Safety and Efficacy of OncoTherad Nano-immunotherapy in Patients with Non-Muscle Invasive Bladder Cancer

J C C Alonso¹², M C Maciel², H A Ferrari², J M Gonçalves¹, B R S Sasaki¹, A A Cintra², N Durán¹*, A Billis³ and W J Fávaro¹**

¹Laboratory of Urogenital Carcinogenesis and Immunotherapy, Department of Structural and Functional Biology, University of Campinas (UNICAMP), Campinas, SP, Brazil.
²Paulínia Municipal Hospital, São Paulo, Brazil
³Department of Pathology, School of Medicine, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil.

*E-mail: nelsonduran1942@gmail.com
**E-mail: favarowj@unicamp.br

Abstract. The new modalities for treating patients with non-muscle invasive bladder cancer (NMIBC) for whom Bacillus Calmette-Guerin (BCG) has failed or is contraindicated are recently increasing due to the development of new drugs. In this scenario, a new perspective is represented by OncoTherad nano-immunotherapy. We carried out a prospective, single-center (Municipal Hospital of Paulínia, São Paulo, Brazil), single-arm phase I/II study (Clinical Trial: RBR-6swqd2) in 29 (18 male, 11 female) patients with BCG-refractory, relapsed or intolerant NMIBC (≥ 1 previous course of BCG therapy). The median age of the 29 patients and follow-up were 65 years (range 34-96) and 24 months, respectively. OncoTherad treatment showed complete response of 79.3% and recurrence-free survival of 22.2 months at 24-month follow-up. Regarding toxicity, 62.1% of adverse events were Grade 1 or 2. The most commonly reported treatment-related adverse events were dysuria, cystitis, pruritus, rash, arthralgia and fatigue. Also, this study demonstrated an important effect of activation of the Toll-like Receptor 4 (TLR4) signaling pathway triggered by OncoTherad in the formation and organization of primary lymphoid follicles in most patients at 24-months follow-up, which may be related to antitumor and immunoprotective effects from this immunotherapy in bladder tissue. In conclusion, OncoTherad nano-immunotherapy seems a safe and effective treatment option for BCG-relapsed and/or intravesical chemotherapy-relapsed NMIBC patients and may provide benefit for preventing tumor recurrence.

1. Introduction

The non-muscle invasive bladder cancer (NMIBC) represents the majority (75%) of newly diagnosed cases of bladder cancer [1,2]. Among NMIBC stages, pTis, high-grade pTa and pT1 tumors are considered to be at high risk for disease recurrence and progression [3]. The standard treatment for high-risk NMIBC is transurethral resection of bladder tumor (TURBT), followed by adjuvant intravesical therapy with Bacillus Calmette-Guérin (BCG) [2, 3]. Although BCG intravesical treatment is the gold standard adjuvant therapy for high-grade NMIBC, its use is limited due to treatment failure, adverse effects and intolerance that occur in more than two-thirds of patients [1,2].
While 84% of patients with NMIBC stage pTis initially achieve a complete response with BCG [4], up to 50% fail to maintain a durable response [5]. Thus, there is a clear unmet need for safe and effective therapeutic options in patients with NMIBC, in whom BCG therapy has failed. Patients with high-grade NMIBC are at increased risk of disease progression. Failure to respond to BCG re-induction is associated with 30% to 50% recurrence and progression risk for muscle invasive disease [6, 7]. Early radical cystectomy has been the standard treatment for improving survival, as therapeutic options are limited. However, cystectomy causes significant changes in morbidity, mortality and quality of life [6]. Currently, there is no gold standard intravesical rescue therapy for patients who do not respond to BCG therapy.

Therapeutic strategies that modulate the tumor microenvironment and reinforce the migration of T cells to the tumor are fundamental in the control of several types of cancer [8]. One of these strategies is the use of innate immunity modulators, such as toll-like receptor (TLR) agonists [8]. Treatment based on the use of these agonists increases the activity of antitumor effector cells, such as cytotoxic T cells and natural killer cells (NK), and at the same time blocks the activity of some types of immunosuppressive cells, such as regulatory T cells (Treg) and myeloid suppressor cells [8].

