OncoTherad: A New Nanobiological Response Modifier, its Toxicological and Anticancer Activities

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Abstract. This study reports the effects of a promising therapeutic option for non-muscle invasive bladder cancer (NMIBC) based on OncoTherad intravesical immunotherapy in an appropriated animal model. OncoTherad is a nanostructured inorganic phosphate complex associated to glycosidic protein, which exhibits immunomodulatory and antitumor properties. Biochemical parameters in rats, mice and rabbits treated intravesically with OncoTherad at doses of 20-100 mg/kg, did not differed statistically from their respective controls, exhibiting no systemic toxic effects. All the target organs did not present inflammation and histopathological changes. NMIBC was induced by treating female Fischer 344 rats with N-methyl-N-nitrosourea (MNU). Bacillus Calmette-Guérin (BCG) were used as positive control in the animal models. The results demonstrated that animals treated with OncoTherad distinctly showed a significant histopathological recovery from the cancer state of animals (80%) when compared to BCG treatment. In addition, BCG and OncoTherad intravesical immunotherapies were able to restore TLR2 levels. However, OncoTherad increased of TLR4 levels when compared to BCG. Thus, the activation of TLR4 by Oncotherad was efficient in reducing urothelial neoplastic progression. All data are indicative that of OncoTherad is a feasible candidate for the NMIBC treatment.

Keywords: Bladder Cancer, OncoTherad, Immunotherapy, Nanomedicine, Inorganic phosphate complex, Toll-like receptor.

1. Introduction

Bladder cancer (BC) in USA is the fourth most incidence tumor in men and the ninth in women, showing high morbidity and mortality rates. The European Association of Urology considers BC as the eleventh most common cancer diagnosed worldwide [1]. American Cancer Society reported on 2017 [2] that 79,030 new cases of BC (60,490 men and 18,540 women) and with 16,879 deaths. More than 70% of BC is superficial (non-muscle invasive bladder cancer): pTis (flat carcinoma in situ)
stage, pTa (papillary carcinoma non-invasive) stage and pT1 (tumor invading mucosa or submucosa of the bladder wall) stage. Unfortunately, despite the prognosis associated with NMIBC tumors, almost 50% of patients will experience recurrence of their disease within 4 years of their initial diagnosis, and 11% will progress to muscle invasive disease (MIBC). It is known that the primary treatment for high-grade NMIBC is based on surgery by transurethral resection of bladder tumor (TURBT), followed by intravesical immunotherapy with Bacillus Calmette–Guerin (BCG) [3].

BCG acts as Toll-like Receptors 2 and 4 (TLRs 2 and 4) agonist, provoking a local inflammatory process with the infiltration of macrophages, granulocytes and lymphocytes [4-6]. After the mentioned events, cytokines are produced, among them interleukins (IL-1, IL-2, IL-6, IL-8, IL-10, IL-12), interferon (IFN) and Tumor Necrosis Factor α (TNF-α) [7].

The response induced by BCG reflects induction of a T-helper type-1 (Th1) being responsible for tumor oblation [7]. For a long time of BCG treatment was associated to several severe side effects that are observed up to 90% of patients, such as chills, fever, fatigue, irritative symptoms, haematuria and sometime important complications as sepsis and death [8].

The treatment of NMIBC remains a challenge since recurrence and progression of the disease, as well as the pronounced side effects still associated to the available therapeutic modalities are still present [9]. In this scenario, compounds that modulate immune system, through TLRs, could be an inestimable strategy for the cancer treatment, either used alone or in combination with existing therapies [10-13]. Some studies have shown that intratumoral administration of certain phosphate compounds activates the immune system in the tumoral microenvironment, leading to significant tumor regression [12].

Thus, this study describes a new therapeutic perspective for NMIBC based on nanostructured inorganic phosphate complex associated to glycosidic protein, known as OncoTherad [14], which exhibits immunomodulatory and antitumor properties. Furthermore, this study reports the histopathological and immunological effects of this immunomodulator in an appropriated animal model for NMIBC.

