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Histopathological Features of Organs in a Rat Model of Mammary Carcinogenesis: A Reference Database

Helena Vala[1,2], Carmen Vasconcelos-Nóbrega[1,2], Adelina Gama[3,4], Rita Ferreira[5], Paula A. Oliveira[1,3], Ana I. Faustino-Rocha[1,6]

[1] Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal; [2] Centre for Studies in Education, and Health Technologies (CI&DETS), Agrarian School of Polytechnic Institute of Viseu, Viseu, Portugal; [3] Department of Veterinary Sciences, UTAD, Vila Real, Portugal; [4] Animal and Veterinary Research Center (CECAV), UTAD, Vila Real, Portugal; [5]Associated Laboratory for Green Chemistry (REQUIMTE), Department of Chemistry, University of Aveiro (UA), Aveiro, Portugal; [6] Department of Zootechnics, School of Science and Technology, Évora, Portugal

Running title: Histopathological features of organs in a rat

Corresponding author: Ana Faustino, DMV, PhD; Universidade de Évora, Núcleo da Mitra, 7002-554 Évora, Portugal; Phone number 00351 266 740 800; E-mail address: anafaustino@uevora.pt

Abstract

Mammary tumors' development was induced through the intraperioneal administration of the carcinogen *N*-methyl-*N*-nitrosourea (MNU). Animals from group control were injected with the vehicle (saline solution). Animals were sacrificed at 25 weeks-old and the organs were histopathologically evaluated. A higher number of lesions was observed in the organs of animals from group MNU. The animals from group control did not present any lesion in lymph nodes. Independently of the experimental group, the internal organs presented hemodynamic alterations, degenerative and inflammatory changes. Hemodynamic changes may be consequence of euthanasia method. As expected, the higher number and the higher grade of the lesions in group MNU were due to the carcinogen administration.

Keywords: histological lesions, *N*-methyl-*N*-nitrosourea, rat model, rodent

Introduction

Animals have been used in biomedical research for a very long time. The discoveries using animals as models of disease are numerous and led to many Nobel Prizes. [1] Together with mice (*Mus musculus*), rats (*Rattus norvegicus*) are among the species more frequently used in research protocols performed in the European Union. According to the last report, approximately 11.5 million animals were used in experimental assays in Europe in the year 2011, and 75% of them were rodents (61% of mice and 14% of rats).[2]

Rats are considered a good fit for modeling human diseases. When compared with other species, like dogs or pigs, the rats have several advantages, namely: they are small animals easy to manipulate and accommodate, they are relatively cheap, their use may be easily approved by ethics committee, and their anatomy, genetic, physiology and biochemistry are well known and similar to humans. Moreover, the rat lifespan is relatively short when

compared with other animals and humans, having a lifespan of approximately one and a half years.[3-6]

Several rat strains are available for modeling different human diseases, like diabetes, obesity, cardiovascular diseases and cancer. The Sprague-Dawley female rats have been used in studies of chemically-induced mammary carcinogenesis.[7-10] Moreover, this model has been frequently used to address the interplay between cancer and potential therapeutic approaches. Despite this, a description of the main lesions found in organs besides the one that harbors the tumor of these animals is lacking. The knowledge of the more frequent lesions is of paramount importance to distinguish between lesions developed naturally due to animals' aging and those related to the carcinogen administration. In this way, we presented a thorough histological description of the lesions found in the main organs of female Sprague-Dawley rats used in an experimental assay of mammary carcinogenesis, thus providing a database for researchers working with this cancer model.

Material and Methods

***Animals*:** Twelve female Sprague-Dawley rats of four weeks of age were used (Harlan Interfauna, Barcelona, Spain). They were placed in the facilities of the University of Trás-os-Montes and Alto Douro (UTAD) under controlled conditions of temperature (23±2°C), humidity (50±10%), air system filtration (10-20 ventilations/hour) and on a 12h/12h light/dark cycle. Animals were fed with a standard laboratory diet (4RF21, Mucedola, Italy) and tap water *ad libitum*. All procedures followed the European and National legislation on the protection of animals used for scientific purposes (European Directive 2010/63/EU and National Decree-Law 113/2013). The experimental protocol was approved by the Ethics Committee of UTAD (CE_12-2013) and by the Portuguese Ethics Committee for Animal Experimentation (Approval no. 008961).

