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Ultrasonography as the Gold Standard for *In Vivo* Volumetric Determination of Chemically-induced Mammary Tumors

ANA I. FAUSTINO-ROCHA[1,2,3,4], ADELINA GAMA[1,3], PAULA A. OLIVEIRA[1,2], ANTONIETA ALVARADO[1,2,5], LIO FIDALGO-GONÇALVES[6], RITA FERREIRA[4] and MÁRIO GINJA[1,2]

[1]*Department of Veterinary Sciences, and* [3]*Animal and Veterinary Research Center (CECAV), School of Agrarian and Veterinary Sciences,*
[2]*Center for the Research and Technology of Agro-Environmental and Biological Sciences (ITAB), and* [6]*Center of Mathematics, Department of Engineering, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal;*
[4]*Organic Chemistry Natural Products and Foodstuffs (QORNA), Mass Spectrometry Center, Department of Chemistry, University of Aveiro, Aveiro, Portugal;*
[5]*Department of Pathology, School of Veterinary Medicine, Central Western University "Lisandro Alvarado", Lara, Venezuela*

Abstract. *Background/Aim: In this study, we evaluated the dimensions and volume of rat mammary tumors and the association of these variables with tumor invasiveness. Materials and Methods: Tumors were measured by caliper and ultrasonography. Volume was determined by water displacement and by application of four formulas using tumor length (L), width (W) and depth (D) or tumor weight. Results: Results confirmed the data obtained in our previous work, where we verified that mammary tumors grow as oblate spheroids. Conclusion: The determination of mammary tumor volume by applying the formula V=(4/3)×π×(L/2)×(L/2)×(D/2) is the best way to evaluate tumor volume in vivo. Beyond volume evaluation by water displacement, the determination on the basis of tumor weight is the most accurate way to evaluate tumor volume after animal sacrifice or tumor excision. According to our results, it is not possible to predict if a tumor is invasive or non-invasive by its dimensions, volume or weight. Future work in chemically-induced mammary cancer should use ultrasonography and water displacement or tumor weight to determine tumor volume in vivo and after animal sacrifice or tumor excision, respectively.*

Correspondence to: Ana I. Faustino, CITAB, Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro, 5001-911, Vila Real, Portugal. Tel: +351 259350000, e-mail: anafaustino.faustino@sapo.pt

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The breast is the most common site of cancer development in women (1). In 2012, breast cancer was responsible for about 521,000 deaths around the world, representing one of the leading causes of death by cancer worldwide (2).

Breast cancer prognosis is based on specific factors, namely on the involvement of axillary lymph nodes and on tumor potential for growth (1). In 1971, Lala reported that tumor growth is the best parameter for obtaining information about the cell population and the effects of different therapeutic approaches on tumors (3). Some years later, in 1979, tumor dimensions were established by the World Health Organization as one of the criteria for the classification of mammary tumors (4). Since then, several reports stated that tumor measurement has an important value in planning and monitoring treatment strategies in patients with cancer (5, 6). In an initial stage of the disease, tumor size is important in choosing the most adequate therapy for each patient (7); it is an important factor to determine whether a woman is or is not a suitable candidate for a specific modality of treatment, such as surgery (partial resection or mastectomy) or chemotherapy (8). During treatment, tumor dimensions are important for evaluating if the selected therapy is having the desired effects (7); a reduction of tumor size (tumor shrinkage) during a treatment suggests that the tumor is vulnerable to it (9). Tumor-growth monitoring is also essential in experimental assays using animal models of different types of cancer for developing and evaluate novel anticancer therapies (10).

Animal models have been widely used in biomedical sciences; they are the intermediate step between *in vitro* cell culture and clinical assays in humans (11). Compared to *in*

vitro cell cultures, animal models provide a three-dimensional (3D) view and a realistic microenvironment where it is possible to study tumor growth and its response to therapy (12). For breast cancer, rodents in particular have an important role due to the biological similarities of breast cancer in these species with that in women, namely epithelial origin and hormonal dependence (13). The model of chemically induced breast cancer in Sprague-Dawley female rats by the carcinogenic agent *N*-methyl-*N*-nitrosourea (MNU) is a well-known model for studying this type of cancer (14).

