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Successful hormonal and chemical induction of prostate cancer in a rat model: practical guidelines

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Abstract

Prostate cancer is a very common cancer in men, affecting approximately 1.40 million men worldwide in 2020. To improve the quality of life and survival of both animals and humans, effective therapeutic approaches have been developed and evaluated using animal models. The rat model of prostate cancer induced by a multi-step protocol that consists of a sequential administration of flutamide, followed by testosterone propionate, then the administration of N-methyl-N-nitrosourea, and finally subcutaneous implantation of tubes filled with crystalline testosterone, is one of the most frequently used for prostate cancer research. However, the lack of standardization in procedures for prostate cancer induction, sample collection, and analysis represents a challenge for researchers. To address this issue, we aim to provide investigators with a detailed, step-by-step guide to implementing a rat model of prostate cancer, based on our extensive experience in this field. First, we briefly review the prostate cancer-induced protocols found in the literature, then we provide a detailed description of the prostate cancer rat model implemented by our team. After, we explore the rats' prostate monitoring during the experiment protocol through imaging modalities, such as ultrasonography, computed tomography, and magnetic resonance imaging. We also describe animal welfare monitoring based on a table of humane endpoints, as well as data collection, such as biological variables and prostate samples. In sum, this article will ensure the quality of results and enable their comparison among different researchers using this rat model.

Introduction

Cancer is one of the main global public concerns. Despite advances in detection and treatment, it remains a prominent cause of death worldwide, accounting for nearly 10.00 million deaths in 2020, or nearly one in six deaths.[1] Prostate cancer affected approximately 1.40 million men worldwide, making it one of the most prevalent tumors in males.[1] The economic impact of cancer is noteworthy and increasing year by year. According to the world health organization, costs related to cancer will reach around 245 billion U.S. dollars in 2023.[2] These predictions emphasize the need for further research into cancer in general, and prostate cancer in particular.

While *in vitro* studies have undoubtedly made significant contributions to biomedical research, animals have been an essential component of research for over 4,000 years.[3] Animals have been invaluable tools in advancing our understanding of the anatomy, physiology etiology, pathogenesis, progression, and genetic and molecular basis of diseases in both animals and humans.

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Animal models have played a key role in the development and evaluation of effective therapeutic strategies for both animals and humans, improving their quality of life and survival. Today, animals remain indispensable tools in biomedical research, aiding researchers in understanding diseases and developing novel treatments that benefit both humans and animals.[4-6]

Several animal species are available as models in biomedical research, such as rabbits, rats, mice, dogs, fish, and non-human primates, but rats (*Rattus norvegicus*) and mice (*Mus musculus*) remain the most frequently used.[7] This is because these mammals share several similarities with humans and other animals in terms of their anatomy, physiology, genetics, and biochemistry. They are also small animals, easily accommodated and manipulated. Their physiology and genetics are well known, they are relaively inexpensive compared to other species, their use is easily approved by Animal Welfare Bodies and National Competent Authorities, and they are susceptible to both hormones and carcinogenic agents.[5] Cancer modeling may use strains that spontaneously develop tumors, implantation of cancer cell lines (syngeneic or xenograft), genetically engineered animals, or induce cancer development in the target organ, through the administration of carcinogenic compounds, irradiation, or hormone administration, depending on the type of cancer.[8]