2. Materials and methods

2.1. Toxicological and Biochemical Analysis

For the toxicological and biochemical analyzes of the nanopharmaceutic OncoTherad, 20 female rats of the Fischer 344 strain, 20 female mice of the C57BL/6 strain and 20 female rabbits of the New Zealand strain were used. The animals were divided into 4 groups for each species, namely: Control Group (n = 5 animals for each species): Received an intravesical dose of 0.9% physiological solution, for 6 consecutive weeks. OncoTherad 20 group (n = 5 animals for each species): Received an intravesical dose of 20 mg/kg OncoTherad, for 6 consecutive weeks. OncoTherad 50 group (n = 5 animals for each species): Received an intravesical dose of 50 mg/kg of OncoTherad for 6 consecutive weeks. OncoTherad 100 group (n = 5 animals for each species) received an intravesical dose of 100 mg/kg OncoTherad for 6 consecutive weeks.

The protocol for the use of the animals in the research was approved by the Ethics Committee on the Use of Animals (CEUA) - UNICAMP (protocols numbers: 4536-1 / 2017; 4579-1 / 2017; 4435-1). After the 6 weeks trial, all animals in each group were euthanized. For the analysis of local and systemic toxicity of the nanopharmaceuticals OncoTherad, urinary organs (urinary bladder, ureter and kidneys), as well as other target organs such as liver, spleen, stomach and pancreas were collected and submitted to histopathological analysis. The histopathology of these organs was evaluated, and the toxicity correlated with the degrees of inflammation. The degree of inflammation was evaluated by a semi-quantitative scale. 0: absent inflammation. 1: Minimal inflammation (less than five lymphocytes in an area of 0.25 mm²). 2: Moderate inflammation (mononuclear inflammatory cells scattered throughout the tissue, but with stromal still visible). 3: Intense inflammation (mononuclear inflammatory cells thickly infiltrating the tissues.
Also, biochemical analyzes were performed to verify the systemic toxicity of this compound, namely: alanine aminotransferase (ALT), a specific marker for hepatic parenchyma lesion; aspartate aminotransferase (AST), non-specific marker for hepatic and / or cardiac injury; alkaline phosphatase; as well as the circulating levels of 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).

2.2. Induction and Treatment of Non-Muscle Invasive Bladder Cancer (NMIBC)

Twenty female Fischer 344 rats, seven weeks old, were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) at the University of Campinas (UNICAMP). The animal experiments were approved by an institutional Committee for Ethics in Animal Use (CEUA/UNICAMP, protocol no. 4536-1/2017). Prior to intravesical catheterisation with a 22-gauge angiocatheter, the rats were anesthetized following a prior report [6]. Five control rats (Control group) received 0.30 ml of 0.9% physiological saline every other week for 14 weeks. For NMIBC induction, 15 rats received n-methyl-n-nitrosourea (MNU; 1.5 mg/kg, dissolved in 0.30 ml of 1 M sodium citrate, pH 6.0) intravesically every other week for eight weeks [7]. Two weeks after the last dose of MNU, all rats were submitted to ultrasonography to evaluate the occurrence of tumours. The ultrasounds were evaluated using a portable, software-controlled ultrasound system with a 10–5 MHz 38-mm linear array transducer.

MNU-treated rats were further divided into three groups (n = 5 per group): the MNU group received 0.30 ml of 0.9% physiological saline, the MNU+BCG group received 10^6 CFU (40 mg) of BCG and the MNU+OncoTherad group received OncoTherad (20 mg/Kg) [14]. All of the rats were treated with saline, BCG or OncoTherad intravesically every other week for six weeks. At the end of the treatments, the rats were killed and the urinary bladders were collected and processed for histopathological analysis and western blotting.

2.3. Induction and Treatment of Non-Muscle Invasive Bladder Cancer (NMIBC): Histopathological Analysis

Samples of urinary bladders (n = 5 per group) were processed as previously described [6]. 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.