***Experimental protocol*:** After one week of quarantine and two weeks of acclimatization to the lab conditions, animals were randomly divided into two experimental groups as follows: group *N*-methyl-*N*-nitrosourea (MNU; n=10) and control (n=2). At seven weeks of age, animals from group MNU received an intraperitoneal injection of the carcinogen agent MNU (Isopac®, Sigma Chemical Co., Madrid, Spain) at a dose of 50 mg/kg. Animals from groups control received an intraperitoneal injection of the vehicle (saline solution 0.9%). The MNU administration was defined as the first day of the experimental protocol.

Animals were observed twice a day in order to monitor their health status during the experiment. Mammary chains from all animals were palpated once a week to detect the mammary tumors' development. Eighteen weeks after the MNU administration (animals with 25 weeks of age), all surviving animals were sacrificed by intraperitoneal injection of ketamine (75 mg/Kg, Imalgene 1000, Merial S.A.S., Lyon, France) and xylazine (10 mg/Kg, Rompun 2%, Bayer Healthcare S.A., Kiel, Germany), followed by exsanguination by cardiac puncture as indicated by the Federation for Laboratory Animal Science Associations.[11] The heart, lungs, liver, spleen, kidneys, adrenal glands, clitoral glands and lymph nodes were removed and immersed in buffered formalin for 24 hours.

Histological analysis*:** After fixation, the organs were included in paraffin and processed for routine histological evaluation. Two μm-thick paraffin sections were stained with hematoxylin and eosin (H&E) and histologically evaluated under a light microscopy. The lesions were scored from 0 to 4, according to the severity grading scheme criteria for various organs: ***grade 1 (Minimal: corresponds to a histologic change that may be barely noticeable to changes considered so minor, small, or infrequent as to warrant no more than the least assignable grade (0-10%). For focal, multifocal or diffusely distributed lesions, this grade is used for processes where <10% of the tissue is involved. For hyperplastic/hypoplastic/atrophic lesions, this grade is used when the affected structure or tissue has undergone <10% increase or decrease in volume); ***grade 2*** (Mild: corresponds to a histological change that is a noticeable but not a prominent feature of the tissue. For focal, multifocal or diffusely distributed lesions, this grade is used for processes with an involvement of 11-20% of the tissue is involved. For hyperplastic/hypoplastic/atrophic lesions, this grade is used when the affected structure or tissue has undergone between an 11% and 20% increase or decrease in volume.); ***grade 3*** (Moderate: corresponds to a histological change that is a prominent feature of the tissue. For focal, multifocal or diffusely distributed lesions, this grade is used for processes where 21-40% of the tissue section is involved. For hyperplastic/hypoplastic/atrophic lesions, this grade is used when the affected structure or tissue has undergone between a 21% and 40% increase or decrease in volume) and ***grade 4*** (Marked: corresponds to a histological change that is an overwhelming feature of the tissue. For focal, multifocal or diffusely distributed lesions, this grade is used for processes where 41-100% of the tissue section is involved. For hyperplastic/hypoplastic/atrophic lesions, this grade is used when the affected structure or tissue has undergone between a 41% and 100% increase or decrease in volume).[12]

Statistical analysis: The descriptive analysis was performed with Microsoft® Excel for MAC, version 16.39 (Microsoft, USA).

Results

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Animals and mammary tumors: Animals from group MNU developed 21 tumors. As expected, animals from control group did not develop any mammary tumor.

Histological analysis: The histological lesions observed in the heart, lungs, liver, spleen, kidneys, adrenal glands, clitoral glands and lymph nodes are summarized in Tables 1 to 6, respectively.

As expected, a higher number of lesions was observed in the heart, lungs, liver, spleen, kidneys, adrenal glands, clitoral glands and lymph nodes of animals exposed to MNU, when compared with non-exposed animals (group control). The animals from group control did not present any lesion in lymph nodes.