Prostate cancer chemically and hormonally-induced. By definition, a chemical carcinogen is any compound with the ability to induce cancer development in living tissues.[9] Depending on the characteristics of the chemical carcinogen, the route of administration, absorption site, organ of metabolism, and excretion, carcinogens can induce tumor development in different organs.[10] In humans, there are several hormone-related cancers, like prostate, breast, ovarium, and endometrium cancers. Exposure to hormones may lead to an increase in cell proliferation, making cells more prone to random genetic errors, which are the origin of carcinogenesis.[11] In laboratory animals, it is possible to induce cancer by simultaneously administering chemical carcinogens and hormones. Regardless of the method used to induce cancer, *in vivo* models of carcinogenesis must be as simple and fast to implement as possible, while also ensuring the safety of researchers. These models must also follow the 3Rs principles of animal experimentation and be reliable, reproducible, and consistent, allowing for accurate and meaningful data collection and analysis.[9,12] The rat model of prostate cancer induced by the administration of chemical carcinogens and hormones (testosterone) is one of the most frequently used for prostate cancer research[13]. Until now, four carcinogens have been described to induce prostate cancer: *N*-nitrosobis (2-oxopropyl) amine, *N*-methyl-*N*-nitrosourea (MNU), 7,12-dimethylbenz[a]-anthracene (DMBA) and 2-amino-1-methyl-6-phenyl-imidazol[4,5-b]pyridine.[14,15] The DMBA is a polycyclic aromatic hydrocarbon that needs to be activated by the cytochrome P-450/P1-450 monooxygenase enzyme systems to exert its carcinogenic effects.[16] Although effective for prostate cancer induction, the DMBA is less used when compared with MNU, because it must be dissolved in oil and administered by gavage, negatively impacting the animals' welfare and survival. The MNU is a potent and direct alkylating agent that reacts with nucleophilic nitrogen and oxygen atoms in the purine and pyrimidine bases and the phosphate group of DNA, producing a wide range of DNA adducts.[17-19] These adducts are responsible for its mutagenic action. In contrast to DMBA, the MNU does not require metabolic activation before its interaction with DNA. This compound is soluble in water and can be administered by several routes, such as intraperitoneally, making it one of the most commonly used carcinogens. As carcinogens, DMBA and MNU should be carefully manipulated and prepared in a laminar flow chamber. The manipulators should use adequate personal protective equipment, including an impermeable lab coat, a surgical cap, a mask, protective glasses, and two pairs of gloves. The residues should be eliminated as Type IV waste, following the national and European guidelines. The model of chemically and hormone-induced prostate cancer was developed by Pollard in Lobound Wistar rats.[20] This protocol involved a single intravenous injection of the carcinogen MNU at a dose of 30.00 - 40.00 mg kg^{-1}, followed by long-term administration of testosterone (10.00 - 40.00 mg) via silastic implants.[21] This protocol induced adenocarcinomas and atypical hyperplastic lesions in the ventral, dorsolateral, and anterior prostate lobes. Over the years, other researchers have improved upon this model, and today, the most commonly used method for inducing prostate cancer involves a sequential treatment with an antiandrogenic drug (flutamide or cyproterone acetate), followed by a single intravenous or intraperitoneal administration of the carcinogen MNU, and long-term treatment with testosterone placed in silastic implants.[13] Marteen Bosland described this experimental protocol in detail: cyproterone acetate (50.00 mg kg^{-1} per day) may be prepared in water and administered by gavage, or dissolved in oil and injected subcutaneously, during 2 - 3 consecutive weeks, followed by subcutaneous administration of testosterone propionate (10.00 - 100 mg kg^{-1}) over 3 consecutive days. Forty-eight hr later, MNU should be intravenously or intraperitoneally injected in doses between 30.00 and 50.00 mg kg^{-1}, and finally, silastic implants filled with testosterone propionate should be placed subcutaneously in the interscapular region by surgical approach under general anesthesia.[13] Although complex to execute, this sequential protocol has been shown to result in a higher incidence of prostate cancer in rats. To achieve the maximal tumor incidence, the experimental protocol should be conducted for up to 50 - 60 weeks after MNU injection.[13]

Despite the many advantages of using rodent models of prostate cancer, there are also some limitations that researchers should be aware of. One such limitation is the anatomical differences between rodents and dogs or humans (e.g. lobulated prostate in rodents versus non-lobulated prostate in dogs and men).[22] Although the dogs' and men's prostates are compact solitary structures, the rodents' prostates consist of four distinct lobes: the ventral, lateral, dorsal, and anterior lobes, according to their relative position to the urinary bladder.[15] Despite these anatomical differences, the dorsal and lateral lobes are considered homologous to the prostate of men and share some similarities in terms of the development and progression of the disease, as weel as in histopathological characteristics, makingthe rat a valid model to study this type of cancer.[23-26]

Experimental protocol. All experimental assays should be evaluated and approved by the Animal Welfare and Ethics Body (ORBEA) and National Competent Authorities prior to bein conducted, to ensure compliance with standards related to animal welfare. The experimental protocol for prostate cancer induction through chemical and hormonal exposure was performed by our research team, to evaluate a non-pharmacological therapeutic approach. This multi-step eprotocol was employed in Wistar rats, as displayed in Figure 1. It was initiated at 12 weeks of age with the subcutaneous administration of the anti-androgenic drug flutamide (50.00 mg kg^{-1}) for 21 consecutive days. The flutamide solution was prepared immediately before use in a laminar flow chamber with sterile material. The flutamide is commercially available as a yellow powder.