2.4. Induction and Treatment of Non-Muscle Invasive Bladder Cancer (NMIBC): Western blotting of TLR2 and TLR4

Urinary bladder samples (n = 5 per group) were weighed and homogenized in 50 µl of RIPA lysis buffer (EMD Millipore Corporation, Billerica, MA, USA) per mg of tissue. Aliquots containing 70 µg of protein were separated by SDS-PAGE on 10% or 12% polyacrylamide gels under reducing conditions. After electrophoresis, the proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham, Pharmacia Biotech, Arlington Heights, IL., USA). The membranes were blocked with TBS-T containing 1% BSA (bovine serum albumin) and incubated overnight at 4 ºC with primary rabbit polyclonal anti-TLR2 (ab13855; abcam, USA) and mouse monoclonal anti-TLR4 (ab30667; abcam, USA), all diluted in 1% BSA. The membranes were then incubated for 2 h with rabbit or mouse secondary HRP-conjugated antibodies (diluted 1:3,000 in 1% BSA; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Peroxidase activity was detected by incubation with a dianimobenzidine chromogen (Sigma Chemical Co., St Louis, MO, USA). Western blots were run in duplicate and urinary bladder samples were pooled from five rats per group for each repetition. Semi-
quantitative densitometric (IOD – integrated optical density) analysis of the bands was done using ImageJ v.1.47 software (National Institutes of Health, Bethesda, MD, USA; available at: http://rsb.info.nih.gov/ij/) followed by statistical analysis. β-Actin was used as an endogenous housekeeping gene for standardization of the intensity of band staining. The results were expressed as the mean ± standard deviation of the ratio of the band intensity relative to that of β-actin [6].

2.5. Statistical Analyses
Quantitative results were expressed as the mean ± standard deviation whenever possible. Toxicological and Western blotting data were compared among groups by one-way analysis of variance (ANOVA) followed by the Tukey test, with the level of significance set at 1% (p<0.01). Histopathological results were compared with a proportion test. The difference between the two proportions was tested using test of proportion with a type-I error of 1%.

3. Results and discussion

3.1. Toxicological and Biochemical Analysis
Serum levels of ALT, AST, alkaline phosphatase, urea and creatinine in rats, mice and rabbits treated intravesically with OncoTherad at doses of 20 mg/kg, 50 mg/kg and 100 mg/kg did not differ statistically from their respective controls (Tables 1, 2 and 3), indicating that this compound did not present systemic toxic effects. The urinary bladder, ureter and kidneys of rats, mice and rabbits of the control group did not present inflammation and histopathological changes (Table 4). OncoTherad-20 rats presented minimal inflammation in the urinary bladder (100.0%), ureter (80.0%) and kidneys (60.0%) (Table 4). Moderate inflammation was observed in the urinary bladder (80.0%), ureter (80.0%), and kidneys (60.0%) of OncoTherad-50 mice (Table 4). OncoTherad-100 rats presented intense inflammation in the urinary bladder (80.0%), ureter (80.0%) and kidneys (60.0%) (Table 4). Mice from the OncoTherad-50 and OncoTerd-100 groups showed flat hyperplasia in the urothelium of the urinary bladder and ureter. The mice on the OncoTherad-20 group presented minimal inflammation in the urinary bladder (100.0%) and ureter (100.0%), and no inflammation in the kidneys (Table 5). Mild inflammation was observed in the urinary bladder (100.0%), ureter (100.0%) and kidneys (100.0%) of the mice of the OncoTherad-50 and OncoTherad-100 groups (Table 5). Flat hyperplasia was verified in the urothelium of the urinary bladder and ureter of the OncoTherad-50 and OncoTherad-100 groups.

The rabbits on the OncoTherad-50 and OncoTherad-100 groups presented moderate inflammation in the urinary bladder (100.0%) and kidneys (100.0%), while the animals of the OncoTherad-20 group showed no inflammation and histopathological alterations in the organs of the urinary system (Table 6). Flat hyperplasia was verified in the urinary bladder urothelium and rabbit ureter of the OncoTherad-50 and OncoTherad-100 groups. Absence of inflammation and histopathological changes were observed in the liver, spleen, stomach and pancreas of all animals of each species (Tables 4, 5 and 6).

### Table 1: Toxicological and biochemical parameters for Rats.

| Groups        | ALT (U/L) | AST (U/L) | Alkaline phosphatase (U/L) | Urea (mg/dL) | Creatinine (mg/dL) |
|---------------|-----------|-----------|---------------------------|--------------|-------------------|
| Control       | 22.9±3.2 a| 85.4±5.3 a| 176.7±15.2 a              | 38.5±2.2 a   | 0.55±0.03 a       |
| OncoTherad-20 | 22.8±4.1 a| 75.6±9.1 a| 183.1±13.4 a              | 40.2±4.0 a   | 0.43±0.04 a       |
| OncoTherad-50 | 265±5.4 a | 73.3±9.1 a| 178.6±31.1 a              | 40.0±1.6 a   | 0.49±0.09 a       |
| OncoTherad-100| 28.1±10.4 a| 73.8±10.6 a| 194.2±14.1 a              | 40.8±3.8 a   | 0.51±0.03 a       |