The ***heart*** mainly exhibited foci of coagulative myocytolysis (focal necrosis of cardiac myocytes), congestion (increase of blood in a particular tissue due to poor venous outflow), hemorrhage (few to many small, irregular foci of extravasated erythrocytes scattered within the myocardium) and hyperemia (localized increase in the blood volume of a particular tissue due to local arteriolar dilation). The number and the grade of the lesions were higher in group MNU, when compared with group control (Table 1). The interstitial inflammation, arteriolosclerosis, arteriosclerosis (proliferative and/or degenerative change to the tunica media and tunica intima of arteries, resulting in loss of elasticity and luminal narrowing), congestion and hyperemia were the main lesions identified in the ***lungs***. The grade of lung interstitial inflammation and arteriolosclerosis was higher in group MNU when compared with group control (Table 2). ***Liver*** presented mainly interstitial inflammation and congestion. The number and histological grade of interstitial inflammation and congestion were higher in group MNU, when compared with group control. Cholestasis was only observed in the liver of group MNU (Table 3). The interstitial inflammation, congestion (characterized by excessive distension of sinuses within the red pulp by erythrocytes), hemossiderosis (intracytoplasmic accumulation of hemosiderin) and hyperemia were the lesions more frequently observed in the ***spleen***. The number and the grade of congestion and hemossiderosis were higher in animals MNU-exposed, when compared with non-exposed ones. The hyperemia was only observed in animals MNU-exposed (Table 4). The ***kidneys*** mainly presented congestion, hyperemia, presence of tubular hyaline casts and cystic dilations. The number and the grade of these kidney lesions were higher in group MNU, when compared with group control. The cystic dilations were only observed in groups MNU (Table 5). The ***adrenal glands*** of group MNU mainly developed hyperplasia (focal to diffuse increase in cell number), congestion and hydropic degeneration. Similarly, to those described in the other organs, the number and histological grade of the lesions was higher in group MNU when compared with non-exposed animals (Table 6). The ***clitoral glands*** of group MNU exhibited ductal dilation, interstitial inflammation and hyperemia. The clitoral glands of animals from group control only exhibited ductal dilation and interstitial fibrosis. Ductal dilation exhibited a higher grade in group MNU (Table 6). ***Lymph nodes*** of group MNU exhibited inflammatory infiltrate and congestion. No lesions were observed in lymph nodes of group control (Table 6).

Discussion

Rats have been used as models of human diseases for a long time. Indeed, they have many advantages when compared with other animals, allowing a simple and not expensive modeling of human diseases. This work intended to identify the histopathological alterations in organs (heart, lungs, liver, spleen, kidneys, adrenal glands, clitoral glands and lymph nodes) of female rats included in an experimental assay of mammary carcinogenesis, providing a database for researchers working with this strain.

The semiquantitative analysis of lesions in toxicologic pathology involves the application of severity grades. Severity grading consists on the application of defined numerical (or numerically equivalent) severity scores of specific lesions. The severity grades are primarily determined by the extent or an estimate of the percent of tissue involvement, as well as, the

magnitude of various components present. The number of severity grades will affect the sensitivity and reproducibility of the evaluation. The lesions should be easily allocated to the severity grades.[12-15]

The group control was used in order to assess if the histological alterations occurred due to the experimental procedures (MNU administration) or animals' aging. The animals were sacrificed with 25 weeks of age. In a general way, they mainly presented hemodynamic vascular changes in almost all organs (heart, lungs, liver, spleen and kidneys), which may be related with the euthanasia. The number of lesions and their histological grade were higher in group MNU, when compared with non-exposed animals (group control), being probably related with the carcinogen administration. The reduced number of lesions and their low grade in group control showed that the age and the procedures were not the probable cause of these lesions.