Taking into account the great importance of tumor size measurement, this study intended to verify the shape of chemically-induced mammary tumors in a rat model and correlate it with tumor dimensions, and to compare tumor volume calculated by different formulas with tumor volume as assessed by water displacement. We also aimed to assess the differences in dimensions, volume and weight between invasive and non-invasive mammary tumors.

Materials and Methods

Animals. Thirteen outbred female Sprague-Dawley rats (*Rattus norvegicus*) of four weeks of age were obtained from Harlan Interfauna Inc. (Barcelona, Spain). Animals were housed in filter-capped polycarbonate cages (1500U Eurostandard Type IV S; Tecniplast, Buguggiate, Italy), using corncob for bedding (Mucedola, Italy). Cages were kept at the animal facilities of the University of Trás-os-Montes and Alto Douro in a ventilated room with controlled conditions of temperature (23±2˚C), relative humidity (50±10%) and light/dark cycle (12 h:12 h). During the experimental protocol, animals had *ad libitum* access to a basic standard diet (4RF21®; Mucedola, Settimo Milanese, Milan, Italy) and tap water.

Chemicals. The carcinogenic agent MNU (Isopac®) was purchased from Sigma Chemical Co. (Madrid, Spain) and stored according to the manufacturer's instructions.

Animal experiments. Before the beginning of the experimental protocol, animals were submitted to a period of quarantine for 1 week. After this, we allowed the animals to acclimate to laboratory conditions for 2 weeks. Then we divided them into two experimental groups: MNU (n=10) and control (n=3). At 7 weeks of age with a mean body weight of 184.99±2.95 g, animals from the MNU-treated group received a single intraperitoneal injection of MNU at a concentration of 50 mg/kg body weight. MNU was dissolved in 0.9% saline solution to a concentration of 11 mg/ml and was used within 1 hour after its preparation. Animals from the group control were used as negative control and were not exposed to MNU. The MNU administration defined the beginning of the experimental protocol (week 0 of the protocol).

Animals were monitored daily to check their general health status. Animal body weight was recorded at the beginning and at the end of the experimental protocol; final body weight was obtained by the subtraction of tumor weight from total body weight. At the end of the experimental protocol (after 18 weeks), we calculated body weight gain applying the formula previously used by Faustino-Rocha and collaborators (15).

All animal procedures were carried out in accordance with the national (Decree-Law no. 113/2013) and European legislation (European Directive 2010/63/EU) on the protection of animals used for scientific purposes. The experimental protocol was approved by the Ethics Committee of the University of Trás-os-Montes and Alto Douro (approval CE_12-2013) and by the Portuguese Ethics Committee for Animal Experimentation (approval no. 008961). Before the beginning of the experimental protocol, an adequate list of humane endpoints was defined.

Mammary tumor evaluation. After the MNU administration, animals were weekly palpated for the detection of mammary tumor development; the time of appearance of the first mammary tumor was recorded. Before the animals were sacrificed, the length (longitudinal axis) and width (transversal axis) of mammary tumors identified by palpation were measured by one researcher using a vernier caliper (Vito; Central Lobão S.A., Santa Maria da Feira, Portugal); these measurements were defined as clinical measurements. Tumor volume (*V*) using these measurements was calculated according to the following formula (16):

$$V=(W^2\times L)/2 \quad \text{(Formula 1)},$$

where *W* is tumor width and *L* is tumor length.

Eighteen weeks after MNU administration, all surviving animals were anesthetized by intraperitoneal injection of ketamine (75 mg/kg, Imalgene®; Merial S.A.S., Lyon, France) and xylazine (10 mg/kg, Rompun® 2%; Bayer Healthcare S.A., Kiel, Germany). Mammary tumors were evaluated by ultrasonography by two experienced examiners. For ultrasonographic examination, animals were placed in supine position. The skin overlying each mammary tumor was shaved using a machine clipper (Aesculap GT 420 Isis; Aesculap Inc, Center Valley, PA, USA) and acoustic gel was applied (Aquasonic; Parker Laboratories, Fairfield, NJ, USA) to mammary tumors. Ultrasonographic evaluation was performed using B-mode ultrasound using a real-time Logic P6 scanner (General Electric Healthcare, Milwaukee, WI, USA) with a 10 MHz linear transducer. A standoff pad (Sonokit; MIUS Ltd, Gloucester, Gloucestershire, UK) made of extremely soft polyvinylchloride especially created for skin-contact sonography was used. We used light pressure when scanning the mammary tumors to avoid distorting their shape. During the ultrasonographic examination, sagittal and transverse views of each mammary tumor were obtained; the probe was rotated until the largest diameter of each view was obtained. Ultrasonographic examinations were recorded in video format. After ultrasonography, the diameter of the sagittal (tumor length) and transverse (tumor width) views and the depth of each mammary tumor were measured by one researcher in a frozen image using the integral calipers of the ultrasound apparatus; the cursors were set at the borders of the tumor. Tumor volume using these measurements was calculated according to the following formulas (15):

