EFFECTS OF ONCOTHERAD® IMMUNOTHERAPY AND ERYTHROPOIETIN ON THE ESTROUS CYCLE, SURVIVAL AND MACROSCOPY OF REPRODUCTIVE ORGANS OF OVARIAN CANCER INDUCED RATS

Keywords: Ovarian Cancer, OncoTherad, Erythropoietin

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BACKGROUND

Ovarian cancer (OC) has the highest mortality among neoplasms of the female reproductive system and ranks fifth in cancer deaths among women (American Cancer Society, 2020). Despite therapeutic efforts and new chemotherapies, 80% of women with advanced OC will have tumor progression or recurrence even after the treatments, which is further aggravated by the acquisition of resistance to chemotherapy (Luvero et al., 2014). The tumor microenvironment of OC involves a complex immunosuppressive network and in this scenario, the need for new therapeutic alternatives to increase response and survival rates is vital, with immunotherapy being a promising strategy (Zhang et al., 2015).

The existence of drugs that act as agonists of Toll like Receptors (TLRs) is extremely important and aiming at its development, our research team developed OncoTherad® or Biological Response Modifier – Inorganic Phosphate Complex 1 (MRB-CFI-1), a synthetic nanopharmaceutical of phosphate and metallic salts associated with a glycosidic protein. OncoTherad® triggered local stimulation of the immune system in the tumor microenvironment and led to antitumor effects mainly due to interferon (IFN) actions (Fávaro; Durán, 2017). The antitumor effects of this nanoimmunotherapy have already been reported in bladder cancer model (Fávaro; Durán, 2017; Durán et al., 2019; Sasaki et al., 2021) and also in veterinary (Böckelmann et al., 2019) and human clinical trials (Fávaro et al., 2019; Alonso et al., 2020).

Erythropoietin (EPO) can exert several non-hematopoietic functions such as immunomodulation (Lifshitz et al., 2010), anti-inflammatory or antioxidant and cytoprotective actions including in the ovary (Mahmoodi et al., 2014; Sayan et al., 2017). In addition, the failure to produce EPO and hypoxia that occur in the tumor process, if treated with exogenous doses of erythropoietin, there may be an improvement in the response to other antitumor treatments (Lombardero et al., 2011). The association of immunotherapy and the use of Erythropoietin might be a real therapeutic scheme in patients anemic due to cancer or chemotherapy treatment. This study is investigating the application of OncoTherad® and EPO for the treatment of OC model and characterized the effects of induced OC and these treatments on the estrous cycle, survival and the reproductive organs characteristics of Fischer rats.

METHODS

OncoTherad® was synthesized, purified and characterized according to Fávaro & Durán (2017). The EPO formulation used was Eritromax®, a lyophilized alpha-epoetin powder (rHuEPO, 4,000 I.U./ml) from Blau Farmacêutica S.A. (Cotia, SP/Brazil). Thirty-five Fischer 344 rats with an average age of 80 days and weight of ±190g were ramdomly distributed into

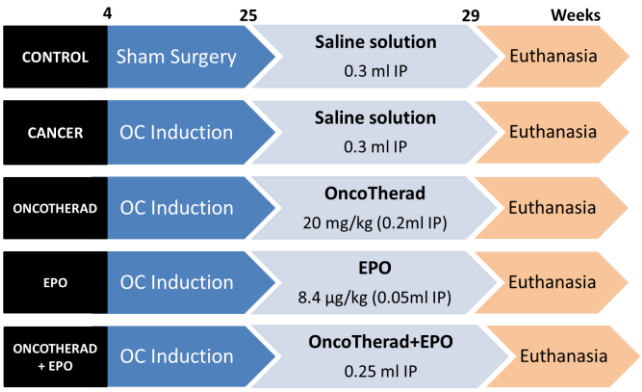

Figure 1. Experimental protocol. In the 3rd and 4th weeks, chemical induction of OC and Sham surgery were performed. The period between the 4th and 25th week corresponds to the tumor development (around 140 days). The period between the 25th and 28th week corresponds to the 4 weeks of treatments with OncoTherad, EPO, and OncoTherad associated to EPO at the same concentrations as the treatments alone. Treatments were carried out twice a week for 4 weeks. The euthanasia took place on 29th week.