In this scenario, a new perspective is represented by OncoTherad nano-immunotherapy. OncoTherad is a nanostructured inorganic phosphate complex associated to glycosidic protein, developed by University of Campinas/ Brazil, which exhibits immunomodulatory and antitumor properties [9]. The aims of this study were to evaluate the safety and efficacy of OncoTherad nano-immunotherapy BCG-refractory or relapsed NMIBC.

2. Materials and Methods

2.1. Pharmacological treatment of patients with NMIBC: use of OncoTherad nano-immunotherapy

A total of 29 patients (18 male and 11 female), coming from Paulinia Municipal Hospital (HMP), São Paulo/ Brazil, with BCG-refractory, relapsed or intolerant NMIBC (≥ 1 prior course of BCG therapy), were included in the study. All included patients were previously submitted to transurethral resection of bladder tumor (TURBT), performed at the HMP.

After diagnosis, all patients signed the Free and Informed Consent Form and received treatment with OncoTherad. The therapeutic regimen with OncoTherad consisted of:
- Induction phase: a weekly application for six consecutive weeks of OncoTherad through intravesical [60 mL (120 mg/mL - keep intravesical for 1 hour) and intramuscular [2 mL (25 mg/mL)] routes [10];
- Maintenance Phase: a biweekly application (intravesical and intramuscular) for 3 months and a monthly application (intravesical and intramuscular) for a further 9 months until completing 1 year of treatment. Then, a quarterly application (intravesical and intramuscular) for another 1 year [10].

The study was approved by the Research Ethics Committee/ UNICAMP (CAAE: 93619718.7.0000.5404) and registered on the Brazilian Clinical Trials Registry platform (RBR-6swqd2 - http://www.ensaiosclinicos.gov.br/rg/RBR-6swqd2).

2.2. Inclusion and exclusion criteria

2.2.1. Inclusion criteria

Included were patients with bladder tumor who relapsed after standard BCG therapy. In addition, BCG-refractory or intolerant patients were included, as well as those refractories to approved first- and second-line chemotherapy, who have a higher risk of progression to invasive and/or metastatic muscle disease. BCG-refractory diseases is defined when there is failure to achieve a disease-free state at 6 months following initial BCG therapy with either maintenance or retreatment at 3 months because of persistent or rapidly recurrent tumor. BCG-relapsing disease when the disease recurs after the patient
is disease-free for 6 months. BCG-intolerant disease when the disease recurs following administration of a less than adequate course of therapy because of serious adverse event or symptomatic intolerance that require discontinuation or further BCG therapy.

- Acceptance of the Informed Consent Form;
- Patients considered ineligible for radical cystectomy;
- Multiple tumors (> two lesions) and/or recurrent and/or large (> 3 cm) TaG1-2;
- Non-muscle invasive tumor (pT1);
- Carcinoma in situ (Cis);
- Grade 3 bladder urothelial carcinoma.

2.2.2. Exclusion criteria
Patients with suspected of malignant bladder neoplasia and who were not previously submitted to TURBT, that is, patients who were not treated for bladder tumor.

- Histology: adenocarcinoma, squamous cell carcinoma.

2.3. Evaluation of therapeutic efficacy of OncoTherad nano-immunotherapy
The main criteria for assessing efficacy were complete response (CR) and recurrence-free survival (RFS). CR was defined as the proportion of patients who showed no evidence of disease or disease progression. RFS corresponded to the time from the date of first dose of OncoTherad treatment to disease recurrence or death as the first event. Recurrence was defined as any tumor recurrence that was histologically proven (any degree) [10].

To assess therapeutic efficacy, all patients were followed up on an outpatient basis (Paulinia Municipal Hospital, São Paulo/ Brazil), undergoing anatomopathological examination, urinary tract ultrasound, cystoscopy and laboratory tests every three months in the first year and, semiannually in the 2nd year [10].

2.4. Histopathological and immunohistochemical analyzes
Samples of urinary bladder from all patients were fixed in 10% formaldehyde for 24 hours and included in paraffin. Subsequently, the bladder samples were sectioned with 5 μm thick in a CUT5062 semiautomatic microtome (SLEE MAINZ, Munich, Germany), stained with Hematoxylin-Eosin and photographed on the Leica DM2500 photomicroscope, camera DFC295 (Leica, Munich, Germany). Urothelial lesions were classified according to the staging proposed by the consensus of the World Health Organization / International Society of Urological Pathology [10].

