New Therapeutic Perspective for Bladder Cancer in Dogs: Toxicological and Clinical Effects of OncoTherad Nanostructured Immunotherapy

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Abstract. Bladder cancer (BC) comprehends around 2% of all spontaneously occurring cancers in dogs. BC treatment remains a challenge since recurrence and progression of the disease, as well as the pronounced side effects associated to the available therapeutic modalities are present. In this scenario, a new perspective is represented by OncoTherad nanostructured immunomodulator. Thus, the aims of this study were to characterize and to evaluate the efficacy and possible toxicological effects of OncoTherad intravesical immunotherapy in 6 dogs with BC. Our results demonstrated that before the first instillation of OncoTherad, all dogs presented irregular tumor mass, mixed echogenicity, and hyperechoic echotexture, with a mean tumor volume of 9.38 cm³. After 6 instillations of OncoTherad, the tumor mass reduced 62.34% of its volume in relation to the initial ultrasound. At the end of 24 instillations, the tumor mass reduced 84.54% of its volume. Hematuria was decreased throughout OncoTherad treatment, disappearing on average after the eighteenth application and not returning after the last application. OncoTherad treatment showed no signs of systemic toxicity at the proposed therapeutic dose. In conclusion, OncoTherad intravesical immunotherapy seems a safe and effective treatment option for spontaneous canine bladder cancer and may provide benefit for preventing tumor recurrence.

Keywords: Bladder Cancer, OncoTherad, Immunotherapy, Nanomedicine, Inorganic phosphate complex.
1. Introduction
Bladder cancer (BC) that affects humans has similar characteristics with invasive urothelial cancer that occurs spontaneously in dogs [1, 2]. According to the National Canine Cancer Foundation, BC accounts for about 2% of all malignancies reported in dogs. With more than 70 million dogs in the USA and the development of cancer in approximately 25% of older dogs, it is estimated that BC affects more than 20,000 dogs annually. Most BC occurs in older dogs, although the disease may occur early in a minority of animals [1, 2].

BC in dogs is often located in the vesical trigone. Bladder tumors range from intermediate to high-grade papillary proliferative lesions, which may lead to partial or complete obstruction of the urinary tract, as well as tumor progression in the kidneys, lymph nodes, lungs and liver [1-3]. The most frequent clinical sign of canine BC is the hematuria. Initially, there is absence of dysuria and polyuria; however, with the progress of disease, these factors appear and become frequent. Urinary tract infections are common and, sometimes cases of hypertrophic osteopathy are also observed [1, 4].

BC treatment remains a challenge since recurrence and progression of the disease, as well as the pronounced side effects associated to the available therapeutic modalities are present [1, 4]. In this scenario, compounds that modulate immune system, through toll-like receptors (TLRs), could be an inestimable strategy for the cancer treatment, either used alone or in combination with existing therapies [5-8]. Some studies have shown that intratumor administration of certain phosphate compounds activates the immune system in the tumor microenvironment, leading to significant tumor regression [7].

Considering the importance of the development of drugs that can be administered intravesically and acting as modulators of the immune system, our research group developed a synthetic nanostructured compound with antitumor and immunological properties called MRB-CFI-1 (Biological Response Modifier - Inorganic Phosphate Complex 1), or OncoTherad [9]. Preclinical studies from our group demonstrated that in the treatment of BC chemically induced in rodents, animals treated with 25 mg/Kg of OncoTherad showed significant inhibition of tumor progression in 70-80% of cases [9]. The aims of this study were to characterize and to evaluate the efficacy and possible toxicological effects of OncoTherad intravesical immunotherapy in 6 dogs with bladder cancer.

2. Material and Methods
The toxicological and antitumor effects of OncoTherad on the progression of bladder cancer were evaluated in 6 dogs, attended at the Veterinary Clinic “Dr. Ronaldo Tizziani” (Campinas, São Paulo, Brazil) presenting macroscopic hematuria. After urothelial carcinoma diagnosis and consent of dog owners, OncoTherad treatment was started. The animal experiments were approved by an institutional Committee for Ethics in Animal Use (CEUA-UNICAMP, protocol no. 4481-1/2017).