five groups (n=7 animals/group) (**Figure 1**) and housed in polypropylene cages containing laboratory-grade pine shavings as bedding in an enriched environment (**Figure 2A**). The OC chemical induction model consisted of a single injection with 7,12-dimethylbenzoanthracene – DMBA (Sigma Chemical Co, St Louis, Mo) into the ovarian bursa (**Figure 2B**) at a dose of 1.5 mg/kg body weight, dissolved in 10 µl of sunflower oil (Chuffa et al., 2018). The rats from the Control group underwent sham surgery, receiving an injection of 10 µl of sunflower oil under the same procedures as the induced groups.

Follow-up and three-dimensional in vivo investigation of tumor development were performed in all animals using X-ray computed microtomography (micro-CT) with the Skyscan 1178 device (SkyScan 1178, Bruker, Brussels, Belgium). The estrous cycle was monitored by cytological examination of vaginal smear and all animals were euthanized during estrus phase. The methodologies were authorized by the Ethics Committee in the Use of Animals – CEUA/UNICAMP (**Registration 5555-1/2020**)

Quantitative data were represented as mean ± standard deviation and evaluated using the analysis of variance ANOVA, complemented with the Tukey test, when they presented normality. In cases of absence of normality, the Kruskal-Wallis non-parametric analysis of variance and the Student-Newman-Keuls test were used. The Kaplan-Meier method was used for survival analysis, and the log-rank test (Mantel-Cox) was performed to assess whether there were differences in survival between groups. Statistical significance was 5% ($p<0.05$).

Figure 2. Experimental procedures. (A) Rats in an enriched environment. (B) Induction of OC via injection of DMBA directly into the ovarian bursa.

RESULTS AND DISCUSSION

X-ray computed microtomography showed that several of the rats induced with ovarian cancer with DMBA had an apparently larger and more visible left ovary, when compared to exams obtained from animals in the Control group (**Figure 3**). Micro-CT in small animals has contrast considered unsatisfactory in soft tissues, which limits the analysis and assessment of intra-abdominal content. Therefore, there were some visualization difficulties due to the size and position of the gonads, as well as the presence of feces and gases, but anatomical structures such as the uterine horns were used to help identify the position of the ovaries.

During the entire experimental period, all rats exhibited the different phases of the estrous cycle (**Figure 4**). The chemical induction of OC through the use of DMBA did not inhibit cyclicity, possibly because the right ovary was not affected by the surgical intervention. However, changes in

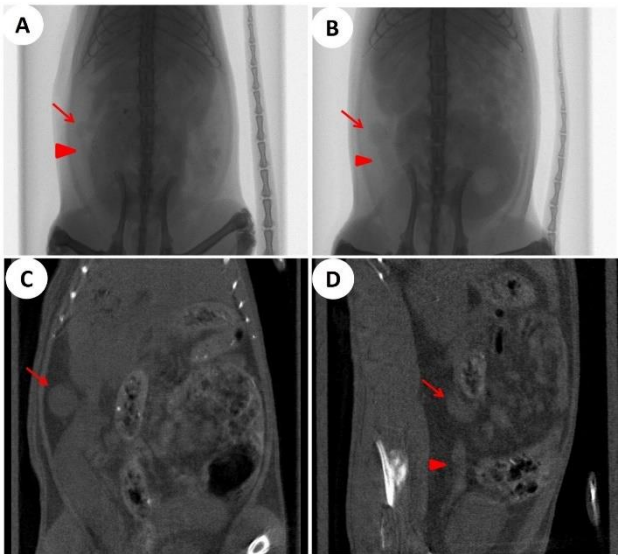

Figure 3. Photographs of micro-CT examination. (A) Healthy Rat (without 3D reconstruction), showing the left ovary (arrow) and the left uterine horn (closed arrowhead). (B) OC-induced rat (without 3D reconstruction), more pronounced left ovary (arrow) and left uterine horn (closed arrowhead). (C, D) Images of OC induced rat after 3D reconstruction. Left ovary (arrow) and uterine horn (closed arrowhead) in coronal (C) and sagittal (D) planes.