It was weighed on a scale based on the animals' body weight and dissolved in 9.00 mL of 96.00% alcohol, 18.00 mL of propylene glycol, and 162 mL of bi-distilled water. The solution was then stirred using a magnetic stirrer, stored, and protected from light. Since precipitation was observed when agitation was stopped, the solution was continuously stirred until it was administered to all animals. For each administration, the animals were wrapped in a cloth and the dorsal region was exposed, where the subcutaneous administration was carried out using 25G needles and 2.00 mL syringes. The solution was shaken in the syringe to avoid flutamide precipitation, and administrated between 8:30 am and 10:30 am, two times per week for 21 consecutive days. Twenty-four hr after the last flutamide administration, testosterone propionate was dissolved in corn oil and subcutaneously administered to the animals at a dose of 100 mg kg^{-1} only one time. Forty-eight hr later, after testosterone propionate administration, the MNU was prepared and intraperitoneally administered at a dose of 30.00 mg kg^{-1}. The MNU was pre-weighed in an appropriate vial, and kept stored at – 20.00 ˚C; just before administration, a 0.10 M phosphate-citrate buffer, pH 4.80, was added at room temperature at $1/15^{th}$ of the final volume, and this was further diluted with normal saline in a ratio of 1/14 (v/v) to make a final concentration of 10.00 mg mL^{-1} (for 100 mL of the final solution, 6.70 mL 0.10 M phosphate-citrate buffer were added along with 92.30 mL of the normal saline; the buffer was prepared by mixing approximately two volumes of a 0.10 M di-sodium phosphate solution to one volume of a 0.10 M citric acid solution and adjust the pH to 4.80; the saline solution is a 0.85% solution of NaCl). The MNU is a potent direct-

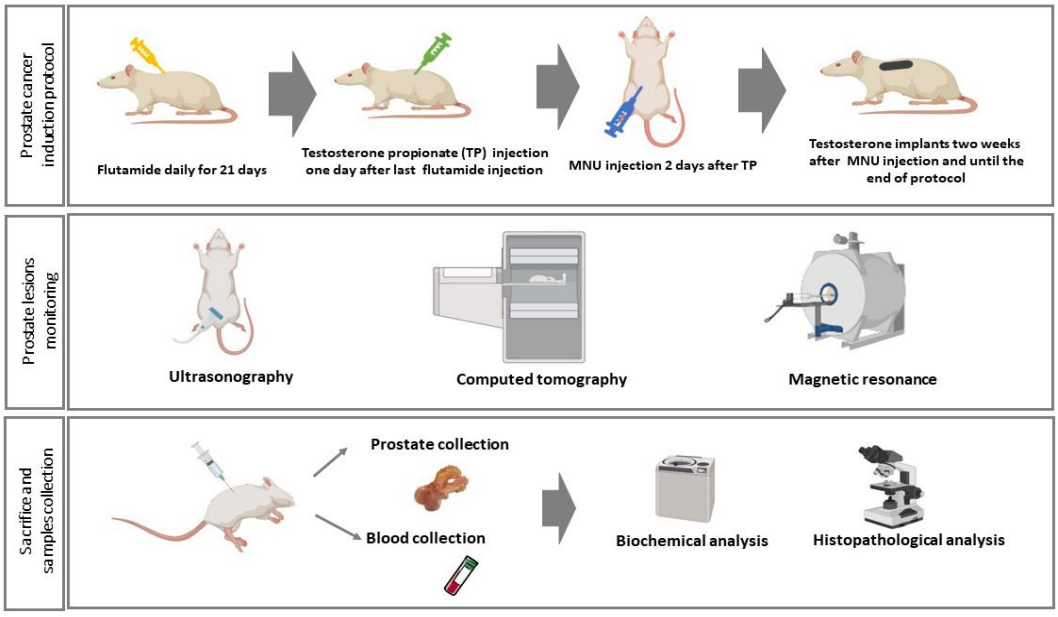


Fig. 1. Schematic representation of a protocol for prostate carcinogenesis chemically and hormonally induced in male rats, beginning with the preparation of the carcinogen agent, and ending with the animals' sacrifice and biochemical and histopathological analysis. Created with BioRender (https://www.biorender.com). MNU: N-methyl-N-nitrosourea.

acting carcinogen that is very unstable at pH 7.00 or higher. All administrations were performed by experienced researchers, who wore protective Tyvek coveralls, head cover, sleeves, double nitrile gloves, and a disposable respirator. The outer gloves were changed as soon as they were possibly contaminated, and all protective clothing was taken off when leaving the animal room, and then submitted for incineration. All personnel must adhere to biosafety and biosecurity measures (e.g. washing hands, arms, and face or even taking a shower). The injection procedure was done on a table, with the person administering the injection seated on a stool and working inside a large tray. The table and tray were covered with plastic-backed absorbent paper, and all overtly contaminated waste was put in a disposable container or plastic bag. Immediately after injection, the animals were placed in a solid bottom cage. They remained in this cage until the first cleaning was necessary (after approximately 7 days). The bedding was disposed of as chemical waste and the cage was cleaned twice in a cage washer. The rats were then placed in their normal cages.

