autolysis of the corpse. In fact, human diseases mainly contributing to mortality (myocardial infarction, brain strokes, disseminated tumors, the majority of infections) [5] fortunately give a very impressive morphological picture, and the diagnosis becomes clear already at the section table [6]. However, even in human pathology some nosologies cannot be determined based only on morphological data, not speaking about their role as the cause of death. Thus, the death from arrhythmias, sudden cardiac arrest (spontaneous and reflectory), the majority of intoxications (e.g. with alcohol), anaphylactic and traumatic shock, anoxia, anemias, the lightning-like course of some infections, especially of viral ones, are not accompanied by development of even a slightly specific macro- and microscopic picture [6-8]. For an accurate diagnosis in the above-presented examples, it is necessary to obtain data on the circumstances of the death (sudden cardiac death, intoxication with natural gas, anoxia, heat shock), clinics (anaphylactic and pain shock, anemia, fulminant infections), functional diagnosis (arrhythmias), bacteriology and virology (fulminant infections), and biochemical, toxicological, and morphological examinations of blood and urine (anemia, intoxications). However, there are situations when even such a complex approach does not help, and for certification of diagnosis and death cause one needs to use the helpless method of exceptions. Such cases described in the International Classification of Disease 10th version (ICD-10) [9] include, in particular, the diagnosis of "sudden infant death syndrome" lacking any positive verification criterion [10-12] or the so-called "senility", which also does not have any diagnostic basis except the patient's age and inability to specify the nosological form resulting in the death [13-15]. In addition to problems of diagnosis, there are problems of interpretation. Thus, in toxic hepatitis associated with taking a high dose of paracetamol on the background of a severe pneumonia, the general pathomorphological picture is sufficiently clear, but macroscopic and histological data do not give the possibility of choosing either of the diseases as the cause of the death. This question can be answered only based on a scrupulous analysis of clinical and paraclinical data. Thus, if the death was accompanied by the clinical picture of respiratory failure and characteristic changes in the lungs on the background of a moderate increase in the level of aminotransferases, it was rather a serious reason for certificating this death as caused by pneumonia even in the presence of morphological signs of a toxic lesion of the liver. It is also well known that diagnosis of a malignant tumor does not definitely mean that just this tumor is the death cause [16]. Unfortunately, conclusions about death cause in such cases that are difficult for interpretation are often based more on subjective impressions than on objective data. One can only hope that just thanatology, on focusing the attention to "*loci minores*" of a healthy and diseased organism and limits of the compensatory abilities on changes in different physiological parameters, will be able in the future to elucidate problems of diagnosis and pathogenesis of the difficult cases mentioned above.

However, in some cases it should not at all specify and analyze the only cause of death since this leads to increasing subjectivity and lowering of information value of the post-mortem examination results. In this connection, it should be noted that now an alternative approach to interpretation of autopsy data based on analyzing multiple causes of death attracts growing attention. In many cases this approach is very productive [17-19].

DIFFICULTIES IN DIAGNOSIS OF DEATH CAUSES OF LABORATORY ANIMALS

As distinguished from human pathology, diseases of laboratory animals except tumors became a subject for serious study only recently, a little more than half a century ago. Laboratory rodents are widely used for modeling human diseases and testing pharmaceuticals, and this forced researchers to consider pathological states specific for these animals and to try to unify criteria of their diagnosis. Certainly, seriously based approaches of medical pathology were taken as models. However, some of such direct introductions concerning in particular thanatological aspects of laboratory animal diseases occurred very difficult for some reasons. First, human pathological anatomy from its very beginning was developing in a complex with clinical medicine that already had accumulated rich material; later a serious reinforcement appeared represented by data of pathophysiology and biochemistry. From the very beginning, clinical medicine was aimed to individualize every case, and that resulted in the creation of a whole system of diagnostic standards allowing a researcher to distinguish from an abundance of data an unambiguous idea about interrelations of different pathological states in the patient. Certainly, no similar problems stood before pathology of laboratory rodents. The animals were usually dissected with specific and very narrow experimental aims, and a spontaneous pathology detected occasionally was not examined systematically and carefully, certainly not if it did not cause massive mortality in the vivarium. An exception although rather conditioned were cases of spontaneous tumors, but in such cases a complex analysis of the clinical picture and pathophysiology was not often performed. In the best case, a pathologist who received for dissection the corpse of a euthanized or deceased animal obtained only scanty