The **myocardial** hemorrhage may be accompanied by other morphological changes in myocardium, like necrosis and inflammation. The heart of these animals also presented coagulative myocytolysis. According to the literature, the presence of hemorrhage and necrosis may be indicative of vascular damage or vasoconstriction of small vessels or represent myocytic toxicity. The congestion observed in the heart generally occurs due to some circulatory pathology in veins downstream of the congested area. The hyperemia is an active process that represents a physiological response to tissue requirements [16-18] (Figure 1A). The **lungs**' vascular alterations, namely arteriolosclerosis and arteriosclerosis, probably occur due to the animals' aging (Figures 1B and C). Aggregates of mixed inflammatory cells may be regularly seen in the **liver** of normal rats. According to Foster [19], the inflammatory infiltrate may be observed in young rats, increases with age, and its etiology remains unknown. According to Rebelatto (2008), the inflammatory lesions are not frequent in the **spleen** of rodents. The congestion is common in this organ and it may be associated with methods of euthanasia and necropsy procedures (exsanguination). Hyperemia may be evident in cases of inflammation.[20] The accumulation of intracytoplasmic pigment, like hemosiderin, within the splenic red pulp macrophages is a common finding in rodents. The hemosiderin pigment results of the macrophage engulfment of red blood cells or free hemoglobin and, like other pigments (ceroid/lipofuscin and melanin), it is common in aging rats and is usually higher in females than in males.[21,22] Considering their role in filtration, metabolism and excretion of compounds, the **kidneys** should be carefully examined. A wide range of spontaneous lesions may be observed in the kidneys. Hyaline casts occur as spontaneous or treatment-related condition in mice and rats. Cystic dilations may be solitary or multiple, and be placed in the renal parenchyma, cortex, medulla or papilla. They may be associated with chronic progressive nephropathy or chemical administration.[23-27] The renal lesions observed may be related with animals' age and probably potentiated by MNU administration (Figure 1D). **Adrenal glands** presented foci of hyperplasia in the cortex and hydropic degeneration (Figure 1E). The hyperplasia may affect only one or both glands. It may be focal or diffuse, in the cortex or in the medulla. In rats and mice, cortical hyperplasia occurs mostly in the zona fasciculata, but may also affect zona reticularis. The hyperplasia may be a spontaneous aging change or a consequence of chemicals administration, and occurs more frequently in rats than in mice. The degeneration of adrenal glands was previously reported in many rat strains. The Sprague-

Dawley strain is one of the most frequently affected by this alteration, probably due to aging or chemicals administration. [28-38] Inflammation is a common finding in the **clitoral gland** that may be associated with treatment.[39] Despite this, one case of interstitial fibrosis and one case of ductal dilation were observed in control group. The fibrosis of the clitoral gland is commonly associated with degenerative lesions, like atrophy, ductal dilation, and inflammation. Ductal dilation is a common finding in aging rodents, and it has been associated with steroids administration. Once no steroids were administered to our animals, ductal dilation may be age-related. Although it is described that the ductal dilation of the adrenal gland is frequently associated with atrophy of the glandular tissue, no atrophy was detected in the clitoral glands of our animals [37,40] (Figure 1F). **Lymph nodes** congestion was previously described in rodents. It may be secondary to another lesions, like neoplasia, or result from the euthanasia method or necropsy. [40,41]

Conclusions

Hemodynamic changes may be consequence of euthanasia method. The higher number and the higher grade of the lesions in group MNU when compared with group control is probably related to the carcinogen administration.

Conflict of interests: None to declare.