After the histopathological diagnosis, the presence of numerous primary lymphoid follicles (PLFs) was found in the urinary bladder samples of 15 patients undergoing treatment with OncoTherad. Thus, to verify the influence of OncoTherad in the formation of PLFs, as well as the role of these structures in the immune modulation of the bladder tissue microenvironment, urinary bladder biopsies prior and after treatment with OncoTherad were subjected to immunohistochemical analyzes immune system mediators: TLR4, TRIF, IRF3, IFN-γ and iNOS. To assess the intensity of the immunoreactivities from different antigens in the bladder tissue, five fields with 400x magnification were selected for each antibody. The immunoreactivities results were analyzed using the Image J software (https://imagej.nih.gov/ij/) in Macro Profile Analysis from the selection of the urothelium and quantification of the positive urothelial cells. Quantitative data were evaluated in two ways: Total Immunoreactivity and Intensity of Immunoreactivity. Total immunoreactivity was obtained as a result of the percentage of negative urothelial and/or stromal cells for a given antibody subtracted from 100%, that is, the values represent the total of urothelial and/or stromal cells in the field that showed immunoreactivity for evaluated antibody. The analysis of the Immunoreactivity Intensity was performed based on the categorization of the immunoreaction occurring in urothelial and/or stromal cells by intensity criterion. The categories defined in the Image J software on a scale of 0-3, and expressed as 0 (absence of immunoreactivity), 0% of positive cells; 1 (weak immunoreactivity), 1-
35% positive cells; 2 (moderate immunoreactivity), 36-70% positive cells; 3 (intense immunoreactivity), > 70% positive cells.

2.5. Toxicological Analysis
To assess the possible local and/or systemic toxics of OncoTherad nano-immunotherapy, all patients were evaluated clinically according to the Toxicity Scale proposed by the 4th Common Terminology Criteria for Adverse Events. For this, blood and urine samples were collected every three months in the 1st year and every six months in the 2nd year, for the following assessments: blood count (hemoglobin and leukocytes); thrombogram; serum levels of: glucose, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), gamma-glutamyl transferase (GGT), urea, creatinine. The local toxic effects were assessed through inflammatory findings during cystoscopy, presence of hematuria and the assessment of Lower Urinary Tract Symptoms (LUTS).

2.6. Statistical Analysis
To verify the response to treatment with OncoTherad according to histological gradation, size and focality of the tumors, frequencies (absolute and percentage) were obtained and Fisher's exact test was used.

For the analysis of recurrence and biochemical serological parameters before and after treatment with OncoTherad, the quantitative variables were presented in values of central tendency and dispersion. The homogeneity of the variance and adherence to the normal curve were assessed by the Kolmogorov-Smirnov test, considering that the variables did not present a normal distribution, a non-parametric test [Kruskal-Wallis Test (Nonparametric ANOVA)] was used.

Descriptive measures (mean, standard deviation, minimum, median and maximum) were obtained for the time of recurrence-free survival (RFS) according to the time of response to treatment (complete response).

Immunohistochemical analyzes for different antigens were evaluated using the proportion test. For these analyzes, type-I error of 5% was considered statistically significant.

The occurrence of adverse reactions (No or Yes) according to the degree of intensity (1-2 or 3-4) was assessed using the McNemar test and descriptive measures were also obtained to test the difference between the medians of the total number of reactions degrees 1-2 versus 3-4 with Wilcoxon test. Each type of reaction was also evaluated individually, and frequencies were obtained (absolute and percentage).

3. Results and Discussions

3.1. Demographic and general characteristic of patients.
Between August 2018 to July 2020, 29 patients were enrolled and started treatment with OncoTherad. During the 24-month follow-up period, no patient was excluded from the protocol. Of total of 29 patients included in the protocol, 18 (62.1%) were men and 11 (37.9%) women, with a mean age of 65 years. Considering the risk factors for bladder cancer, current smokers represented 24.1% of the total patients; ex-smokers (65.5%) and, non-smokers 10.4%. Most of the included patients (86.2%) used 2 prior courses of intravesical BCG. Seven included patients (24.1%) had previously used intravesical chemotherapy with Gemcitabine, after failure or lack of BCG. In relation to BCG failure, 55.2% of patients were refractory to BCG, 37.9% relapsing and, 6.9% intolerant (Table 1).
Table 1. Baseline demographic and general characteristics of patients