indications of a veterinary about one or two of the most pronounced symptoms observed on examination of the living mouse, and results of weighing and general palpation if the experiment had supposed tumors to be detected. Thus, the necropsy and pathohistological examination were usually the only full-value approach for analyzing a disease in dead animals, and this approach was supplemented by serological, bacteriological, and virological investigations only seldom. From the narrow utilitarian standpoint, this approach was reasonable for a long time because it allowed virtually any tumor and infection to be diagnosed. Some other diseases, neither infections nor tumors, could also be revealed by this approach, but up to a certain time they were of little interest for researchers. This situation was clearly not favorable for deep penetration into pathophysiology and thanatology of rodents' diseases. The situation changed, first, when requirements for maintenance conditions of laboratory animals were increased and so-called "specific pathogen free" (SPF) animals appeared in which only rare spontaneous infections occur caused by conventionally-pathogenic microflora, second, when researchers used genetically engineered mice and rats that often died because of metabolic disorders and not because of infections or tumors, and third, when large-scale long-term toxicological studies were started of pharmaceutical preparations, potential carcinogens, and geroprotectors that required the survival of rodents until their natural death. Under conditions of SPF-vivaria, the death of the animals was mainly caused by non-infectious pathologies and not only by tumors. Diagnosis of such pathologies is difficult and often not trivial. Now only a few laboratories in the world have sufficient funds and developed structures for complex examination of experimental animals that would be comparable with the careful examination in clinical medicine; in the majority of cases the former standard remains – routine pathomorphology, scarce notes of a veterinary in an arbitrary form, and only rarely bacteriological and serological data. Moreover, another circumstance discriminating the pathology of rodents from human pathology should be taken into account. The matter is that the pathology spectrum of mice and rats is much different from that of human. If the main death causes in humans, such as infarctions, thromboembolisms, and brain strokes are of acute character, have distinct morphological manifestations, and affect vitally important organs (the heart, lungs, brain), the damage of which rapidly and obviously leads to death [5, 6], similar lesions in rodents are described as a casuistry [20-22]. Many non-tumoral diseases of SPF-rodents – chronic cardiomyopathy, chronic glomerulopathy, progressive nephropathy, vasculitis, and senile and autoimmune anemias – have a chronic course, and the association between the morphological picture and the death is often rather unclear [21]. For instance, in mice the "nutmeg" liver can be virtually never found, and the brown induration of lungs is detected very seldom, whereas these observations are very characteristic for chronic heart failure in humans [6]. Such diseases as arterial thrombosis or obstructive uropathy, when the disease association with the animal's death can be easily established at the necropsy, are only a small fraction, usually 5-10%, of the total mortality. Tumors in rodents are much less prone to metastasizing than in humans; therefore, it is more difficult to prove the association of a tumor with a death. In a number of cases (10-30% in different studies and in different strains), the death of animals that survived until their natural death occurred without definite morphological changes that could be associated with it [23]. Moreover, there are data that death in mice can be caused by a sudden heart stop without visible histological alterations in this organ [21].

"FATAL" AND "INCIDENTAL" PATHOLOGY: THE BAD SOLUTION OF THE PROBLEM

Just such was the situation when the group of R. Peto under the aegis of IARC [24] proposed not only to ascertain some or other pathological changes or even nosologies in a deceased or euthanized laboratory animal, but, imitating human pathology, to establish their causal relation with the death. Initially this proposal concerned only tumors, but later there were attempts to extent it also to other diseases of rodents. Depending on the relation with the animal's death, Peto et al. proposed to ascribe tumors to "fatal", i.e. causing the death, or "incidental", not causally related with the death. Rubrics of "probably fatal" and "probably incidental" tumors were also foreseen. The authors did not formulate distinct parameters for ascribing a tumor to these rubrics; just the presence of the rubrics "probably fatal" and "probably incidental" diseases expressively emphasizes the utter subjectivity of this approach (not speaking that in the case of euthanizing a diseased animal, scientific argumentations about the death cause appear rather strange and fictitious). The proposal of Peto's group was aimed to increase the informativity and reliability at statistical assessment of data on testing preparations influencing the development of tumors in rodents.