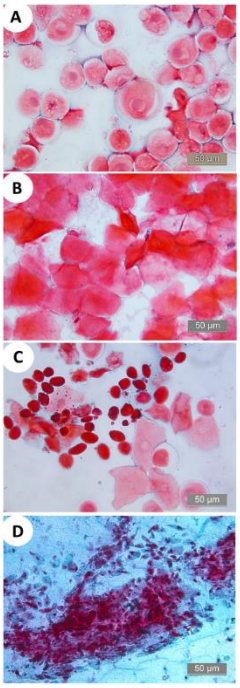

Figure 4. Estrous Cycle stages. (A) Proestrus; (B) Estrus; (C) Metaestrus; (D) Diestrus. Shorr stain. Bars=50 µm.

cyclicity, such as the rhythm and duration of specific phases were observed. Prior to the beginning of OC induction surgeries, the rats of all groups did not present significant differences ($p>0.05$) in the duration of the estrous cycle, number of days in estrus and in diestrus (**Figure 5A-C**). The average duration of the cycle was between six and seven days, and around three days in the estrus phase and from 5 to 6 days in the diestrus, considering a period of 15 days.

At the end of the tumor development period, it was possible to observe an increase in absolute numbers in the duration of the estrous cycle and in the number of days of estrus in the OC-induced rats (**Figure 5D, E**), although these differences were not statistically significant ($p>0.05$). Regarding the end of the treatment period (prior to euthanasia of the animals), all groups in which rats were induced to OC (Cancer, OncoTherad, EPO and OncoTherad+EPO) exhibited an increase ($p<0.05$) in cycle duration estral compared to the Control (**Figure 5G**). This drag on the cycle was confirmed by the greater ($p<0.05$) number of estrus days in these same groups when compared to the Control group (**Figure 5H**). Possibly, the persistent estrus was due to changes promoted by the induction surgery and the time of tumor development, since this trend both in terms of the longer cycle duration and the increase in the number of estrus had already been noticed before the treatment phase.

Furthermore, it was possible to notice that the treatments did not alter the estrous cycle or accentuated the changes resulting from induction, since the same cycle pattern was observed in the Cancer group without treatment before euthanasia. Regarding diestrus, there were no statistically differences ($p>0.05$) between groups at the end of treatments (**Figure 5C, F, I**).

Thus, at the established doses and treatment time, the drugs did not drastically alter hormonal control to prevent the incidence of ovulation and maintenance of the cycle.

Regarding the macroscopy evaluation, the animals of Control group did not present any visible structural alteration in the reproductive tract (**Figures 6A-C**). On the other hand, in the Cancer group (**Figures 6D-F**) the left ovaries showed diversified alterations such as the presence of a cystic nodular lesions and a more vascularized appearance (**Figures 6E, F**). In general, the lesions had a solid appearance with cystic areas around them, yellowish color, rounded shape with the smallest diameter of 3 mm and the biggest diameter of 5 mm, (**Figure 6F**). Other abnormalities observed in some of the animals of Cancer were peritoneal implants and more edematous appearance of the right uterus and ovaries.