2.1. Histopathological analysis
Samples of bladder tumors (n= 6 dogs) were fixed in Bouin solution for 12 h and then washed in 70% ethanol, dehydrated in an increasing series of alcohols, cleared in xylene for 2 h and embedded in plastic polymer (Paraplast Plus, St. Louis, MO, USA). Subsequently, 5-µm thick sections were cut on a rotary microtome (Slee CUT5062 RM 2165; Slee Mainz, Mainz, Germany), stained with hematoxylin-eosin and photographed with a Leica DM2500 photomicroscope (Leica, Munich, Germany). A senior uropathologist analyzed the urinary bladder lesions based on the criteria of the Health/World International Society of Urological Pathology Organization [10].

2.2. OncoTherad intravesical treatment
The dogs received 25 mg of OncoTherad dissolved in 2.0 mL of 0.9% physiological saline by cystocentesis [9]. These animals received a weekly dose of OncoTherad for six consecutive weeks. For maintenance therapy, the animals received a dose of OncoTherad every 15 days for 6 months and a monthly dose for another 6 months, totalling 24 intravesical instillations of OncoTherad.
2.3. Ultrasonography and urine analyzes
Therapeutic effects of OncoTherad were evaluated by ultrasonography and urinalysis (Urine 1 – red blood cells) during treatment cycle. Ultrasound and urine evaluations were performed at the following time points: before the first instillation, after the first instillation and after 3, 6, 18 and 24 instillations of OncoTherad. Tumor volumes were calculated using the formula of an ellipsoid V (Tumor Volume) = 0.5235 x (small diameter)^2 x large diameter [9].

2.4. Toxicological and biochemical analyzes
The biochemical analyzes were performed to verify the possible systemic toxicity of OncoTherad intravesical immunotherapy, namely: alanine aminotransferase (ALT), a specific marker for hepatic parenchyma lesion; erythrogram (hemoglobin); leukogram (leukocytes); thrombogram (platelets); and creatinine and urea to check renal function. Spectrophotometric measurements were performed on a Pharmacia Biotech spectrophotometer with temperature controlled cuvette chamber (UV/ visible Ultrospec 5,000 with Swift II software applications for computer control, 97-4213, Cambridge, England, UK). All chemical reagents were from LaborLab (Guarulhos, São Paulo, Brazil). Biochemical analyses were performed at the following time points: before the first instillation and after 6 and 24 instillations of OncoTherad.

2.5. Statistical Analysis
Exploratory data analysis was performed through summary measures (mean, standard deviation, minimum, median and maximum). The comparisons between the times of the therapeutic regimen with the tumor volume and with the biochemical parameters (clinical toxicology) were evaluated through ANOVA for repeated measures with the variables transformed into stations. The level of significance was 5%. The software used was: The SAS System for Windows (Statistical Analysis System), version 9.4. SAS Institute Inc, Cary, NC, USA.

3. Results and Discussion
3.1. OncoTherad intravesical immunotherapy was effective in reducing bladder tumors and hematuria
Before the first instillation of OncoTherad, all dogs presented irregular tumor mass, mixed echogenicity, and hyperechoic echotexture, with a mean tumor volume of 9.38 cm^3 ± 4.43 (Figure 1; Table 1). After 6 instillations of OncoTherad, the tumor mass reduced 62.34% ± 16.38% (3.06 cm^3 ± 1.46) of its volume in relation to the initial ultrasound (Figure 1; Tables 1 and 2). At the end of 24 instillations, the tumor mass reduced 84.54% ± 5.23% (1.37 cm^3 ± 0.78) of its volume (Figure 1; Tables 1 and 2).

Hematuria (presence of red blood cells in the urine) was decreased throughout OncoTherad treatment, disappearing on average after the eighteenth application and not returning after the last application (Table 3). Thus, the reduction of hematuria was related to the reduction of tumor volume (Table 3).