The paradigm of "fatal and incidental tumors" was taken by pathologists rather ambiguously, but it did find its supporters. In 2001 additional recommendations were given about the use of the Peto classification, which determined the causative relation of tumors with the death (or killing!) of the animal [25]. According to these recommendations, neoplasms were to be divided into: 1) "detected lifetime" – tumors found in a living animal on examination or palpation; 2) "incidental" – neoplasms found only at necropsy and believed by the researcher to be unrelated with the death (killing) of the animal; 3) "fast-fatal" – tumors found only at the necropsy but in the pathologist's opinion capable of resulting in the

death; 4) "non-fast-fatal" – tumors found only at necropsy that seemed to be unable to rapidly lead to the death. Note that the authors emphasized that the arbitrariness in determination of these rubrics was dangerous for subsequent statistical treatment of the data, but at the same time they continually resorted to subjectivity using such words as "it appears" and "pathologists believe". This impression is still increased due to examples presented by them. Ependymoma of the brain lateral ventricle seems to them to be not "fast-fatal", whereas ependymoma of the aqueduct of Sylvius (without hydrocephalus signs!) seems to be fast-fatal; femoral osteosarcoma is not thought to be a fast-fatal tumor, whereas even a small sarcoma of the temporo-mandibular joint seems to be a "fast-fatal" neoplasm. The authors do not take into consideration that the rate of growth and metastasizing can be very different even in neoplasms of the same structure and localization. Moreover, it is clear that the decision is significantly determined by features of the personal experience and fancy of the researcher. It is even more interesting that on finding two tumors, a pathologist may consider both "fast-fatal" if he believes them to be able to result in the rapid death of the animal or to serve a reason for its euthanasia.

In practice, tumors were recorded by some researchers from the "fatal-incidental pathology standpoints" [26-29], notwithstanding the absence of generally adopted principles for this classification. Work [27] seems to be the most detailed example – the authors attempted to retrospectively determine death causes in mice and rats from ten different studies (4800 animals in total) using their own criteria for both tumors and non-tumoral pathologies. It is important that they rejected at once "probable" rubrics, and the pathological changes in the animals were divided into only two categories. The authors think that they have succeeded in such division for about 80% of rats and 70% of mice, and the lower percent of the established death causes they associated with the smaller size of mice in comparison with that of rats. The authors mentioned the difficulty in the verification of the death cause in animals with two diseases, but such cases were not frequent in their material. The criteria for considering diseases as fatal were given in the Supplement; in addition to tumors, they included only progressive nephropathy and polyarthritis in rats and glomerulopathy and ulcerative dermatitis in mice. "Disorders in vitally-important functions" was declared as the major principle of fatal pathology; however, it did not follow from the proposed criteria. All these criteria are morphological, rather arbitrary, and very unclear. Thus, the diagnosis of the death caused by progressive nephropathy in rats or by glomerulopathy in mice was based only on detection in the kidneys of histological manifestations of far-developed disease. But why clearly pronounced nephropathy has to be considered as the death cause but not a moderate cardiomyopathy (which is common in old Wistar rats [30] but not mentioned by the authors), or a concomitant tumor, or even sudden cardiac death – the parameters used cannot decide. Moreover, the authors similarly declare (quite arbitrarily, "based on previous experience") that liver tumors in mice are fatal if their size is larger than 12 mm. In addition, there is a reasonable question – if the size of 12 mm is fatal, why in some other dead animals larger tumors have been found? Lung tumors had no quantitative limits that would determine their role as the death cause except the note that "the tumor size was considered more significant than their malignancy". As to the "fatality" of ulcerative dermatitis – it was said, "mice with large and persisting skin damages were euthanized".

Thus, nearly all "fatality" criteria proposed by the authors do not withstand critics and are simply useless for practice. There were no attempts to propose standards for determination of death causes in animals, although in work [26] it was suggested that careful pathomorphological analysis of animals euthanized at different times during a long-term toxicological study could be useful for verification of fatal and incidental changes.

It is not surprising that in recommendations of "US National Toxicology Program" published in 2002 [31], it was specially emphasized that for statistical assessment of the results it was not necessary to subdivide tumors into fatal and incidental.

IT IS DIFFICULT BUT PRINCIPALLY POSSIBLE TO DETERMINE DEATH CAUSE OF LABORATORY ANIMALS

Taking into consideration all that has been said above, is it reasonable to question the cause of death of a laboratory rodent under conditions of a chronic experiment?

