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Implementation of Humane Endpoints in a Urinary Bladder Carcinogenesis Study in Rats

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Abstract. *Background/Aim: This study aimed to evaluate the utility of several biological parameters for the prediction of tumor development and animal welfare in a rat model of urinary bladder cancer. Materials and Methods: The control group (n=9) received tap water while the test group (n=12) received the carcinogen N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) in drinking water. A score sheet with biological variables was used to monitor animals' welfare. Body weight, food and drink consumption and rectal temperature were measured weekly. Blood and urine samples were collected. Results: Animals from the control group exhibited a slightly higher body weight and body weight gain. The final urine volume was higher in BBN group ($p<0.05$). All animals from the BBN group exhibited macroscopic hematuria at 35th week. Four animals were anemic in the last week of the experiment. Conclusion: The routine control of hematuria was a useful non-invasive biomarker of disease progression that may be used as a potential earlier humane endpoint. Animals did not show clinical signs of suffering that justified their sacrifice before the end of the study.*

Over the last years animal models have been essential to understand the growth and subsistence of tumors, leading to the development of new and more effective therapies capable to prevent and treat cancer. Although the scientific community is concerned about the use of animals in experimental studies, they have been widely employed in cancer studies (1-4), since alternatives are not available. Concerned with the inflicted suffering in animals, William Russell and Rex Burch presented the 3R's in 1959, proposing the replacement of animals by *in vitro* methods, the reduction in number of animals used, and the introduction of refinements to decrease the incidence or severity of procedures in research animals (5). Although replacement and reduction are readily applied concepts, the refinement of procedures to reduce animals' suffering without producing a number of unwanted variables to the research, represents a bigger challenge.

Apart from being a serious ethical concern, pain and stress are undesirable features. Furthermore, they are a potential source of experimental errors as many physiological, immunological, endocrine and behavioral parameters may change as a consequence of such conditions, which ultimately might misrepresent the results of the trials (6, 7). Thereby, it became a legal and ethical obligation for the investigator to have the utmost care to keep animals in a state of optimal wellness (4, 8), once they are completely dependent on him (9, 10). Researchers should always aim to minimize animals' suffering, with good practices in the recognition and alleviation of pain and distress in animals, protecting animal welfare and encouraging good science (11, 12). When there is the possibility to cause animals' pain or suffering, investigators need to refine techniques and implement experimental endpoints, allowing early intervention, minimizing data loss and relieving animals'

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suffering (13). Thus, the application of appropriate and objective humane endpoints is crucial when refining *in vivo* cancer research.

Defining humane endpoints in cancer research can be challenging (14). According to the National Centre for the Replacement, Refinement and Reduction of Animals in Research, humane endpoints are defined as the criteria that allow early termination of experiments before animals experience significant harm while still meeting the experimental objectives (15). The Canadian Council on Animal Care (15) suggests the use of a Scoring System (Checklist) specific for each scientific procedure and species, which is based on general observations that animals might display during the experiment (16). Animals' behavior and physiological parameters should be monitored (14) and based on these predictions animals should be removed from the study when the proposed endpoints are reached (15, 17-19). A major obstacle to implement humane endpoints is a general lack of biomarkers that can be used as specific indicators of disease progression (20). Although sometimes tumor burden and disease progression can be directly measured using calipers, such as in assays of mammary carcinogenesis or in assays using xenograft models were the tumor cell lines are subcutaneously implanted (21, 22), many other tumors are not externally visible and consequently their growth cannot be monitored, as in the case of urinary bladder tumors.

In order to address these questions, the main goal of this study was to define humane endpoints in an experimental protocol using a rat model of chemically-induced urinary bladder cancer addressing several biological parameters.

Materials and Methods

Ethics statement. All animal procedures were performed in accordance with the European Directive 2010/63/EU and National Decree-Law 113/3013 on the protection of animals used for scientific purposes. All experiments and procedures were carried out under Direção Geral de Alimentação e Veterinária, Approval no. 008961.

Animals. Twenty-one male Wistar rats (Rattus norvegicus) with four weeks of age were obtained from Harlan Interfauna Inc. (Barcelona, Spain). Animals were group-housed in filter-capped polycarbonate cages (1500UEurostandard Type IV S, Tecniplast, Buguggiate, Italy) with corncob for bedding (Mucedola, Settimo Milanese, Milan, Italy), under controlled conditions of light/dark cycle (12 h/12 h), temperature (22˚C±2°C) and relative humidity (50±10%). All animals had free access to a standard diet (4RF21®, Mucedola, Settimo Milanese, Milan, Italy) and tap water was supplied through capped water bottles (ACBT0502, Tecniplast, Buguggiate, Italy) *ad libitum* throughout the study. Cages were cleaned and water was changed once *per* week.