The uses of chemotherapeutic agents and cyclooxygenase (COX) inhibitors, or combinations thereof, are the therapeutic pillar of primary and metastatic bladder cancer in dogs [1]. Remission rates are less than 20% versus single agent use, and 35-50% with the combination of chemotherapy and COX inhibitors. Chemotherapy drugs based on platinum, cisplatin and carboplatin are most commonly used in the treatment of canine bladder cancer, especially when combined with COX inhibitors [1]. However, the use of these platinum-based chemotherapeutics is limited, as they promote a number of serious side effects, as well as severe toxic effects on the gastrointestinal tract, urinary tract and bone marrow [11, 12]. The use of vinblastine and gemcitabine produces very low rates of remission of the disease, around 25% and 36%, respectively [13, 14].
**Table 1**: Comparison of bladder tumor volume throughout the treatment schedule (ANOVA for repeated measures with the transformed response variable in posts).

| Variable                             | Number | Mean  | S.D.  | Minimum | Median | Maximum | p-value  |
|--------------------------------------|--------|-------|-------|---------|--------|---------|----------|
| Tumor volume before 1st Instillation | 6      | 9.38a | 4.43  | 4.07    | 9.00   | 14.93   | <.0001   |
| Tumor volume after 1st instillation  | 6      | 6.59b | 2.05  | 3.75    | 6.55   | 9.12    |          |
| Tumor volume after 3rd instillation  | 6      | 5.68b | 2.20  | 3.51    | 5.56   | 9.15    |          |
| Tumor volume after 6th instillation  | 6      | 3.06c | 1.46  | 1.60    | 2.67   | 5.77    |          |
| Tumor volume after 18th instillation | 6      | 2.09d | 0.89  | 1.17    | 1.91   | 3.79    |          |
| Tumor volume after 24th instillation | 6      | 1.37e | 0.78  | 0.63    | 1.21   | 2.83    |          |

Different lowercase letters (a, b, c, d, e) indicate significant differences (p <0.0001) throughout the treatment.

S.D.: standard deviation

**Table 2**: Comparison of percentage of reduction of bladder tumor volume throughout the treatment schedule (ANOVA for repeated measures with the variable transformed in posts).

| Variable                             | Number | Mean  | S.D.  | Minimum | Median | Maximum | p-value  |
|--------------------------------------|--------|-------|-------|---------|--------|---------|----------|
| Tumor reduction after 1st instillation | 6      | 22.84a| 20.15 | 3.03    | 15.36  | 52.84   | <.0001   |
| Tumor reduction after 3rd instillation | 6      | 33.57a| 21.68 | 5.20    | 38.31  | 66.29   |          |
| Tumor reduction after 6th instillation | 6      | 62.34b| 16.38 | 44.71   | 58.94  | 88.17   |          |
| Tumor reduction after 18th instillation | 6      | 73.56c| 13.77 | 50.36   | 74.59  | 91.35   |          |
| Tumor reduction after 24th instillation | 6      | 84.54d| 5.23  | 79.30   | 83.36  | 93.93   |          |

Different lowercase letters (a, b, c, d) indicate significant differences (p <0.0001) throughout the treatment.

S.D.: standard deviation
Table 3: Comparison of presence of hematuria (red blood cells in the urine) throughout the treatment schedule (ANOVA for repeated measures with the transformed response variable in posts).

| Variable                        | Number | Mean   | S.D.   | Minimum | Median | Maximum | p-value |
|---------------------------------|--------|--------|--------|---------|--------|---------|---------|
| Tumor volume before 1st instillation | 6      | 277,583.3a | 37,3197.9 | 1,500.00 | 95,000.0 | 960,000.0 | <.0034  |
| Tumor volume after 1st instillation | 6      | 80,166.67b  | 81,290.63 | 1,000.00 | 60,000.0 | 200,000.0 |         |
| Tumor volume after 3rd instillation | 6      | 43,750.00b  | 44,451.94 | 2,500.00 | 40,500.0 | 98,000.00 |         |
| Tumor volume after 6th instillation | 6      | 15,833.33c  | 19,054.31 | 500.00   | 9,500.00 | 50,000.00 |         |
| Tumor volume after 18th instillation | 6      | 3,866.67d   | 4,501.85  | 200.00   | 2,000.00 | 1,2000.00 |         |
| Tumor volume after 24th instillation | 6      | 1,266.67d   | 1,072.69  | 200.00   | 1,050.00 | 3,000.00  |         |

Different lowercase letters (a, b, c, d, d) indicate significant differences (p <0.0034) throughout the treatment.