This question can in principally be answered in the affirmative but with some reservations. The "fatal–incidental" paradigm is faulty not because it is absolutely impossible or unwanted to establish the vital importance of a pathology, but because this idea is clearly premature and does not correspond to the current level of diagnosis of diseases in laboratory animals. In fact, there are some situations when the verification of the death cause is significant. Such information would be useful for many toxicological studies, for phenotyping rodents with genetic modifications, and especially for gerontological studies associated with searches for new geroprotectors. The geroprotective effect of a preparation is recorded based on its ability to decelerate the age-associated death of animals; it is clear that the understanding of the deceleration causes will be incomplete without the establishment of the death mechanism of the experimental and control animals. Studies on death causes become extremely important if the so-called non-aging animals (e.g. the

naked mole rat *Heterocephalus glaber* (Ruppell, 1842)) and new species of laboratory animals are introduced into practice [32, 33].

Reservations and conditions associated with a possibility of establishment of the death cause can be represented as the following positions: 1) it is necessary to initially submit with an idea that in some animals it will be impossible to determine correctly the death cause even under ideal conditions. It has been mentioned above that even in modern medicine there are situations when it is impossible to detect conclusively the disease resulting in a human's death; 2) for determination of death cause it is necessary to provide for lifetime individual monitoring of the animals' health, the conditions comparable with those existent for a patient's examination in clinics. This protocol must include daily objective examination (survey, weighing, palpation, thermometry) by a qualified veterinary with recording all signs in a special record and periodic complete general and biochemical analyses of blood and urine, ultrasonic diagnosis, electrocardiography, X-ray examination, blood pressure measurement, determination of lung functions, bacteriological and virological investigation of excretions, and studies on the functional and biochemical parameters are especially important immediately before the death; 3) a complete qualified examination of the corpse with modern methods sometimes including electron microscopy is extremely important; 4) a complex analysis of the resulting data by specialists in clinics, pathophysiology, and pathomorphology of laboratory animal diseases would be essential.

Certainly, such studies are very expensive and at present can be organized only in a few laboratories. However, with time they might become a standard for gerontological and toxicological studies. The widening of such a complex approach will promote developing in the future clinico-pathological criteria for determination of the death cause of animals with the same accuracy as in the modern hospitals and to remove the existing great number of white spots in pathology and thanatology of laboratory rodents. For example, up to now it is unclear whether such a severe disease as progressive nephropathy can lead mice to death or what set of pathological changes is sufficient for verification of anemia as the death cause of laboratory animals.

Thus, it is clear that wide studies on fatal and incidental character of pathologies are more likely a matter for the future. In all fairness, it must be noted that in some cases the death cause in rodents can be reliably determined by routine necropsy supplemented by a histological investigation. First, it concerns common infections with a clear morphological picture (such as ectromelia) and cases of disseminated tumors. However, sometimes it can be successful also in other pathologies. Thus, in mice with kidney amyloidosis, it is easy to establish the causal relation with the death in the case of subtotal damage of glomeruli and clearly pronounced exudates in serous cavities indicating terminal renal failure (certainly, in the absence of heart diseases with non-amyloid character) [21-23]. Similarly, on hemangioma rupture with bleeding and hemoperitoneum an error in the death cause determination is hardly likely even for an inexperienced pathologist. Obviously, this can be performed only at a rather careful pathomorphological examination on corpses with minimal manifestations of autolysis. If histological data can be supplemented with results of the premortal blood biochemistry, the resolution ability of the examination significantly increases. Thus, it was established that the death of animals with sarcomas without metastases was usually associated with development of hypoglycemia [34], which could be of significant help for determination of the role of the tumor in the development of fatal disorders in homeostasis. However, the question remains how to take data of such a thanatological analysis on considering their obvious incompleteness and the obvious presence of a large rubric of lesions non-classified with respect to the death cause. It seems that every time this question has to be solved individually, depending on the relative number of necropsies when it is impossible to determine objectively the death cause and certainly on the purpose of the study; and the publication of such analysis criteria also seems to be necessary in every case. It is possible that the most suitable solution of the problem is not the requirement to determine indisputably the only definite death cause, but using statistical analysis of the detected changes as "multiple death causes" – the approach that has been well proved in studies on human pathology.

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