$$V=(4/3)\times\pi\times(L/2)\times(L/2)\times(D/2) \quad \text{(Formula 2)},$$

$$V=(1/2)\times L\times W\times D \quad \text{(Formula 3)},$$

where *L* is the length, *W* is the width and *D* is the depth of the tumor.

Animal sacrifice and necropsy. After ultrasonographic examination, anesthetized animals were sacrificed by exsanguination by cardiac puncture as indicated by the Federation of European Laboratory Animal Science Associations (17). All animals were scalped (all skin was removed) and the skin was carefully observed under a light in order to detect mammary tumors. All mammary tumors were excised and three measurements of each mammary tumor (length, width and depth) were made using a vernier caliper (Vito; Central Lobão S.A.) by one researcher; these measurements were defined as anatomopathological measurements. The volume of mammary tumors was calculated using these measurements according to formulas 1 and 3 presented above. Then mammary tumors were weighed in a top-loading scale (Mettler PM 4000; LabWrench, Midland, Canada) and their volume was calculated using the following formula:

V=Tumor weight/1.056 (Formula 4),

considering the tumor density to be similar to that of soft tissue (1.056 g/cm^3) (18). Mammary tumor volume was also determined by water displacement by immersing each tumor in a beaker with saline solution; this volume was defined as the true tumor volume. Immediately after this procedure, mammary tumors were immersed in phosphate-buffered formaldehyde for 24 hours.

Histology. After fixation, mammary tumors were cut, embedded in paraffin and 2 μm-thick sections were routinely stained with hematoxylin and eosin (H&E). Histological slides were observed blindly under light microscopy by an experienced pathologist. Mammary tumors were classified according to the classification previously established by Russo and Russo (19).

Data analysis. A descriptive analysis was performed for all the variables included in the study. Data were statistically analyzed using SPSS® (Statistical Package for the Social Sciences, version 23 for Windows; SPSS Inc., Chicago, IL, USA). Continuous data are expressed as the mean±standard deviation (S.D.) or the mean±standard error (S.E.). We used independent sample *t*-test to compare the mean initial and final body weight, and body weight gain between both the two groups of rats, and to compare true volume and tumor weight between invasive and non-invasive mammary tumors. We used the paired *t*-test to compare the mean initial and final body weight in each group. We used ANOVA with the Bonferroni correction to assess the differences in tumor length, width and depth measured using a vernier caliper (clinical and anatomopathological measurements) and by ultrasonography (ultrasonographic measurements); and to compare tumor volume calculated by different formulas. The mean values were considered to be statistically significant when $p<0.05$. Using Matlab® (version 7.12.0.635; The MathWorks Inc., Natick, MA, USA), we created two 3D models of mammary tumors using tumor length, width and depth measured by ultrasonography and by caliper at anatomopathological analysis.

Results

General observations. Immediately before the begining of the experimental protocol, one animal from the control group exhibited signs of disease; the animal was lethargic and exhibited an orthopneic position, its body weight decreased, and it had a very rough coat, piloerection and moderate chromodachryorrhea, its mucosae were moderately anemic and its eyes were partial closed. Taking into account the humane endpoints established for this experimental protocol, the animal was humanely sacrificed and data were excluded from our results.

Table I. *Initial and final body weight (g) and body weight gain (%) in animals from both the N-methyl-N-nitrosourea (MNU)-treated and control groups (mean±S.E.).*

Group	Body weight		
	Initial (g)	Final (g)	Gain (%)
MNU (n=10)	184.99±2.95[a]	299.58±5.86	38.16±0.86
Control (n=2)	183.82±2.84[a]	295.98±9.78	37.86±1.10

[a]Statistically significant different from final body weight ($p<0.05$).