Experimental protocol. After one week of acclimatization, the rats were randomly divided into two groups: control (n=9) and *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) (n=12). Animals from control group only received tap water along the experimental protocol. Animals from BBN group drank water with BBN (Tokyo Kasei Kogyo, Tokyo, Japan) at a concentration of 0.05%, during the first 20 weeks of the experimental protocol. After this period, animals were maintained with simple water until the end of the experiment (Figure 1).

Score sheet. A form for registration of biological variables of each animal, such as body weight, body condition, mental status, coat and grooming, eyes, ears and whiskers, skin and mucosa, response to manipulation, breathing, hydration status, body temperature, urine color and urine volume, was elaborated by our team based on previous studies in animal welfare (9, 17) (Table I). A numerical value was attributed for each parameter, and the values' sum was used to determine the endpoints. The animals were daily observed and body weight, food and drink consumption and rectal temperature, were measured weekly. Food and water consumption were determined using a toploading scale (METTLER PM 4000, LabWrench, Midland, Canada). Mean food consumption for each animal placed in a cage in a group of five animals was calculated as the difference between the weight of the food container at the beginning of the week and the weight of food container at the end of the week, divided by the number of animals in the cage times the number of days. Mean water consumption for each animal was calculated as the difference between the weight of the water bottle at the beginning of the week and the weight of the water bottle at the end of the week, divided by the number of animals in the cage times the number of days (23). The following formula was used to calculate the body weight gain (%): (Final body weight-Initial body weight)/Final body weight×100 (21). Animals were daily observed and the data was weekly filled in the scoring sheet.

Urine and blood collection. Urine collection was performed in several time points along the experimental protocol as suggested by Talhada and collaborators (24). For this, animals were placed in metabolic cages during 24 h. After this period of time, urine from each animal was collected; the volume was measured and urine was macroscopically evaluated to detect the presence of hematuria. The degree of hematuria of each animal was scored with a value from 0 to 5, where the number 0 corresponded to the absence of hematuria and 5 corresponded to severe hematuria. Blood samples (150 to 200 µl *per* animal) were taken in several time points along the experimental protocol to microhematocrit tubes (Hirschmann® Laborgerate, Eberstadt, Germany). The last blood samples were taken by cardiac puncture at the time of animals' sacrifice. After each blood collection, microhematocrit was measured, the values were evaluated according to the reference values for the specific species defined by Havenaar *et al*. (25).

Animals' sacrifice. Thirty-five weeks after the start of the protocol, animals were sacrificed by intraperitoneal overdose of sodium pentobarbital (Eutasil, CEVA, Libourne, France): when deeply anaesthetized animals underwent exsanguination by cardiac puncture as indicated by Federation for Laboratory Animal Science Associations (26). A complete necropsy was carried out in all animals and all organs (heart, lungs, liver, kidneys, spleen and urinary bladder) were collected and macroscopically evaluated. The urinary bladder of each animal was fixated *in situ* with buffered formalin and collected as described by Teixeira *et al*. (27) for further histopathological evaluation.

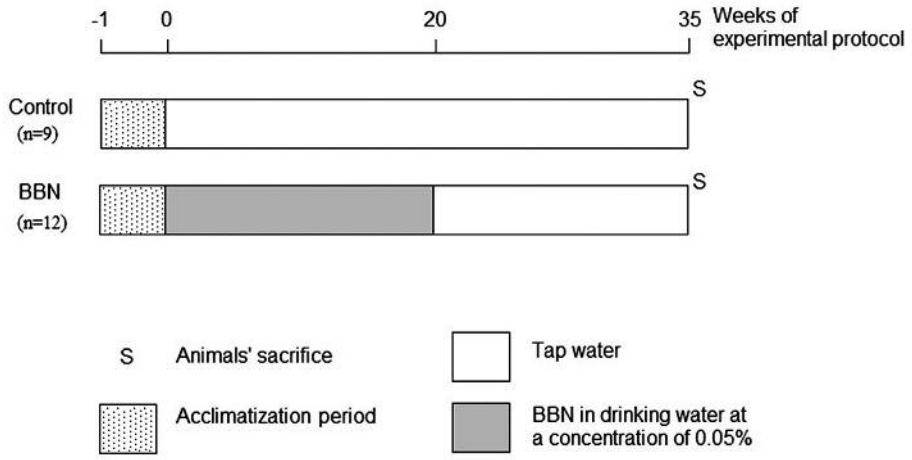

Figure 1. *Experimental protocol*.