The personnel biosafety and biosecurity precautions were in effect until after the rats were moved to regular cages. All equipment used and the animal room were cleaned thoroughly with an alkaline detergent. After 2 weeks, testosterone implants were subcutaneously implanted in the interscapular region of animals anesthetized with ketamine (75.00 mg kg^{-1}) and xylazine (10.00 mg kg^{-1}) and remained for 18 or 44 weeks. The animals were shaved on their backs in the thoracic region, and the area was wiped down with 70.00% alcohol. Using a sterile technique, the implants were placed under the skin and the incision was closed using surgical staples. The testosterone implants were made from silastic tubing (Dow Corning; VWR Scientific, Mississauga, Canada) sealed with RTV-108 adhesive sealant (General Electric Co., Waterford, USA). The implants were soaked in 70.00% alcohol for 30 min and washed with a sterile buffer using semi-sterile techniques. The implants were soaked in phosphate buffer (pH 7.00) for 2 days before implantation. The buffer was changed twice daily and once approximately 1 hr before implantation. About 4.00 cm long pieces of tubing was cut and inserted on one end of a blue pipet tip filled with cotton that was attached to a 1.00 mL syringe. This syringe was attached to a tube that leads to a vacuum pump and a small hole was made in the syringe to regulate the suction with a finger. Crystalline testosterone was then sucked up from a small dish until there was about 3.00 cm of filling. The tube was then detached using a metal rod to compact the testosterone in the tube. A small glass bead was inserted on either end, ensuring that the distance between the beads was 3.00 cm. Both ends were sealed with silicone glue and allowed to cure for 24 hr. The ends were cut so that the excess glue was removed and there was a smooth end on both sides of the tube. Of note, more than the precise amount of testosterone inside the implant, it is the length of the filling that determines how much testosterone is released, together with the wall thickness and diameter of the silastic tube. The testosterone implants must be prepared inside a fume hood, while wearing a lab coat, using double glove procedures, and wearing a disposable respirator as a secondary barrier. Gloves should be worn while handling the implants and the washing fluid, as well as during implantation.

Follow-up of prostate cancer development.The rats' prostate modeling can be monitored during the experiment through imaging modalities, namely ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI; Fig. 1). The ultrasonographic examination was performed several times during the experimental protocol, while the CT and MRI examinations were only performed once. This is because the ultrasonographic examination is a more accessible and less expensive technique and can be performed in awake animals if they are adapted to the handling, while the CT and MRI examinations require the animals' anesthesia. As our animals are frequently handled, we performed ultrasonographic examinations in awake animals. If the animals are not adapted to the handling, they should be sedated with an intraperitoneal administration of ketamine (37.50 mg kg^{-1}) and xylazine (5.00 mg kg^{-1}). Immediately before the exam, the animals' abdominal region was shaved using a clipper machine. During the ultrasonographic exam, the animals were restrained in a supine position, and the exam was made as quickly as possible by an experienced researcher. A linear high-frequency probe (10.00 to 12.00 MHz) and ultra-sound transmission gel were used in our examinations. The prostate lobes' appearance and size can be monitored using B-mode, while prostate vascularization may be assessed by using Power Doppler, B Flow, Pulsed Doppler, and Contrast-enhanced ultrasound (CEUS).

During the ultrasonographic examination, sagittal and transverse images of the prostate lobes can be obtained, and measured using electronic cursors integrated into the ultrasound machine and set at the borders of the prostate lobes. Several changes in prostate size and echogenicity were observed throughout the experiment, in response to flutamide, testosterone, and MNU administration. For example, the ventral prostate area of the prostate cancer-induced groups decreased due to flutamide administration and increased after androgen and carcinogen administration. Additionally, in the last ultrasonographic examination, hypoechoic and anechoic lesions were observed in the prostate cancer-induced group.[27] Moreover, in the control group, the size of the prostate lobes gradually increased from the first to the last exam, as a consequence of animal growth.[28] The prostate gland and prostate tumors' vascularization may be assessed by the

quantification of color pixels in Power Doppler and B Flow images in Adobe Photoshop or GIMP (GNU Imaging Manipulation Program) through the formula: [29]

$$Color\ pixels\ density\ (\%) = \frac{Number\ of\ colored\ pixels\ in\ the\ tumor}{Number\ of\ total\ pixels\ of\ the\ tumor}$$