All remaining animals exhibited a normal health status during the experimental protocol. In both groups, the mean initial body weight was statistically different from the mean final body weight ($p<0.05$). The mean initial and final body weight and the body weight gain were similar between the MNU and control groups ($p>0.05$) (Table I).

Mammary tumor numbers and histological evaluation. At the end of the experimental protocol, 22 masses were palpated in six animals from the MNU-treated group; none of the animals from the group control developed any mass.

The first mass that was subsequently classified as a mammary tumor by histopathology was palpated in the 8th week of the experimental protocol (Figure 1 and 2A). At histopathological analysis, we verified that one of the masses that were palpated was not a mammary tumor, it was histologically classified as a reactive lymph node; this mass was excluded from the study (Table II). Therefore, at the end of the protocol, a total of 21 mammary tumors were counted in six out of 10 animals from the MNU-treated group (incidence of 60%; mean number of approximately 4 mammary tumors per animal) (Figure 1). Of these tumors, one was classified as a benign lesion (fibroadenoma) and the remaining were classified as malignant lesions, papillary non-invasive carcinoma being that most frequently identified (Table II).

Tumor dimension. From the 21 mammary tumors, data were only collected from caliper (clinical and anatomo-pathological) and ultrasonography in 13 tumors. Although at anatomopathological analysis, all tumors were measured using calipers; not all were previously evaluated by clinical or ultrasonographic analysis due to their small size (tumors smaller than 0.5 cm in diameter) and hence were not

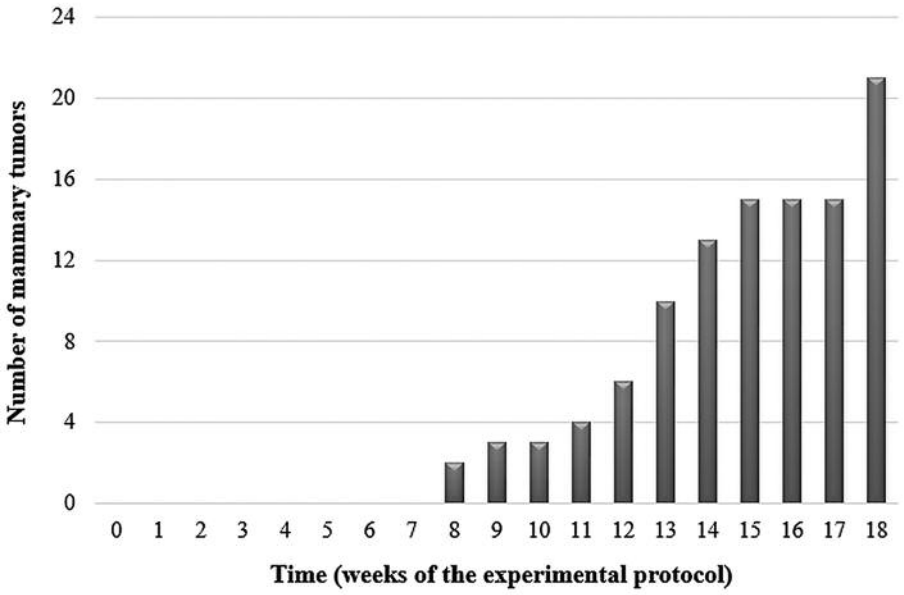

Figure 1. *Number of mammary tumors in animals from the N-methyl-N-nitrosourea-treated group (n=6) during the experimental protocol; the first mammary tumor was identified by palpation at week 8 of the protocol.*

Table II. *Histological classification of mammary tumors identified in animals from the N-methyl-N-nitrosourea-treated group according to the classification established by Russo and Russo (19).*

Histological classification		Number of lesions
Benign lesion	Fibroadenoma	1
Malignant lesion	Papillary non-invasive carcinoma	12
	Cribriform non-invasive carcinoma	1
	Papillary invasive carcinoma	3
	Cribriform invasive carcinoma	3
	Comedo carcinoma	1
	Total	21

Table III. *Measurement of length, width and depth by caliper (before and after necropsy) and ultrasonography in 13 mammary tumors identified in animals from group N-methyl-N-nitrosourea (mean±S.D.).*