Reference value for red blood cells in urine: up to 6,000/mL

S.D.: standard deviation
Figure 1: Ultrasonography representative of the urinary bladder from Dog 1 (as example) at the following times: before the first instillation (a), after the first instillation (b) and after 3 (c), 6 (d), 18 (e) and 24 (f) instillations of OncoTherad.
3.2. *OncoTherad intravesical immunotherapy does not cause systemic toxicity at the therapeutic dose*

Serum ALT, urea and creatinine analyzes did not show significant differences prior to OncoTherad treatment, as well as, after 24 instillations (Table 4). Furthermore, erythrogram, leukogram, and thrombogram analyzes in all dogs also did not show significant differences before OncoTherad treatment, as well as, after 24 instillations (Table 4). So, these results indicated that OncoTherad treatment showed no signs of systemic toxicity at the proposed therapeutic dose.

### Table 4: Clinical Evaluation of Dogs, Before and After OncoTherad Instillations.

| Therapeutic Schedule | Hemoglobin (g/dL) | Leukocytes (mm$^3$) | Platelets (mm$^3$) | ALT (U/L) | Urea (mg/dL) | Creatinine (mg/dL) |
|----------------------|--------------------|---------------------|--------------------|-----------|--------------|------------------|
| Dog 1                |                    |                     |                    |           |              |                  |
| Before 1st Instillation | 10.6              | 29,900              | 355,000           | 95.0      | 36.0         | 1.20             |
| After 6th Instillation | 11.8              | 16,300              | 400,000           | 88.0      | 51.0         | 0.85             |
| After 24th Instillation | 12.1             | 13,000              | 375,000           | 81.0      | 42.0         | 0.98             |
| Dog 2                |                    |                     |                    |           |              |                  |
| Before 1st Instillation | 12.6              | 28,000              | 455,000           | 188.0     | 25.0         | 1.10             |
| After 6th Instillation | 12.0              | 16,300              | 350,000           | 129.0     | 35.0         | 0.80             |
| After 24th Instillation | 13.1             | 11,000              | 400,000           | 68.0      | 22.0         | 1.00             |
| Dog 3                |                    |                     |                    |           |              |                  |
| Before 1st Instillation | 12.0              | 7,900               | 950,000           | 46.0      | 30.0         | 1.16             |
| After 6th Instillation | 13.9              | 8,600               | 759,000           | 58.0      | 45.0         | 0.94             |
| After 24th Instillation | 14.7              | 12,900              | 300,000           | 41.0      | 50.0         | 0.90             |
| Dog 4                |                    |                     |                    |           |              |                  |
| Before 1st Instillation | 11.4              | 15,800              | 430,000           | 64.0      | 54.0         | 1.15             |
| After 6th Instillation | 12.3              | 18,800              | 390,000           | 80.0      | 53.0         | 0.78             |
| After 24th Instillation | 12.0              | 17,000              | 410,000           | 84.0      | 56.0         | 0.66             |
| Dog 5                |                    |                     |                    |           |              |                  |
| Before 1st Instillation | 12.7              | 10,500              | 260,000           | 33.0      | 31.0         | 0.62             |
| After 6th Instillation | 12.1              | 9,600               | 334,000           | 50.0      | 40.0         | 1.15             |
| After 24th Instillation | 12.5              | 11,200              | 420,000           | 45.0      | 52.0         | 0.77             |
| Dog 6                |                    |                     |                    |           |              |                  |
| Before 1st Instillation | 14.2              | 9,100               | 260,000           | 52.0      | 34.0         | 0.85             |
| After 6th Instillation | 13.9              | 8,300               | 360,000           | 40.0      | 31.0         | 0.88             |
| After 24th Instillation | 14.7              | 7,900               | 330,000           | 48.0      | 33.0         | 0.92             |

Reference Values: Hemoglobin (12 – 18 g/dL); Leukocytes (6,000 – 18,000/mm$^3$); Platelets (150,000 – 500,000/mm$^3$); ALT (10 – 88 U/L); Urea (10 – 65 mg/dL); Creatinine (0.5 – 1.5 mg/dL).
4. Conclusions
Although the use of chemo and immunotherapy represents a clear advance in the treatment of canine bladder cancer, the management of this disease, mainly for high grade tumors remains a challenge, because the high rates of recurrence and progression to metastatic stages. Our study demonstrated that OncoTherad intravesical immunotherapy seems a safe and effective treatment option for spontaneous canine bladder cancer and may provide benefit for preventing tumor recurrence.

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