The blood flow can also be characterized in Pulsed Doppler mode, by determining the pulsatility and resistive indexes using the software of ultrasonographic apparatus. Usually, immediately before the animals' sacrifice, they are anesthetized them by an intraperitoneal administration of ketamine (75.00 mg kg^{-1}) and xylazine (10.00 mg kg^{-1}) and the tumor's vascularization is evaluated by CEUS. For this, 0.10 mL of the contrast agent SonoVue (Bracco, Milan, Italy) in injected into the tail vein after its cannulation, followed by a flush of 1.00 mL of saline solution, with no risks for the animal. The use of contrast allows the visualization of small vessels, not previously detected by B Flow or Doppler. The quantitative analysis of the CEUS images can be performed using the time-intensity curve analysis of the ultrasound apparatus.[30] According to our experience, an ovoid region of interest can be drawn in the most enhanced/vascularized area of the tumor. The CT and MRI also allow the detection of changes in the prostate, such as alterations in size and morphology, including the detection of lesions.[28]

Animal welfare monitoring. In compliance with the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA), it is mandatory to monitor animals' welfare during experiments to ensure data reliability and quality. In our experimental protocol, we applied the Table of Humane Endpoints (Table 1) previously described by our team for a rat model of chemically induced mammary cancer,[31] This table includes the following parameters to be monitored: general appearance and mental status, body condition, body weight, food and water intake, posture, coat and grooming, mucosal, eyes, ears and whiskers, mental status, behavior (response to external stimuli), hydration status, respiratory and heart rate, body temperature, hematocrit, and urine specific gravity. A score from zero to three was attributed to each parameter, and a sum equal to or greater than four was indicated for animal sacrifice. According to our experience, this model does not induce significant changes that justify the animals' sacrifice before the end of the experimental protocol.

Body weight, food and water consumption. The monitoring of animals' body weight, and food and water consumption, is an indirect way to assess animals' welfare. In our experiment, in addition to daily observation, the animals were individually weighed once a week, using a top-loading scale. We then determined the ponderal weight gain (%), by applying the following formula:[32]

$$Weight\ gain\ (\%) = \frac{Final\ body\ weight - Initial\ body\ weight}{Final\ body\ weight} \times 100$$

Similarly, the food and water were also weighed every week, and the mean food and water consumption per animal was calculated. The data is always compared between control animals and those exposed to carcinogens and hormones.

Samples collection. Although some samples may be collected throughout the experimental protocols, such as blood samples from the tail vein, and urine and feces samples using metabolic cages, the sacrifice is the ideal moment for the collection of a high diversity of samples. The animals should be sacrificed following FELASA guidelines. Usually, our animals are sacrificed by an intraperitoneal overdose of ketamine and xylazine, followed by exsanguination by cardiac puncture, but they can also be sacrificed by a pentobarbital overdose. Immediately after collection, the blood samples are processed for hematological or biochemical evaluations. Afterward, a complete necropsy is performed. For this, the animals are placed in the supine position and an incision is made in the median ventral line. All organs are carefully observed *in situ* (Fig. 2), and then the lungs, heart, liver, spleen, kidneys, and male accessory glands are collected and weighed on a top-loading scale.

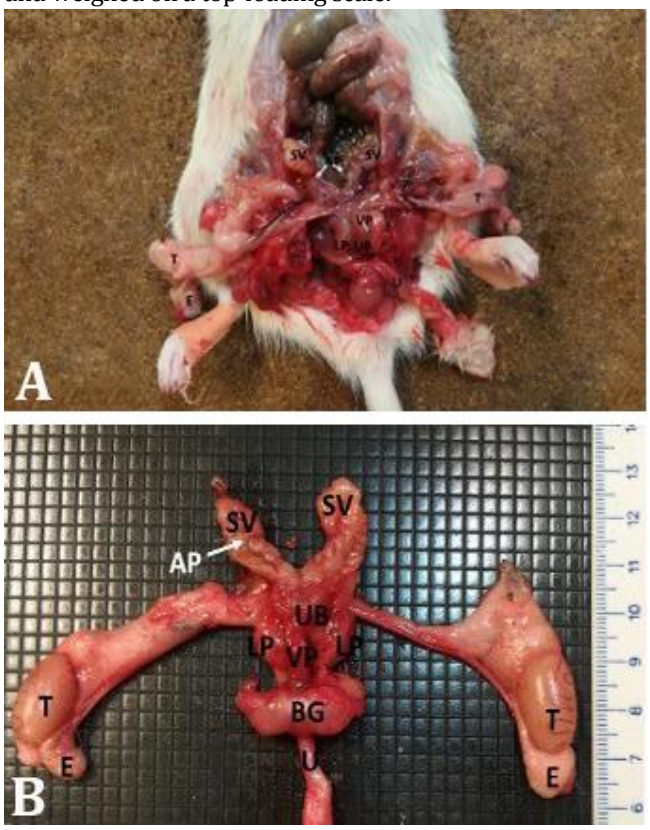
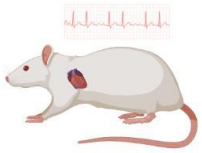