Data expressed as average ± SD (n=5 animals for groups). a) Two averages followed by the same lowercase letter do not differ statistically by the Turkey test (P <0.05).
Table 2: Toxicological and biochemical parameters for Mice.

| Groups          | ALT (U/L) | AST (U/L) | Alkaline phosphatase (U/L) | Urea (mg/dL) | Creatinine (mg/dL) |
|-----------------|-----------|-----------|---------------------------|-------------|-------------------|
| Control         | 11.7±3.1  | 92.4±5.5  | 154.1±9.3                | 36.0±7.1    | 0.27±0.04         |
| OncoTherad-20   | 13.4±2.7  | 94.8±5.5  | 179.7±7.8                | 32.3±4.9    | 0.23±0.05         |
| OncoTherad-50   | 14.1±2.7  | 93.7±5.5  | 185.3±10.7               | 25.9±3.7    | 0.34±0.14         |
| OncoTherad-100  | 13.7±2.0  | 94.4±5.4  | 187.5±8.8                | 26.6±3.5    | 0.31±0.08         |

Data expressed as average ± SD (n=5 animals for groups). a) Two averages followed by the same lowercase letter do not differ statistically by the Turkey test (P <0.05).

Table 3: Toxicological and biochemical parameters for Rabbits.

| Groups          | ALT (U/L) | AST (U/L) | Alkaline phosphatase (U/L) | Urea (mg/dL) | Creatinine (mg/dL) |
|-----------------|-----------|-----------|---------------------------|-------------|-------------------|
| Control         | 20.9±2.6  | 42.0±1.4  | 129.0±12.7               | 39.7±4.7    | 0.90±0.06         |
| OncoTherad-20   | 30.5±2.1  | 47.0±1.4  | 126.0±9.9                | 43.0±4.2    | 0.85±0.15         |
| OncoTherad-50   | 33.4±0.6  | 41.0±1.3  | 137.5±10.6               | 40.2±5.4    | 0.94±0.06         |
| OncoTherad-100  | 35.7±1.8  | 47.5±2.1  | 126.0±19.8               | 44.0±4.2    | 0.95±0.08         |

Data expressed as average ± SD (n=5 animals for groups). a) Two averages followed by the same lowercase letter do not differ statistically by the Turkey test (P <0.05).

Table 4: Semi-quantitative evaluation of inflammation in the urinary bladder, ureter, kidneys, liver, spleen, pancreas and stomach for the 5 rats from each experimental group.

| Organs         | Groups                 |
|----------------|------------------------|
|                | Control (n=5) | OncoTherad-20 (n=5) | OncoTherad-50 (n=5) | OncoTherad-100 (n=5) |
| Urinary bladder| 1  2  3  4  5        | 1  2  3  4  5       | 1  2  3  4  5       | 1  2  3  4  5       |
| Ureter         | 0  0  0  0  0         | 0  1  1  1  1       | 2  2  2  1  3       | 3  3  3  3  2       |
| Kidney         | 0  0  0  0  0         | 0  1  1  1  0       | 2  2  2  1  3       | 3  3  3  3  2       |
| Liver          | 0  0  0  0  0         | 0  0  0  0  0       | 0  0  0  0  0       | 0  0  0  0  0       |
| Spleen         | 0  0  0  0  0         | 0  0  0  0  0       | 0  0  0  0  0       | 0  0  0  0  0       |
| Pancreas       | 0  0  0  0  0         | 0  0  0  0  0       | 0  0  0  0  0       | 0  0  0  0  0       |
| Stomach        | 0  0  0  0  0         | 0  0  0  0  0       | 0  0  0  0  0       | 0  0  0  0  0       |

Note: 0, absent inflammation, 1, minimal inflammation (less than five lymphocytes in an area of 0.25 mm²), 2, moderate inflammation (mononuclear inflammatory cells scattered throughout the tissue but with still visible stroma), 3, intense inflammation (mononuclear inflammatory cells densely infiltrating the tissues.)
Table 5: Semi-quantitative evaluation of inflammation in the urinary bladder, ureter, kidneys, liver, spleen, pancreas and stomach for the 5 mice from each experimental group.