Histological analysis. After fixation, urinary bladders from both control and BBN groups were processed for routine histological evaluation. Paraffin 2-μm-tick sections were stained with hematoxylin and eosin (H&E) and histologically evaluated under a light microscope by an experienced researcher (Oliveira PA) taking into account the lesion with the highest histological grade, following the WHO classification for urinary bladder tumors (28).

Statistical analysis. The statistical analysis was performed by SPSS® program (Statistical Package for the Social Sciences Inc., Chicago, IL, USA). Data were analyzed using the independent sample *t*-test. Differences between groups were tested using the independent *t*-test. Analysis of variance (ANOVA) with the Bonferroni correction test was used to evaluate the variables throughout the study. Data are showed as mean±standard deviation (S.D.). The differences were considered statistically significant at $p<0.05$.

Results

General observations. No deaths were recorded during the experimental protocol. All animals displayed a mental status, eyes, ears and whiskers position, response to handling, breathing and hydration within the parameters considered normal for the species. BBN-exposed animals exhibited pale mucous membranes in the last two weeks of the experimental protocol. No animal showed signs of pain or distress that implied their sacrifice before the end of the study.

Food and drink intake. We compared the mean food and water intake for control rats and for rats receiving BBN as measured at the end of the first week of the animals' arrival in the facility, and at the end of the study. The food intake was similar between groups ($p>0.05$), with an initial mean consumption of 17.11±0.32 g and 16.89±0.90 g for control and BBN groups, respectively. A final consumption of 22.02±2.81 g (control group) and 21.95±0.37 g (BBN group) was registered ($p>0.05$). An initial drink intake of 28.27±2.72 g (control group) and 25.66±1.51 g (BBN group) was noticed ($p>0.05$). The final drink intake was higher in BBN group (40.75±1.52 g) when compared with control group (34.08±5.14 g), this difference was not statistically significant ($p>0.05$).

Body weight. The animals from control group exhibited a slightly higher final body weight (477.24±39.89 g *versus* 473.52±48.90 g) and body weight gain (71.26±3.10% *versus* 71.24±2.18%) when compared with animals from BBN group. However, the differences did not reach the level of statistical significance ($p>0.05$). According to the score sheet, there was no loss of body weight, score of 0 for this parameter for all animals.

Body temperature. During the experimental protocol, no differences were observed in rectal temperature between groups ($p>0.05$). An initial rectal temperature of 35.98±1.04˚C (control group) and 36.13±1.24˚C (BBN group), and a final rectal temperature of 37.06±0.37˚C (control group) and 37.17±0.62˚C (BBN group) were registered.

Urine analysis. Although the urine volume at the beginning of the experiment was similar between groups (14.06±5.24 ml for control group and 14.88±5.34 ml for BBN group) ($p>0.05$), the final urine volume was higher in BBN group when compared with the control one (21.33±11.42 ml *versus* 13.78±1.92 ml) ($p<0.05$). The urine from control animals did not show any macroscopic changes throughout the experimental protocol, it exhibited a clear yellow color. The urine of animals from BBN group exhibited a clear yellow color during the first 14 weeks of the experiment. Hematuria was observed for the first time at the 15th week of the experimental protocol in an animal from the

Table I. *Scoring sheet for urinary bladder cancer chemically-induced by N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN).*

Parameters	Score	Status
		Body weight
Body	0	Normal
	1	Weight loss <10%
	2	Weight loss 10-15%
	3	Weight loss >15%
		Body condition
General appearance	0	Well-conditioned
	1	Under conditioned
	2	Emaciated
		Mental status
	0	Normal (alert, curious, eyes bright)
	1	Lethargic
	2	Stupor
	3	Moribund / Coma
		Coat and grooming
	0	Normal
	1	Lack of grooming
	2	Rough coat, chromodachryorrhea
	3	Very rough coat, piloerection, severe chromodachryorrhea
		Eyes, ears and whiskers
	0	Normal
	1	Partially closed eyes, droopy ears, forward whiskers
	2	Completely closed eyes, droopy and curved ears, forward and bunched whiskers
		Skin (ears, hands and feet) and mucosa (nasal and oral) (if anemic see microhematocrit)
	0	Normal
	1	Mild Anemic
	2	Moderate Anemic
	3	Severe Anemic
		Response to manipulation
	0	Normal
	1	Stress response to manipulation (signs of discomfort, vocalization)
Behavior	2	Absence of response (lethargic animal)
		Breathing
	0	Normal
	1	Tachypnea
		Hydration status (skin pinch test)
	0	Normal
	1	Abnormal
Clinical signs		**Body temperature**
	0	Normal (35.6- 38.9°C)
	1	Hyperthermia (> 38.9°C)
	2	Hypothermia (< 35.6°C)
		Urine color
	0	Normal
	1	Hematuria (+)
	2	Hematuria (++)
Urine	3	Hematuria (+++)
	4	Hematuria (++++)
	5	Hematuria (+++++)
		Urine volume
	0	Normal
	1	Oliguria
	2	Anuria