| Characteristics                              | No. (%)                      |
|---------------------------------------------|------------------------------|
| Follow-up time                              | 24 months                    |
| Age, median (years)                         | Between August 2018 and July 2020 |
| Gender                                      |                              |
| Male                                        | 18 (62.1%)                   |
| Female                                      | 11 (37.9%)                   |
| Smoking                                     |                              |
| Never                                       | 03 (10.4%)                   |
| Former                                      | 19 (65.5%)                   |
| Actual                                      | 07 (24.1%)                   |
| Prior BCG cycles                            |                              |
| 1                                           | 04 (13.8%)                   |
| 2                                           | 25 (86.2%)                   |
| BCG failure                                 |                              |
| BCG refractory                              | 16 (55.2%)                   |
| BCG relapsing                               | 11 (37.9%)                   |
| BCG intolerant                              | 02 (6.9%)                    |
| Intravesical chemotherapy                   | 07 (24.1%)                   |

3.2. OncoTherad nano-immunotherapy with was effective in the treatment of NMIBC, promoting high rates of complete response and increase in recurrence-free survival in the 24-month follow-up

Regarding staging and initial histological grading, after treatment with BCG, most tumors were high-grade pTa (55.2%) followed by high-grade pT1 (34.5%) and pTis (10.3%). Most tumors presented as single lesions in 58.6% of patients and multifocal lesions in 41.4%. Tumors> 3 cm occurred in 89.7% of patients, whereas tumors <3 cm occurred in 10.3%.

The mean recurrence after a previous course of BCG was 2.2 recurrences per patient. The mean and median time of the last recurrence, considering only the last 24 months of treatment with BCG, were 10.3 and 8.0 months, respectively.

After 3 to 9 months of OncoTherad treatment, no patient had recurrence (Table 2). However, a total of 06 patients had recurrence over the 24-month follow-up, with 3 patients in the 10 to 12-month follow-up, 02 in the 13 to 18-month follow-up and 01 in the 19 to 24-month follow-up (Table 2).

In relation to histological grade, low-grade pTa, high-grade pT1 and high-grade pTa tumors occurred in 50.0%, 33.3% and 16.7% of patients who relapsed to OncoTherad (Table 2). OncoTherad immunotherapy significantly reduced histological grading compared to previous treatment with BCG (Table 2). High-grade tumors occurred in 100% of patients who underwent previous treatment with BCG, while on OncoTherad treatment, 50.0% were high-grade and 50% low-grade (Table 3).

The patients, who relapsed to OncoTherad treatment, presented single lesions <3 cm (Tables 2 and 3). Similar to histological grade, OncoTherad immunotherapy significantly reduced the size and focality of the lesions compared to previous treatment with BCG (Table 3).
A total of 23 patients (79.3%) had a complete response (CR) after 24-month follow-up with OncoTherad (Table 2). In the 24-month follow-up, the RFS was 22.2 months (Table 3).

Our study with OncoTherad nano-immunotherapy was consistent with the guidelines of Food and Drug Administration (FDA) and International Bladder Cancer Group, since we used the CR and RFS as the primary outcome, in addition to quarterly clinical monitoring in the first year and semiannual in the second year. Thus, considering the data together, OncoTherad nano-immunotherapy seems a safe and effective treatment option for BCG-failure and or intravesical chemotherapy-relapsed NMIBC patients and may provide benefit for preventing tumor recurrence.

3.3. OncoTherad nano-immunotherapy stimulated the TLR4 signaling pathway for interferon production and induced the formation of primary lymphoid follicles after 24-month follow-up

OncoTherad nano-immunotherapy induced the formation of primary lymphoid follicles in most patients (51.7%) after 24-month follow-up. Thus, considering the importance of the formation of primary lymphoid follicles (PLFs) in the bladder tissue, the immunohistochemical reactions and analyzes after 24-month follow-up with OncoTherad, were performed in the urinary bladder regions from 15 patients (51.7%) who presented PLFs.