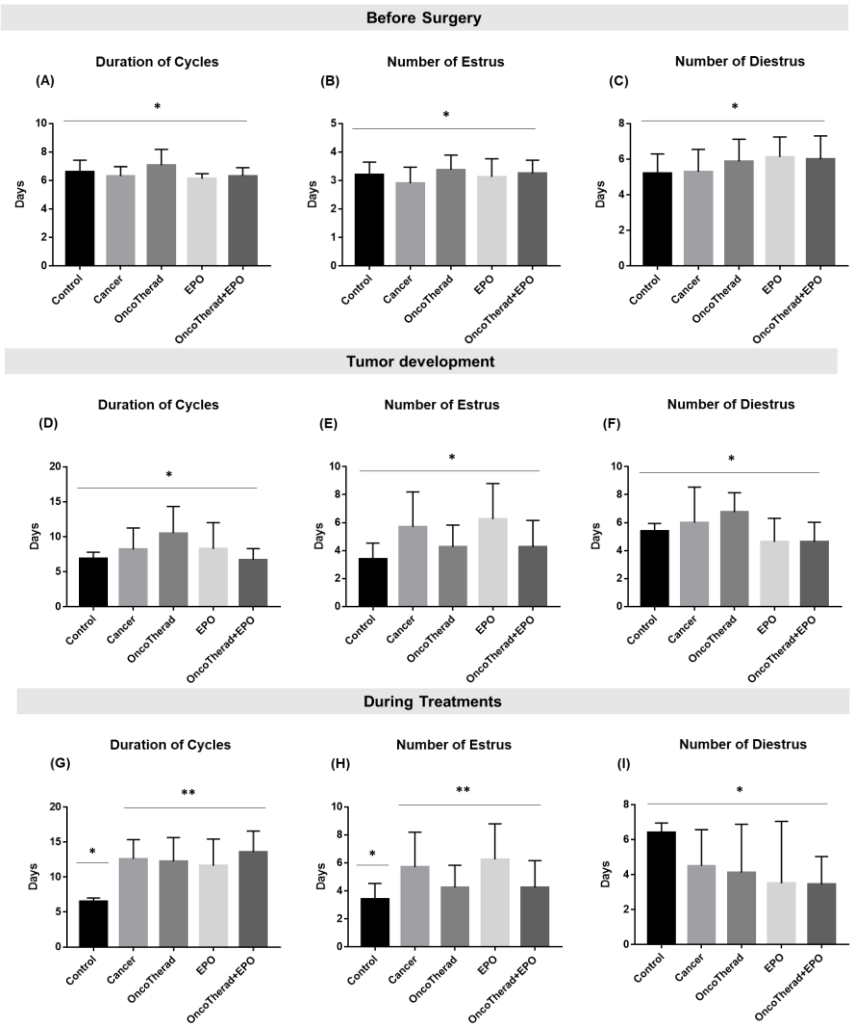

Figure 5. Estrous Cycle Parameters. Values expressed as mean ± standard deviation. */** Distinct symbols indicate a statistically significant difference ($p<0.05$). (A, B, D, F, G, H, I) Kruskal-Wallis, Student-Newman-Keuls test. (C, E) ANOVA.

Regarding the OncoTherad group (**Figures 6G-I**) it was possible to notice that most of the left ovaries had a normal appearance and presence of corpora lutea and, in some animals, the gonads were slightly more atrophied (**Figure 6I**). In the most of the rats of this group, the uterus and right ovaries have a healthy macroscopic appearance (**Figures 6H, I**), including some with the presence of functional ovarian cysts (**Figure 6I**).

In the EPO group (**Figures 6J-L**), it was possible to identify left ovaries with a more swollen appearance (**Figure 6L**), slightly atrophied, as well as some with a normal appearance. Two rats exhibited possibly neoplastic implants in the intraperitoneal fat (**Figure 6J**). The swollen appearance of the ovaries of rats treated with EPO might be partly related to the increased expression of angiogenic factors, as well as the maintenance of ovarian function.

The OncoTherad +EPO group (**Figures 6M-O**) showed left ovaries with different aspects were identified as macroscopically normal with corpora lutea, more edematous, slightly atrophied or with adhered nodules. Two rats had cystic nodular lesions, yellowish and approximately 3 x 4 mm diameter, attached to left ovary (**Figures 6N, O**).