Fig. 2. Necropsy of a male rat. **A)** Macroscopic observation of the organs *in situ* and **B)** collection of male accessory sex glands together. Anterior prostate lobe (AP), lateral prostate lobe (LP), ventral prostate lobe (VP), seminal vesicles (SV), urinary bladder (UB), bulbourethral gland (BG), urethra (U), testes (T), and epididymis (E).

Table. 1. Humane endpoints applied to the rat model of chemically and hormone-induced prostate cancer. Adapted from: Faustino-Rocha *et al.*[31] and created with BioRender.

Parameters	Classification	Score
Body condition 	Well-conditioned	0
	Altered body condition	1
	Emaciated	2
Body weight 	Normal	0
	Loss of < 10.00%	1
	Loss of 10.00 - 20.00%	2
	Loss of > 20.00% (Euthanasia)	3
Food intake 	Normal (~ 5.00 – 10.00 g per 100 g of body weight)	0
	Decreased (< 5.00 g per 100 g of body weight)	1
Water intake 	Normal (~ 10.00 – 15.00 mL per 100 g of body weight)	0
	Decreased (< 10.00 mL per 100 g of body weight)	1
Posture 	Normal	0
	Curved	1
Coat and grooming 	Normal	0
	Lack of grooming	1
	Rough coat, chromodachryorrhea	2
	Very rough coat, piloerection, severe chromodachryorrhea	3
Mucosal color 	Normal	0
	Mild anemic	1
	Moderate anemic	2
	Severe anemic (Euthanasia)	3
Eyes, ears and whiskers 	Normal	0
	Partial closed eye, droopy ears, forward whiskers	1
	Complete closed eye, droopy and curved ears, forward and bunched whiskers	2

Parameters	Classification	Score
Mental status 	Normal (alert, curious, eyes bright)	0
	Lethargic	1
	Stupor	2
	Moribund / Coma (Euthanasia)	3
Behavior (response to external stimuli) 	Normal	0
	Mild response	1
	Moderate response with vocalization	2
	Violent response	3
Hydration status 	Normal skin pinch test (< 2 sec)	0
	Abnormal skin pinch test (> 2 sec)	1
Respiratory rate 	Normal (66 - 115 breaths per min)	0
	1 Abnormal (decreased: < 66 breaths per min or increase: > 115 breaths per min)	1
	Abdominal breathing (Euthanasia)	2
Heart rate 	Normal (250 - 450 beats per min)	0
	Abnormal (decreased: < 250 beats per min or increased: > 450 beats per min)	1
Body temperature 	Normal (35.60 - 38.90 ˚C)	0
	Abnormal (hypothermia: < 35.60 ˚C or hyperthermia: > 38.90 ˚C)	1
Hematocrit 	Normal (35.00 – 51.00%)	0
	Abnormal (decreased: < 35.00% or increased: > 51.00%)	1
Urine specific gravity 	Normal (1.040 - 1.070)	0
	Abnormal (decreased: < 1.040 or increased: > 1.070)	1

The urinary bladder should be fixated *in situ* and the male accessory sex glands are removed together, as previously described by Talhada *et al.*[33] Then, the prostate lobes should be separated and weighed individually. The organs may be immersed in 10.00% buffered formalin for 24 - 48 hr or frozen at –80.00 ˚C for posterior histopathological and biochemical analysis. In our protocol, the animals were sacrificed at two different times: 35 or 61 weeks of age, which correspond to 4.5 or 10.6 months after MNU administration (23 or 49 weeks of age, respectively) after the beginning of the experiment, to detect neoplastic lesions in different stages.