| Organs          | Control (n=5) | OncoTherad-20 (n=5) | OncoTherad-50 (n=5) | OncoTherad-100 (n=5) |
|-----------------|---------------|---------------------|---------------------|----------------------|
| Urinary bladder | 0 0 0 0 0 0 1 1 1 1 2 2 2 2 2 2 2 2 |                      |                     |
| Ureter          | 0 0 0 0 0 0 1 1 1 1 2 2 2 2 2 2 2 2 |                      |                     |
| Kidney          | 0 0 0 0 0 0 0 0 0 0 0 2 2 2 2 2 2 2 |                      |                     |
| Liver           | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |                      |                     |
| Spleen          | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |                      |                     |
| Pancreas        | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |                      |                     |
| Stomach         | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |                      |                     |

Note: 0, absent inflammation, 1, minimal inflammation (less than five lymphocytes in an area of 0.25 mm²), 2, moderate inflammation (mononuclear inflammatory cells scattered throughout the tissue but with still visible stroma), 3, intense inflammation (mononuclear inflammatory cells densely infiltrating the tissues).

Table 6: Semi-quantitative evaluation of inflammation in the urinary bladder, ureters, kidneys, liver, spleen, pancreas and stomach for the 5 rabbits from each experimental group.

| Organs          | Control (n=5) | OncoTherad-20 (n=5) | OncoTherad-50 (n=5) | OncoTherad-100 (n=5) |
|-----------------|---------------|---------------------|---------------------|----------------------|
| Urinary bladder | 0 0 0 0 0 0 0 0 0 0 0 2 2 2 2 2 2 2 |                      |                     |
| Ureter          | 0 0 0 0 0 0 0 0 0 0 0 2 2 2 2 2 2 2 |                      |                     |
| Kidney          | 0 0 0 0 0 0 0 0 0 0 0 2 2 2 2 2 2 2 |                      |                     |
| Liver           | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |                      |                     |
| Spleen          | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |                      |                     |
| Pancreas        | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |                      |                     |
| Stomach         | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |                      |                     |

Note: 0, absent inflammation, 1, minimal inflammation (less than five lymphocytes in an area of 0.25 mm²), 2, moderate inflammation (mononuclear inflammatory cells scattered throughout the tissue but with still visible stroma), 3, intense inflammation (mononuclear inflammatory cells densely infiltrating the tissues).

3.2. Non-Muscle Invasive Bladder Cancer (NMIBC): Histopathological Analysis

The Control group displayed no histological changes in bladder tissue (Figures 1a, 1b; Table 7). It is known that three different cell types composed the normal bladder urothelium: 1) basal cell layer, 2) intermediate cell layer and 3) surface cell layer (umbrella cells) (Figures 1a, 1b).

In contrast, MNU group demonstrated deep microscopic changes in the urinary bladder tissue such as, pTa (60%) (Figures 1c, 1d), pTis (20%) and pT1 (20%) (Table 7). In the case of MNU+BCG group the most frequent neoplastic lesions were pTis (Figures 1e, 1f) and pTa in 60% and 20% of the animals, respectively (Table 7). The rest of the animals (20%) showed pre-neoplastic lesions, such as low-grade intraurothelial neoplasia, denoting that this immunotherapy promoted inhibition of tumor progression in 20% of the animals (Table 7). Animals treated with OncoTherad intravesical immunotherapy neatly showed better histopathological recovery from the cancer state than those
observed in the MNU+BCG group, with a decrease of bladder neoplastic lesions progression in 80% of the animals (Table 7). Benign lesions, such as, flat hyperplasia (Figures 1g, 1h), and low-grade intraurothelial neoplasia (pre-neoplastic lesion) were found in 60% and 20% of the animals, respectively (Table 7). The most frequent neoplastic lesions found in this group were pTis in 20% of the animals (Table 7).

Compounds as also molecules that bind to and activate TLRs are the matters of deep research and development for the cancer’s treatment, including NMIBC [5,6]. TLRs activation causes tumor regression by increasing vascular permeability and over the recruitment of leukocytes, which determines tumor cell lysis by natural killer (NK) and cytotoxic T cells. Epithelial and immune cells express TLRs, as is known, play important role in activating immune system [15]. NMIBC show decreased TLRs expression [16,17]. The BCG antitumor activities in the NMIBC are related to local immunological mechanisms, since after BCG instillation were found in the urine of patients within 24 hours several cytokines and activated immunocompetent leukocytes. However, BCG use is limited in NMIBC by treatment failure, adverse effects and intolerance that occur in over two-thirds of all patients and consist largely of irritative voiding symptoms including haematuria, dysuria and urgency [8].