Immunoreactivities for TLR2 were significantly weak (Table 4) on biopsies prior to treatment with OncoTherad. Similarly, TLR4, TRIF, IRF-3, IFN-γ and iNOS immunoreactivities were significantly weak (Table 4) in the biopsies prior to treatment with OncoTherad. In contrast, TLR2, TLR4, TRIF, IRF-3, IFN-γ and iNOS immunoreactivities were significantly intense (Table 4) in the regions of PLFs from patients treated with OncoTherad, after 24-month follow-up. Thus, the present study demonstrated an important effect of the activation of the TLR4 signaling pathway triggered by OncoTherad in the formation and organization of PLFs, which may be related to the immunoprotective effects on bladder tissue.

3.4. OncoTherad nano-immunotherapy caused mild and moderate adverse reactions in the 24-month follow-up

A total of 22 patients (75.9%) had adverse reactions resulting from OncoTherad treatment, while 7 patients (24.1%) had no adverse effects. Grade 1-2 adverse reactions were significantly more frequent than Grade 3-4 reactions (Table 5). A total of 18 patients (62.1%) had only Grade 1-2 adverse reactions, while 4 patients (13.8%) had both Grade 1-2 and Grade 3-4 adverse reactions.

The most common Grade 1-2 adverse reactions (≥ 20%) were dysuria (51.7%), pruritus (44.8%), cystitis (34.5%), fatigue (27.6%) and rash (27.6%) (Table 5).

The most common Grade 3-4 adverse reactions (≥ 2%) were diarrhea, abdominal pain, skin rash, cough and dyspnea (Table 5).

In addition, biochemical serological analyzes for glucose, hemoglobin, leukocytes, platelets, GOT, GPT, GGT, urea and creatinine showed no significant differences before and after treatment with OncoTherad. Thus, these results indicated that OncoTherad treatment did not show signs of systemic toxicity at the proposed therapeutic dose.
Table 2. Frequencies of categorical variables (historical grade, tumor focality, tumor size and complete response) and descriptive measures of quantitative variables (number of relapses and recurrence-free survival) during 24 months of OncoTherad treatment

| Parameters                      | N   | %    | Mean | Standard Deviation | Minimum | Median | Maximum |
|---------------------------------|-----|------|------|--------------------|---------|--------|---------|
| **Number of Relapses**          |     |      |      |                    |         |        |         |
| 3 to 9-month follow-up          | 0   | 0    | 0    | 0                  | 0       | 0      | 0       |
| 10 to 12-month follow-up        | 03  | 50.0 | 0.10 | 0.30               | 0       | 0      | 1       |
| 13 to 18-month follow-up        | 01  | 16.7 | 0.03 | 0.18               | 0       | 0      | 1       |
| 19 to 24-month follow-up        | 02  | 33.3 | 0.07 | 0.26               | 0       | 0      | 1       |
| Absent Frequency= 23            |     |      |      |                    |         |        |         |
| **Total**                       | 06  | 20.7 | 0.20 | 0.40               | 0       | 0      | 1.0     |
| **Histological Grade**          |     |      |      |                    |         |        |         |
| High-grade pT1                  | 02  | 33.3 | -    | -                  | -       | -      | -       |
| High-grade pTa                  | 01  | 16.7 | -    | -                  | -       | -      | -       |
| Low-grade pTa                   | 03  | 50.0 | -    | -                  | -       | -      | -       |
| Absent Frequency= 23            |     |      |      |                    |         |        |         |
| **Tumor Focality**              |     |      |      |                    |         |        |         |
| Multifocal                      | 01  | 16.7 | -    | -                  | -       | -      | -       |
| Single                          | 05  | 83.3 | -    | -                  | -       | -      | -       |
| Absent Frequency= 23            |     |      |      |                    |         |        |         |
| **Tumor Size**                  |     |      |      |                    |         |        |         |
| <3 cm                           | 06  | 100  | -    | -                  | -       | -      | -       |
| >3 cm                           | 0   | 0    | -    | -                  | -       | -      | -       |
| Absent Frequency= 23            |     |      |      |                    |         |        |         |
| **Complete Response (CR)**      | 23  | 79.3 | -    | -                  | -       | -      | -       |
| **Recurrence-Free Survival (24-month follow-up)** | 29  | 100  | 22.2 | 4.1                | 10.0    | 24.0   | 24.0    |
Table 3. Evaluation of histological grade, tumor size and tumor focality before (BCG) and after OncoTherad treatment