Prostate tumors' histological analysis. The histological analysis is one of the last steps of our experiments, but it is crucial for assessing the effects of carcinogens and hormones on prostate tissue, as well as for addressing the efficacy of the tested therapies. After collection and fixation, the prostate samples should be cut, embedded in paraffin, and 3.00 µm-thick trimmed sections stained with Hematoxylin and Eosin (H&E) for histopathological evaluation by experienced pathologists in a blind essay. The prostate lesions should be classified according to the classification established by the "Guides for Toxicologic Pathology, STP/ARP/AFIP".[34] At histological analysis, acute and/or purulent inflammation, small foci of stromal chronic inflammation, atypical hyperplasia, prostatic intra-epithelial neoplasia (PIN), and micro-invasive carcinomas were observed in the prostate of our animals (Fig. 3). Furthermore, in addition to the routine histopathological analysis, the samples may also be immunostained to assess the expression of hormone receptors, vascularization, proliferation, and apoptosis, and studies of tumor microenvironment using specific antibodies.

In conclusion, despite all the advances in disease modeling, animal models will continue to constitute an important tool for the investigation of cancer biopathology and the search for new tailored therapies. Therefore, considering our experience in cancer modeling, we are providing readers with a complete and step-by-step description of the procedures to implement the rat model of prostate cancer, guaranteeing the quality of the results, and facilitating comparison among different protocols.

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Conflict of interest

There are no conflicts of interest to declare for any of the authors.

References

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021;71(3): 209-249.
2. Cancer Today. International agency for research on cancer, WHO. Globocan 2020: Estimated cancer incidence, mortality and prevalence worldwide in 2020. Available at: http://gco.iarc.fr/today/home. Accessed Aug 18, 2023.
3. Ericsson AC, Crim MJ, Franklin CL. A brief history of animal modeling. Mo Med 2013; 110(3): 201-205.
4. Clarke R. Animal models of breast cancer: their diversity and role in biomedical research. Breast Cancer Res Treat 1996; 39(1): 1-6.
5. Iannaccone PM, Jacob HJ. Rats! Dis Model Mech 2009; 2(5-6): 206-210.
6. Liska J, Macejova D, Galbavy S, et al. Treatment of 1-methyl-1-nitrosourea-induced mammary tumours with immunostimulatory CpG motifs and 13-cis retinoic acid in female rats: histopathological study. Exp Toxicol Pathol 2003; 55(2-3): 173179.

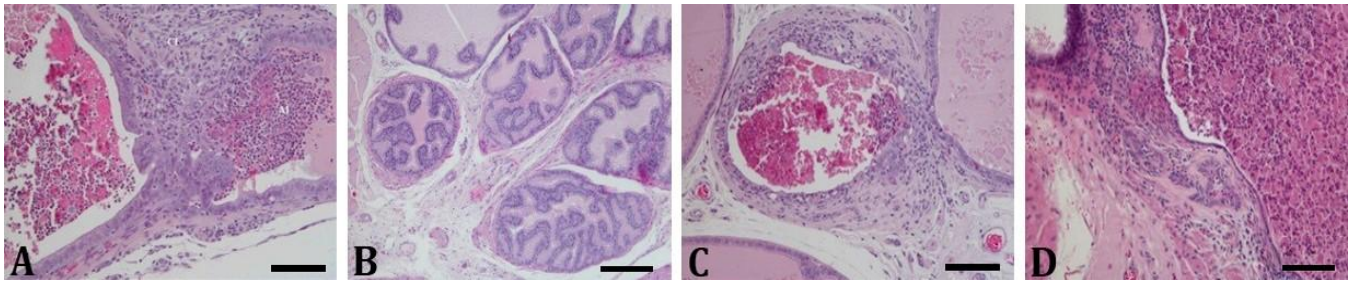

Fig. 3. Histopathological lesions in rat prostate using H&E staining. **A)** prostate with chronic inflammation and acute inflammation areas (bar = 100 µm); **B)** atypical hyperplasia (bar = 200 µm); **C)** prostatic intraepithelial neoplasia, (bar = 500 µm) and **D)** micro-invasive carcinoma (bar = 100 µm).