**Table 7:** Percentage of histopathological changes of the urinary bladder of rats from different experimental groups.

| Histopathology                  | Control (n=05) | MNU (n=05) | MNU+BCG (n=05) | MNU+OncoTherad (n=05) |
|---------------------------------|---------------|------------|----------------|-----------------------|
| Normal                          | 05 (100%)*    | -          | -              | -                     |
| Flat Hyperplasia                | -             | -          | -              | 03 (60%)*             |
| Low-grade Intraurothelial Neoplasia | -          | -          | 01 (20%)*      | 01 (20%)*             |
| High-grade Intraurothelial Neoplasia – Flat Carcinoma *in situ* (pTis) | -      | 01 (20%)* | 03 (60%)*      | 01 (20%)              |
| Papillary Carcinoma *in situ* (pTa) | -          | 03 (60%)* | 01 (20%)       | -                     |
| Tumor invading mucosa or submucosa of the bladder wall (pT1) | -          | 01 (20%)* | -              | -                     |

The histopathological alterations are expressed as a percentage of the number of rats (n) examined in each group. *P<0.0001 (proportions test). Benign lesions: flat hyperplasia; Pre-neoplastic lesions: low-grade intraurothelial neoplasia; Neoplastic lesions: pTis, pTa and pT1.
Figures 1a – 1h: Representative photomicrographs of urinary bladder from Control (a, b), MNU (c, d), MNU+BCG (e, f) and MNU+OncoTherad (g, h) groups. (a), (b) Three different cell types composed the normal bladder urothelium: basal cell layer (arrowhead), intermediate cell layer (arrow) and surface cell layer (or umbrella cells, open arrowhead). (c), (d) pTa tumor: cancer cells show slender papillae with frequent branching, minimal fusion, and variations in nuclear polarity, size, shape, and chromatin pattern and with the presence of nucleoli (asterisks). (e), (f) pTis tumor (circle): flat lesion in the urothelium surface characterized by large and pleomorphic cells, severe nuclear atypia (asterisks) and loss of cellular polarity. (g), (h) Flat hyperplasia characterized by thickening of the urothelium without cellular atypia. **Lp** – lamina propria, **M** – muscle layer, **Ur** – urothelium.

3.3. Non-Muscle Invasive Bladder Cancer (NMIBC): Western blotting of TLR2 and TLR4
It was demonstrated here that chemically induced bladder tumors showed decreased TLRs 2 and 4 protein levels (Figures 2A, 2B). In addition, BCG and OncoTherad intravesical immunotherapies were able to restore TLR2 levels (Figure 2A). However, Oncotherad treatment led to distinct activation of immune system TLRs-mediated, coming out in increased TLR4 levels when compared to BCG (Figure 2B). Thus, the activation of TLR4 by Oncotherad was more effective in reducing urothelial neoplastic lesions progression.
Figure 2: Western blot analysis of TLR2 (A) and TLR4 (B) in urinary bladder tissues. Representative protein profiles pooled from five rats/group. The graphs represent the relative expression of integrated optical density for the TLR2 and TLR4 proteins, normalized by β-actin and expressed as mean ± standard deviation. Different lowercase letters (a, b, c, d) indicate significant differences (p<0.01) between the groups after the Tukey test.
4. Conclusions

Thus, our data indicated an important role as antitumoral effect of OncoTherad immunotherapy, based on increase of TLR2 and TLR4 protein levels in the NMIBC animal model. Therefore, increased TLR4 levels by OncoTherad led to important antitumor effects, probably activating interferon signaling pathway. Considering all our results will encourage the further investigation of OncoTherad as a potential candidate for the treatment of bladder cancer.

New studies concerning the TLRs 2 and 4 and its signaling pathways are being planned to understand the immunological mechanism of OncoTherad. Also, other studies acting in combination with BCG, chemotherapies and monoclonal antibodies are actually in progress, beside the vaccine behavior before induction of NMIBC. This knowledge may give us useful data to complement the understanding of the OncoTherad’s mechanism of action.

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