| Parameters | BCG | OncoTherad | P-value |
|------------|-----|------------|---------|
| **Historical Grade** | | | |
| High-grade | 29 (100) | 03 (50.0 *) | 0.0055 (Fisher) |
| Low-grade | 0 (0) | 03 (50.0 *) | |
| Absent Frequency= 23 | | | |
| **Tumor Size** | | | |
| <3 cm | 03 (10.3) | 05 (83.3 *) | 0.0055 (Fisher) |
| >3 cm | 26 (89.7) | 01 (16.7 *) | |
| Absent Frequency= 23 | | | |
| **Tumor Focality** | | | |
| Multifocal | 12 (41.4) | 01 (16.7 *) | |
| Single | 17 (58.6) | 05 (83.3 *) | 0.0055 (Fisher) |
| Absent Frequency= 23 | | | |

*Statistical significance

Table 4. Semiquantitative analysis of immunolabelled antigens of the urinary bladder before and after OncoTherad treatment

| Antigens | Before OncoTherad Treatment (n=15) | After OncoTherad Treatment (n=15) |
|----------|-----------------------------------|----------------------------------|
| TLR2     | 1 (9.8%)                          | 3 (88.5%)*                       |
| TLR4     | 1 (11.9%)                         | 3 (95.2%)*                       |
| TRIF     | 1 (17.4%)                         | 3 (87.6%)*                       |
| IRF-3    | 1 (12.1%)                         | 3 (92.3%)*                       |
| IFN-γ    | 1 (21.2%)                         | 3 (94.8%)*                       |
| iNOS     | 1 (8.6%)                          | 3 (96.9%)*                       |

0, no reactivity; 1, weak immunoreactivity (1% - 35% positive cells); 2, moderate immunoreactivity (36% - 70% positive cells); 3, intense immunoreactivity (> 70% positive cells). *Statistical significance (proportion test, P <0.0001)
## Table 5. Adverse Reactions in Patients with NMIBC Submitted to OncoTherad Intravesical and Intramuscular Treatment

| Adverse Reactions                      | OncoTherad N= 29 | 1-2 Grades N (%) | 1-2 Grades N (%) |
|----------------------------------------|------------------|------------------|------------------|
| **General**                            |                  |                  |                  |
| Fatigue                                | 8 (27.6%)        | 0                |
| Peripheral edema                       | 3 (10.35%)       | 0                |
| Pyrexia                                | 5 (17.25%)       | 0                |
| **Gastrointestinal**                   |                  |                  |                  |
| Diarrhea                               | Diarrhea         | Diarrhea         | Diarrhea         |
| Nausea                                 | Nausea           | Nausea           | Nausea           |
| Vomiting                               | Vomiting         | Vomiting         | Vomiting         |
| Constipation                           | Constipation     | Constipation     | Constipation     |
| Abdominal pain                         | Abdominal pain   | Abdominal pain   | Abdominal pain   |
| **Urinary Tract**                      |                  |                  |                  |
| Dysuria                                | 15 (51.7%)       | 0                |
| Cystitis                               | 10 (34.5%)       | 0                |
| **Musculoskeletal and Connective Tissue** |              |                  |                  |
| Arthralgia                             | 8 (27.6%)        | 0                |
| **Skin and Subcutaneous Tissue**       |                  |                  |                  |
| Pruritus                               | 13 (44.8%)       | 0                |
| Rash                                   | 8 (27.6%)        | 2 (6.9%)         |
| **Respiratory, Thoracic, and Mediastinal** |            |                  |                  |
| Cough                                  | 4 (13.8%)        | 1 (3.45%)        |
| Dyspnea                                | 2 (6.9%)         | 1 (3.45%)        |

### 4. Conclusions

OncoTherad nano-immunotherapy seems a safe and effective treatment option for BCG-unresponsive NMIBC patients and may provide benefit for preventing tumor recurrence. Also, this study demonstrated an important effect of activation of the TLR4 signaling pathway triggered by OncoTherad in the formation and organization of PLFs, which may be related to antitumor and immunoprotective effects from this immunotherapy in bladder tissue.

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