BBN group. After this, more animals showed hematuria and at the 35th week all animals from BBN group exhibited hematuria (urine from all of them showed a red color) (Table II).

Microhematocrit. The microhematocrit values can be consulted in Table III. Except in weeks 0 and 3, the microhematocrit values were lower in animals from the BBN group when compared with the control group. Statistically significant differences were only found between groups at the beginning (week 0) and at weeks 8, 29 and 35 ($p<0.05$). It is worth to note that the hematocrit value increased between the first and the last week of the experiment in control group and the inverse was observed in BBN-exposed animals ($p<0.05$). The degree of hematuria and the mean values of microhematocrit of each animal from BBN group in the last week of the experiment can be seen in Table IV. According to the reference values indicated by Havenaar *et al*. (25), four animals were considered anemic in the last week of the experiment.

Tumors induction and histological analysis. The urinary bladder of animals from control group and the remaining organs from both experimental groups did not show any macroscopic alteration. At the end of the experiment, all animals from BBN group developed urinary bladder tumors (100% of incidence), exhibiting different levels of development that were classified as different histological grade. Two animals presenting invasive carcinoma and two animals with high-grade papillary urothelial carcinoma were those with the higher degree of hematuria and lower microhematocrit (Table IV).

Table II. *Number of animals from N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) group with hematuria during the experimental protocol.*

Time (weeks)	Number of animals with hematuria (n=12)
5	0
15	1
20	5
24	11
29	10
35	12

Table III. *Microhematocrit values (%) (mean±S.D.) in both control and N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) groups throughout the experimental protocol.*

Time (weeks)	Group	
	Control	BBN
0	43.25±2.25	47.58±2.91*
3	48.89±1.45	49.42±2.07
8	51.33±1.87	49.67±1.07†
14	52.89±2.09	51.67±1.92
18	52.11±2.03	51.83±1.64
24	50.44±1.33	49.75±1.76
29	51.56±1.59	48.92±2.57‡
35	48.67±2.18	41.83±8.02#

*$p=0.002$; †$p=0.034$; ‡$p=0.010$; #$p=0.014$ from control group.

Discussion

Urinary bladder cancer remains a major concern due to its high rates of incidence and mortality (29). Since chemical carcinogens were discovered, several studies were carried out with rats and mice in the last twenty years in order to analyze the effectiveness of antineoplastic drugs in urinary bladder tumors chemically-induced or implanted (1). However, the animal welfare is often neglected, and the studies reporting the occurrence of pain and stress in animals used as cancer models are rare (30, 31). To overcome the lack of studies in this area, this work intended to implement humane endpoints in a rat model of urinary bladder cancer chemically-induced by BBN. This model is widely used by investigators to study the pathophysiology of this disease due to their similarities to human urinary bladder cancer (32).

According to our knowledge this is the first study that examined a rat male model of BBN-induced urinary bladder tumors during a long period of time (35 weeks). Since urinary bladder cancer is more frequent in men than in women, male rats were used in our protocol (29). BBN was administered orally in drinking water at a concentration of 0.05%, since it was previously verified by other investigators that this administration method and dose are the most efficient in the induction of this type of tumors (33). After 35 weeks of the beginning of the experiment, all animals from BBN group developed urinary bladder tumors (100% of incidence). These results are in accordance with those reported in previous studies performed with male rats exposed to BBN during eight weeks and sacrificed 28 weeks after the start of the protocol (33,34). Similarly to the results observed by Wanibuchi and colleagues (33), no animals died or exhibited major clinical signs during our experimental protocol.