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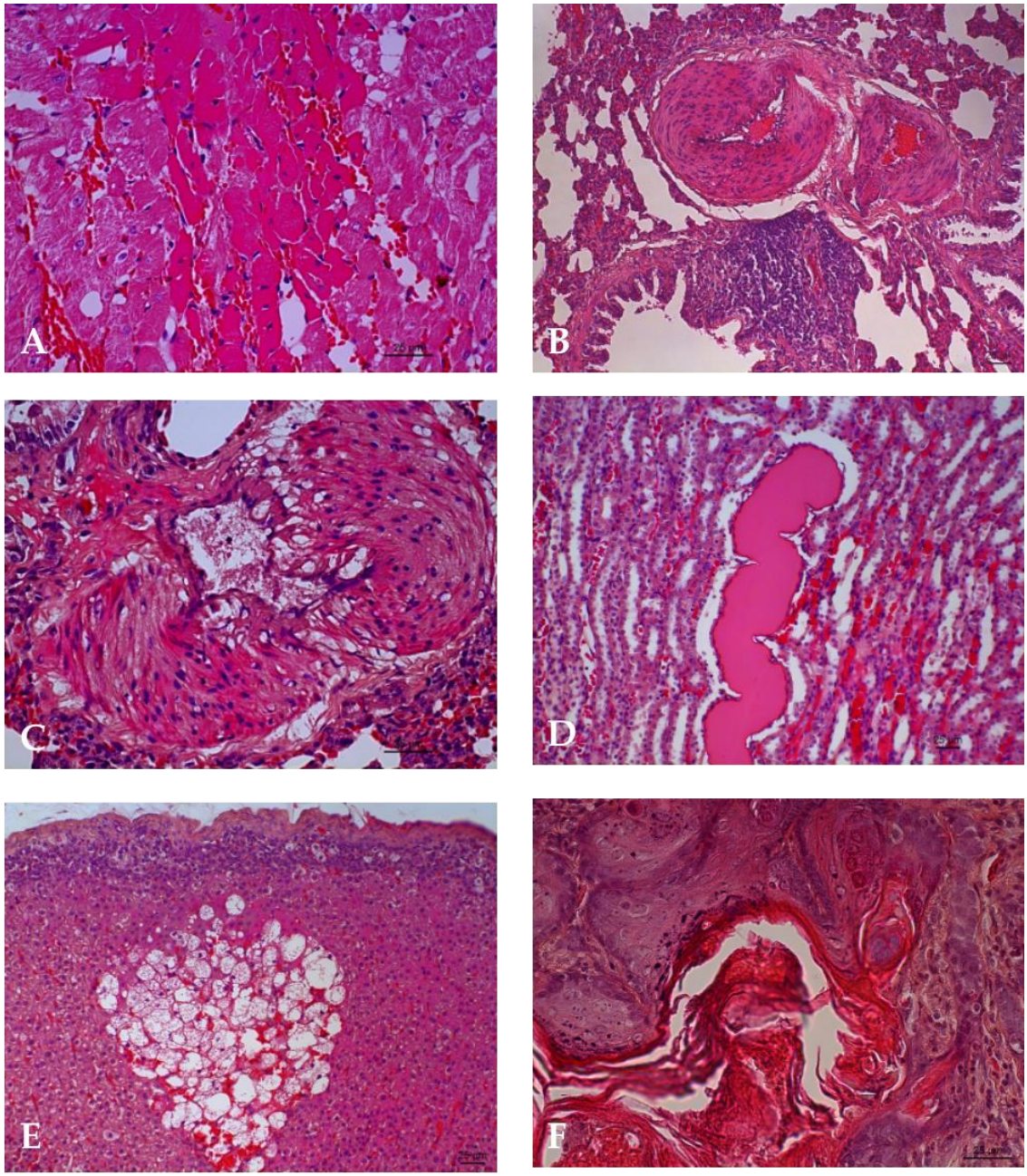

Figure 1. Histological lesions in the systemic organs of animals from all experimental groups. Hematoxylin and eosin staining. **(A)** Heart, group MNU. Congestion (grade 2) and coagulative myocytolisis (grade 3). **(B)** Lung, group MNU. Hyperemia (grade 3), mononuclear inflammatory infiltrate (grade 2). **(C)** Lung, group MNU. Arteriosclerosis (grade 3). **(D)** Kidney, group control. Hyaline casts (grade 1). **(E)** Adrenal gland, group MNU. Congestion (grade 3). **(F)** Clitoral gland, group MNU. Ductal dilation (grade 3) and mixed inflammatory infiltrate (grade 2).

Table 1. Histological lesions in the heart of animals from all experimental groups.