7. Report from the commission to the European parliament and the council 2019 report on the statistics on the use of animals for scientific purposes in the Member States of the European Union in 2015-2017. Brussels: European Commission; 2020: 21.
8. Alvarado A, Faustino-Rocha AI, Colaço B, et al. Experimental mammary carcinogenesis - Rat models. Life Sci 2017;173: 116-134.
9. Oliveira PA, Colaço A, Chaves R, et al. Chemical carcinogenesis. An Acad Bras Cienc. 2007; 79(4): 593-616.
10. Knowles M, Selby P. Introduction to the cellular and molecular biology of cancer. 4th ed. Oxford, UK: Oxford University Press 2005; 549.
11. Henderson BE, Feigelson HS. Hormonal carcinogenesis. Carcinogenesis 2000; 21(3): 427-433.
12. Arcos JC, Argus MF, Woo YT. Chemical induction of cancer: modulation and combination effects an inventory of the many factors which influence carcinogenesis. Boston, USA: Birkhäuser Boston; 1995; 711.
13. Bosland MC, Schlicht MJ, Horton L, et al. The MNU plus testosterone rat model of prostate carcinogenesis. Toxicol Pathol 2022; 50(4): 478-496.
14. Nascimento-Gonçalves E, Seixas F, Ferreira R, et al. An overview of the latest in state-of-the-art murine models for prostate cancer. Expert Opin Drug Discov 2021; 16(11): 1349-1364.
15. Nascimento-Gonçalves E, Faustino-Rocha AI, Seixas F, et al. Modelling human prostate cancer: Rat models. Life Sci 2018; 203: 210224.
16. Currier N, Solomon SE, Demicco EG, et al. Oncogenic signaling pathways activated in DMBA-induced mouse mammary tumors. Toxicol Pathol 2005; 33(6): 726-737.
17. Bonkhoff H, Remberger K. Widespread distribution of nuclear androgen receptors in the basal cell layer of the normal and hyperplastic human prostate. Virchows Arch A Pathol Anat Histopathol 1993; 422(1): 35-38.
18. Magee PN, Barnes JM. Carcinogenic nitroso compounds. Adv Cancer Res 1967; 10: 163-246.
19. Singer B, Kuśmierek JT. Chemical mutagenesis. Ann Rev Biochem 1982; 51: 655-693.
20. Shirai T, Takahashi S, Cui L, et al. Experimental prostate carcinogenesis - rodent models. Mutat Res 2000; 462(2-3): 219226.
21. Bosland MC. Chemical and hormonal induction of prostate cancer in animal models. Urol Oncol 1996; 2(4): 103-110.
22. Cekanova M, Rathore K. Animal models and therapeutic molecular targets of cancer: utility and limitations. Drug Des Devel Ther 2014; 8: 1911-1921.
23. Shirai T. Significance of chemoprevention for prostate cancer development: experimental *in vivo* approaches to chemoprevention. Pathol Int 2008; 58(1): 1-16.
24. Bosland MC. Animal models for the study of prostate carcinogenesis. J Cell Biochem Suppl 1992; 50(16H): 89-98.
25. Lucia MS, Bostwick DG, Bosland M, et al. Workgroup I: rodent models of prostate cancer. Prostate. 19980; 36(1): 49-55.
26. Nascimento-Goncalves E, Seixas F, DA Costa RMG, et al. Appraising animal models of prostate cancer for translational research: future directions. Anticancer Res 2023; 43(1): 275-281.
27. Fautino-Rocha AI, Seixas F, Ferreira R, et al. Ultrasonographic follow-up of the multistep protocol for prostate cancer induction in Wistar rats. *In Vivo* 2020; 34(4): 1797-1803.
28. Ginja M, Pires MJ, Gonzalo-Orden JM, et al. Anatomy and imaging of rat prostate: practical monitoring in experimental cancer-induced protocols. Diagnostics (Basel) 2019; 9(3): 68. doi: 10.3390/ diagnostics9030068.
29. Denis F, Bougnoux P, de Poncheville L, et al. *In vivo* quantitation of tumour vascularisation assessed by Doppler sonography in rat mammary tumors. Ultrasound Med Biol 2002; 28(4): 431437.
30. Faustino-Rocha AI, Gama A, Oliveira PA, et al. Effects of lifelong exercise training on mammary tumorigenesis induced by MNU in female Sprague-Dawley rats. Clin Exp Med 2017; 17(2): 151-160.
31. Faustino-Rocha AI, Ginja M, Ferreira R, et al. Studying humane endpoints in a rat model of mammary carcinogenesis. Iran J Basic Med Sci 2019; 22(6): 643-649.
32. Arantes-Rodrigues R, Henriques A, Pires MJ, et al. High doses of olive leaf extract induce liver changes in mice. Food Chem Toxicol 2011; 49(9): 1989-1997.
33. Talhada D, Andrade A, Faustino-Rocha AI, et al. Recommendations for urine and urinary bladder collection in chemical carcinogenesis assays with rodents. Scand J Lab Anim Sci 2015; 41: 1-8.
34. Bosland MC, Tuomari DL, Elwell MR, et al. Proliferative lesions of the prostate and other accessory sex glands in male rats. In: Guides for toxicologic pathology. Washington DC, USA: STP/ARP/AFIP 1998; 1-20.