Weight loss is a common clinical marker of disease severity. However, Franco and collaborators (20) suggested that alterations in body weight as a single indicator of disease progression may lead to misinterpretations, namely in cancer investigation due to the tumors' weight. A reliable interpretation of animals' physiological and psychological state can be achieved by combining body weight with other behavioral observations, such as food and water consumption. According to Stasiak *et al*. (35), food and water consumption are considered useful indicators of pain. Furthermore, Kelley and collaborators (36) suggested that its reduction could be

Table IV. *Hematuria degree, microhematocrit (%) and histological classification of urinary bladder tumors in each N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-exposed animal in the last week of the experimental protocol.*

Animal	Hematuria degree	Microhematocrit (%	Histological classification
1	3	47	High-grade papillary urothelial carcinoma
2	0	48	High-grade papillary urothelial carcinoma
3	2	44	High-grade papillary urothelial carcinoma
4	4	32*	High-grade papillary urothelial carcinoma
5	1	48	Low-grade papillary urothelial carcinoma
6	1	47	High-grade papillary urothelial carcinoma[1]
7	3	38*	Invasive carcinoma[1]
8	4	38*	High-grade papillary urothelial carcinoma[1]
9	3	47	Low-grade papillary carcinoma
10	0	46	Invasive carcinoma
11	5	22*	Invasive carcinoma[1]
12	1	45	Invasive carcinoma[1]

*Animals with anemia [1]These tumors occupied the whole bladder lumen.

seen as a general non-specific sign of disease. The analysis of these three parameters as a whole, namely a reduced food and water intake combined with a noticeable decrease in body weight may have considerable negative consequences for the animals, since malnutrition may lead to cachexia, and eventually, morbidity and mortality (37). Animals from BBN group showed a lower final mean body weight when compared with animals from control group ($p>0.05$). This is in accordance with data previously published by Iatropoulos *et al.* (38) and Padrão *et al.* (39). The body weight loss can be associated with the development of urinary bladder tumors due to the BBN exposition and to the related host responses (40). Although statistically significant differences in mean food intake were not observed in this study, animals from BBN group exhibited a slightly lower food intake.

Similar to that observed by Oliveira (41), at the end of the experiment, the mean drink intake was higher in the BBN group ($p>0.05$) conducing to an increase of the final urine volume ($p<0.05$). Renal macroscopic alterations were not observed in our study, which may be justified by the maintenance of the renal function due to the rapid passage of BBN trough the kidneys when compared with the time that it is in contact with urinary bladder (42).

Franco *et al.* (20) suggested that changes in body weight and temperature, along with biomarkers easily measured in blood samples, should be carefully investigated as potential early predictors of death/survival. One potential disadvantage of temperature as a marker of disease progression and humane endpoint is the natural variability in body temperature. Indeed, the body temperature may be influenced by a large number of factors, including time, activity and activation of the sympathetic nervous system (43). However, unlike to that observed in breast cancer (44), differences in body temperature between BBN and control animals were not observed.

The animal welfare scoring sheet used in this study was predictive of the tumor aggressiveness (histological grade). As expected, the urine from control animals did not show any alteration. Inversely, the number of animals from BBN group with macroscopic hematuria was increasing throughout the study due to the development of urinary bladder tumors (45-48). At 35 weeks after the beginning of the experiment, all animals from this group presented macroscopic hematuria. Accordingly, animals with hematuria showed a lower microhematocrit value (47). In alternative to the use of hematuria as a biological parameter of tumor progression and animal welfare, more advanced methodologies, such as imaging are available to monitor tumors' growth. However, some imaging modalities require repeated anesthesia, which may affect experimental purposes and have a welfare cost. Besides that, a financial cost, specialist skills and equipment are necessary (49).

To assess the animal welfare during this experimental protocol of urinary bladder carcinogenesis, several biological variables were registered. Although alterations in physiological parameters, namely in the urine volume and color and microhematocrit values were observed, no changes in the other parameters of the score sheet that justified the animals' sacrifice before the end of the study were registered. In complement to that proposed by Morton and Griffiths (19), the decision to sacrifice the animals should not only depend on sum values, but mainly on the individual evaluation of each animal as a whole.

Conclusion

The rodent animal model and experimental procedures used in this protocol were adequate to induce urinary bladder cancer. We conclude that the score sheet and evaluated

physiological parameters are adequate to monitor animals' welfare during an assay of urinary bladder carcinogenesis. Furthermore, this work suggests that urine evaluation is a useful parameter in urinary bladder carcinogenesis studies to determine the general health and physiological status of the animals, with the particularity of being obtained non-invasively.

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Conflicts of Interest

The Authors declare that they have no conflicts of interest.

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