Organ	Lesion	Score	Group (number of animals with lesions)	
			MNU (n=10)	Control (n=2)
HEART	*Hyalinization*	1		1 (50%)
	Coagulative myocytolysis	2	4 (40%)	1 (50%)
		3	3 (30%)	
	Congestion	1		2 (100%)
		2		
		3	4 (40%)	
		4	3 (30%)	
	Hemorrhage	1		1 (50%)
		2		1 (50%)
	Hyperemia	1		
		2		
		3	7 (70%)	
Total number of animals with lesions			21	6

Table 2. Histological lesions in the lungs of animals from all experimental groups.

Organ	Lesion	Score	Group (number of animals with lesions)	
			MNU (n=10)	Control (n=2)
LUNGS	*Interstitial inflammation*	1		1 (50%)
		2	8 (80%)	1 (50%)
		3	2 (20%)	
	Arteriolosclerosis	2		2 (100%)
		3	10 (100%)	
	Arteriosclerosis	1	9 (90%)	
		2		
	Congestion	2	1 (10%)	
		3	9 (90%)	2 (100%)
		4		
	Hyperemia	2		
		3	10 (100%)	1 (50%)
		4		1 (50%)
Total number of animals with lesions			49	8

Table 3. Histological lesions in the liver of animals from all experimental groups.

Organ	Lesion	Score	Group (number of animals with lesions)	
			MNU (n=10)	Control (n=2)
LIVER	*Interstitial inflammation*	1	8 (80%)	1 (50%)
		2		
		3		
	Congestion	1		2 (100%)
		2		
		3	7 (70%)	
		4	1 (10%)	
	Cholestasis	1	1 (10%)	
		2		
Total number of animals with lesions			17	3

Table 4. Histological lesions in the spleen of animals from all experimental groups.

Organ	Lesion	Score	Group (number of animals with lesions)	
			MNU (n=10)	Control (n=2)
SPLEEN	*Interstitial inflammation*	1	9 (90%)	1 (50%)
		2		
		3		
	Congestion	2		
		3	1 (10%)	
		4	8 (80%)	2 (100%)
	Hemossiderosis	2		1 (50%)
		3	7 (70%)	1 (50%)
		4	2 (20%)	
	Hyperemia	1		
		2	6 (60%)	
		3	3 (30%)	
Total number of animals with lesions			36	5

Table 5. Histological lesions in the kidneys of animals from all experimental groups.

Organ	Lesion	Score	Group (number of animals with lesions)	
			MNU (n=10)	Control (n=2)
KIDNEYS	*Congestion*	1		2 (100%)
		2		
		3	3 (30%)	
		4	6 (60%)	
	Hyperemia	1		1 (50%)
		2		
		3	9 (90%)	
	Blebbing	1		1 (50%)
	Hydropic degenerescence	1		2 (100%)
	Hyaline casts	1		1 (50%)
		2	4 (40%)	
		3		
		4	1 (10%)	
	Cystic dilations	1	1 (10%)	
		2	1 (10%)	
		3	7 (70%)	
Total number of animals with lesions			32	7

Table 6. Histological lesions in the adrenal glands, clitoral glands and lymph nodes of animals from all experimental groups.

Organ	Lesion	Score	Group (number of animals with lesions)	
			MNU (n=10)	Control (n=2)
ADRENAL GLANDS	*Hyperplasia*	1	5 (50%)	
		2	2 (20%)	
		3		
	Congestion	1		2 (100%)
		2		
		3	6 (60%)	
		4	1 (10%)	
	Hydropic degeneration	1	7 (70%)	
		2		
		3		
Total number of animals with lesions			21	2
CLITORAL GLANDS	*Interstitial fibrosis*	1		
		2		1 (50%)
	Ductal dilation	1		1 (50%)
		2		
		3	4 (40%)	
		4	4 (40%)	
	Interstitial inflammation	1		
		2	2 (20%)	
		3	5 (50%)	
	Hyperemia	1	8 (80%)	
		4		
Total number of animals with lesions			23	2
LYMPH NODES	*Infiltrate*	1	2 (20%)	
		2	1 (10%)	
	Congestion	2	2 (20%)	
		3	1 (10%)	
Total number of animals with lesions			6	0