Measurement (cm)	Measurement method		
	Clinical	Ultrasonographic	Anatomopathological
Length	2.29±0.66[a,b]	2.05±0.75[a,b]	2.12±0.67[a,b]
Width	1.79±0.67[b]	1.98±0.51[a,b]	1.94±0.63[a,b]
Depth	-	1.08±0.43	0.97±0.33

[a]Statistically significant difference from ultrasonographic depth ($p<0.05$); [b]Statistically significant different from anatomopathological depth ($p<0.05$).

identified during clinical palpation and consequently they were not evaluated by ultrasonography, or because their size exceeded 4 cm and we were unable to measure them owing to the size of our probe. The tumors that were not identified by palpation during the experimental protocol due their small size were identified during the observation of the skin under a light after sacrifice.

Looking at the data from these 13 mammary tumors, we verified that the measurement of the length, width and depth was similar using the different methods of measurement employed in this experimental protocol (clinical, ultrasonographic and anatomopathological) ($p>0.05$) (Table III).

In all methods of measurement, we verified that the tumor lengths and widths were similar ($p>0.05$). In ultrasonographic and anatomopathological measurements, we also observed that tumor length and width were greater than tumor depth ($p<0.05$) (Table III). These data suggest that mammary tumors grown as oblate spheroids (Figure 2B and C).

Tumor volume and weight. The tumor volume was determined by water displacement and using different formulas previously published by us and by other researchers. In the statistical analysis, we found no statistically significant differences among tumor volumes when calculated by different methods ($p>0.05$) (Table IV). However, we verified that the mean tumor volume determined by water displacement, considered as the

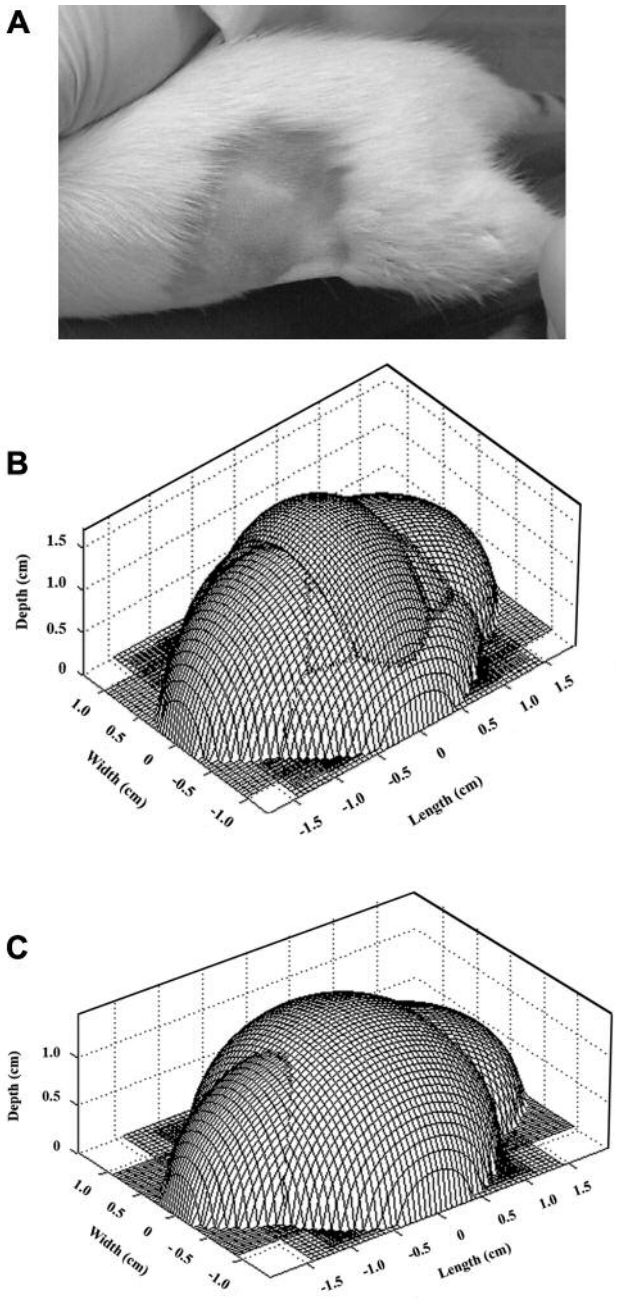

Figure 2. *A: Mammary tumor in an animal from the N-methyl-N-nitrosourea-treated group. B: 3D Model of mammary tumor as measured by ultrasonography. C: 3D Model of mammary tumor as measured by caliper at anatomopathological analysis.*

true volume, was more similar to the tumor volume calculated using formula 2 and formula 4 than with that calculated using formulas 1 and 3 (Table IV). We also observed that true tumor volume and tumor volume calculated using formulas 2 and 4 were very similar to tumor weight ($p>0.05$) (Table IV).

Table IV. *Volume and weight of 13 mammary tumors calculated using different formulas (mean±S.D.).*

		Mean±S.D. (cm^3)	Range (cm^3)
Formula 1	Clinical	4.66±3.37	0.27-10.48
	Anatomopathological	4.66±3.23	0.45-11.57
Formula 2	Ultrasonographic	3.40±3.16	0.23-10.87
Formula 3	Ultrasonographic	2.61±1.93	0.20-5.65
	Anatomopathological	2.35±1.74	0.21-5.89
Formula 4		3.49±2.29	0.40-7.20
True volume		3.63±2.37	0.50-7.50
Tumor weight (g)		3.68±2.42	0.38-7.58

Statistically significant differences were not found ($p>0.05$).

Table V. *Comparison of tumor length, width, depth, volume and weight between non-invasive and invasive mammary tumors (mean±S.D.).*

Parameter		Mammary tumors	
		Invasive (n=5)	Non-invasive (n=8)
Length (cm)	Clinical	2.12±0.41	2.41±0.81
	Ultrasonographic	1.93±0.63	2.13±0.86
	Anatomopathological	1.89±0.28	2.29±0.84
Width (cm)	Clinical	1.84±0.47	1.76±0.81
	Ultrasonographic	2.01±0.53	1.96±0.53
	Anatomopathological	2.07±0.45	1.84±0.75
Depth (cm)	Ultrasonographic	1.17±0.40	1.01±0.46
	Anatomopathological	1.06±0.16	0.91±0.41
True volume (cm^3)		3.50±1.70	3.71±2.88
Weight (g)		3.56±1.67	3.76±2.97

Statistically significant differences were not found ($p>0.05$).

Invasive and non-invasive mammary tumors. At the end of the experimental protocol, five invasive and eight non-invasive mammary tumors were compared (Table V). We verified that the length, width and depth of mammary tumors measured by caliper or ultrasonography were similar between invasive and non-invasive mammary tumors ($p>0.05$). Although the differences did not reach the level of statistical significance, we noted that tumor length was slightly greater in non-invasive tumors than in invasive ones ($p>0.05$). We observed the converse for tumor width and depth, where these measurements were higher in invasive tumors than in non-invasive ones ($p>0.05$) (Table V).

Although the mean true tumor volume and tumor weight were slightly higher in non-invasive than in invasive mammary tumors, we verified that there were no statistically significant differences between invasive and non-invasive tumors ($p>0.05$) (Table V).

Discussion

In experimental protocols using animal models of several types of cancer, namely prostate, skin, mammary and liver, the accurate and efficient determination of tumor size will determine the success of the experimental protocol (20). Tumor volume is also frequently used as an endpoint in experimental protocols that aim to evaluate the efficacy of anticancer drugs (21). Tumor size can be assessed by different modalities, namely physical examination (measurement with a caliper or a ruler), by mammography, ultrasonography and magnetic resonance. According to Pain and collaborators, the three most used methods of diagnosis, the physical examination, mammography and ultrasound, had a similar accuracy for predicting the pathological size (22).

In our study, the mean initial and final body weight were similar between groups; it was expected that the animals from the MNU-treated group would have a lower final body weight once they developed mammary tumors. This difference was not observed probably because the experimental protocol may not have been long enough and animals that developed mammary tumors did not develop cachexia associated with carcinogenesis. These results are in accordance with those that were observed in a previous protocol in mammary carcinogenesis (15).

The first mammary tumor was detected 8 weeks after the injection of MNU; in our previous protocol in mammary carcinogenesis (15) where we used the same carcinogen at same dose, in animals of the same strain and age, we detected the first mammary tumor later, at 10 weeks after the MNU administration. Conversely to our previous study, where all animals exposed to the carcinogen developed mammary tumors, in this protocol, only six animals exposed to MNU developed mammary tumors. At 18 weeks after MNU administration, we counted a total of 21 mammary tumors in six out of 10 animals (incidence of 60%; mean number of approximately 4 mammary tumors per animal); in our previous study, at the same timepoint, we only observed a total of five mammary tumors in five out of 11 animals (incidence of 45%; mean number of 1 mammary tumor per animal). Once we used outbred animals, the differences in results can be related to the individual differences among animals that were used. As expected and similarly to what was observed in the last experimental protocol of mammary cancer performed by our research team, we did not detect any mass in the surviving animals from the control group. Contrary to what we would expect when working with the same animal model more than once, the results will always be different among protocols; hence we should be aware that we are working with living experimental animals and not mathematics, and there are many biological factors that can influence the results.

Similarly to what was described by Russo and Russo (19) and in accordance with what we previously observed (15), we verified that the number of malignant mammary tumors was higher than the number of the benign ones; of these, the papillary non-invasive carcinoma was the most frequently identified lesion.

Tumor dimensions (length, width and depth) were measured by only one researcher to avoid interobserver variations. It was described in a previous publication that measurements made by more than one person can lead to different results (23). During the ultrasonographic evaluation, the probe, and consequently the beam, was maintained perpendicular to the skin surface to avoid artifacts and to minimize variations in pressure of the transducer which might modify tumor dimensions, especially the depth (24). We verified that tumor length, width and depth were similar among the methods that were used (caliper before and after animal sacrifice and ultrasonography). We also observed that the tumor length was similar to tumor width and greater than tumor depth. Taking these data into account, we can state that mammary tumors grow as a circular surface with small depth, resembling the shape of an oblate spheroid, where the length and width are similar and greater than the depth. These data are in accordance with those we obtained in our previous study (15). We suppose that this shape of development of mammary tumors in rats is due to the fact that these animals do not have a developed mammary gland and their skin is thin, with a low quantity of fat, conversely to that in women.

Tumor volume was determined by water displacement similarly to that previously performed by Tomayko and collaborators (25) and using different formulas. Water displacement is a more direct method of volume measurement, but it is difficult to perform when the volume of the tumors is smaller than 0.5 cm^3 (26). In this work, we used formulas 1 and 2, which according to our previous work (15), are the best formulas for determining tumor volume using two tumor dimensions measured by caliper (length and width) and ultrasonography (length and depth), respectively. We had also used formula 4 to determine tumor volume on the basis of tumor weight. Additionally to our previous work, and in accordance with other investigators (27), which have stated that using the three measurements (length, width and depth) is the most accurate way to determine tumor volume, in this study, we also calculated tumors volume using these three dimensions as obtained by ultrasonography and by using a caliper (anatomopathology). For this, we used formula 3, which was previously used by Tomayko and Reynolds (25) to determine the volume of subcutaneous tumors in a xenograft mouse model. Looking at our results, we can conclude that formula 2 previously developed by us and formula 4 are the most accurate for assessing tumor volume. According to our results, formula 1 overestimates the tumor volume, whereas formula 3 underestimates it.

In this work, we also compared tumor dimensions, real volume and weight between invasive and non-invasive mammary tumors, finding no statistically significant differences.

Conclusion

The results of this work confirm the data obtained in our previous work, where we verified that mammary tumors chemically induced by the carcinogen MNU in female rats grow as oblate spheroids (15). We can conclude that the determination of mammary tumors volume by the application of formula 2 using two ultrasonographic dimensions (length and width) is the best way to evaluate tumor dimensions *in vivo*. Beyond the determination of tumor volume by water displacement, the determination of volume on the basis of tumor weight by the application of formula 4 is the best way to evaluate tumor volume after animal sacrifice or after tumor excision. According to our results, it is not possible to predict if a tumor is invasive or non-invasive by its dimensions, volume or weight.

In future work of chemically induced mammary cancer, we recommend the following methodology for assessing tumor volume: the use of ultrasonography to assess tumor volume *in vivo* by the application of formula 2; the use of water displacement or tumor weight by the application of formula 4 to determine tumor volume after animal sacrifice or tumor excision.

Conflicts of Interests

None to declare.

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