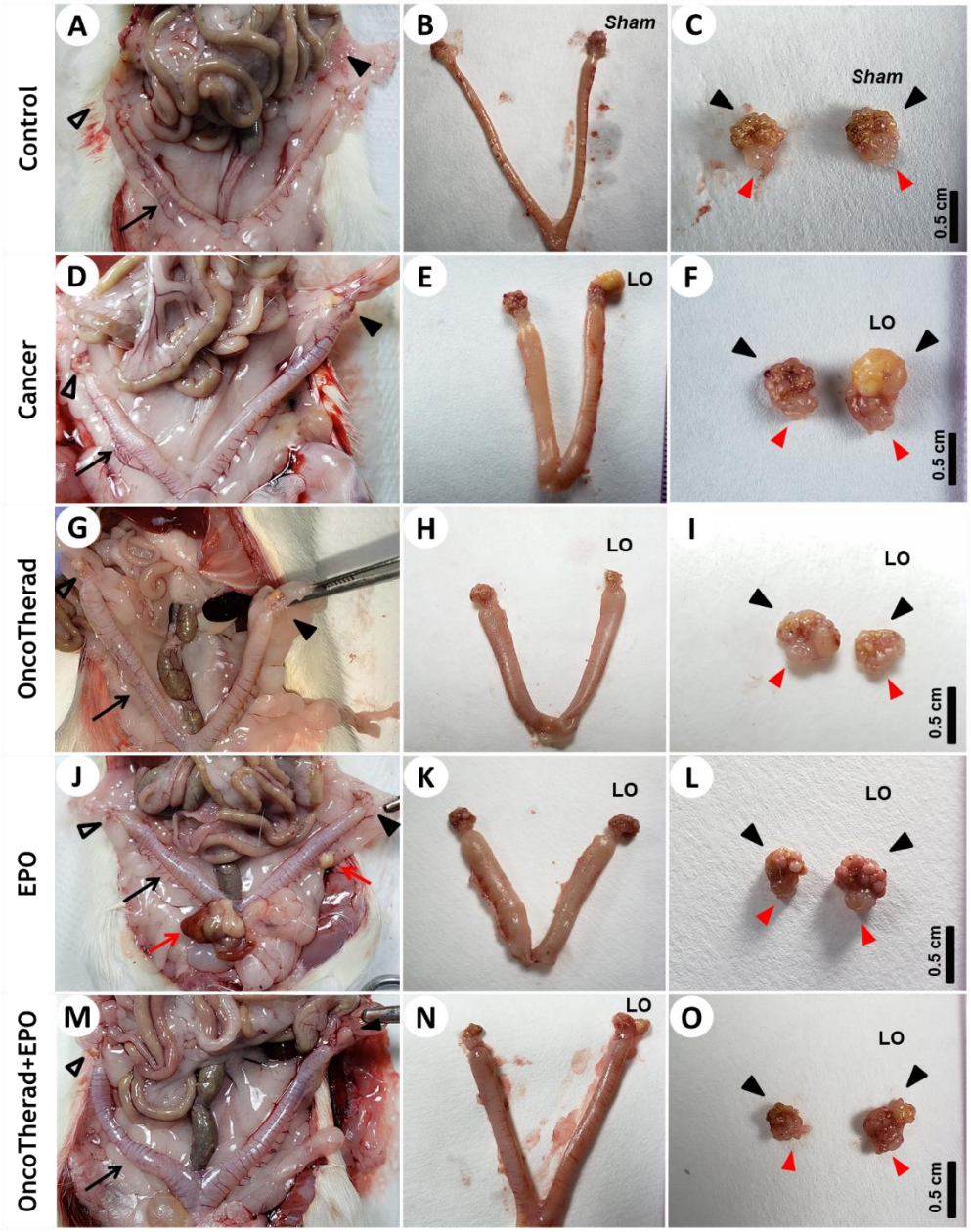

Figure 6. Mascroscopic description of the reproductive organs of females from the Control (A-C), Cancer (D-F), OncoTherad (G-I), EPO (J-L) and OncoTherad+EPO (M-O) groups. (A) Exposure of the reproductive tract, highlighting the right ovary (open arrowhead), left ovary undergoing Sham surgery (closed arrowhead) and uterus (arrow). **(B)** Detail of healthy-looking reproductive tract. **(C)** Detail of the right and left ovaries (black arrowhead) and uterine tubes (red arrowhead). **(D)** Exposure of the reproductive tract of OC induced rats, showing the presence of structural changes in the left ovary (closed arrowhead). **(E)** Detail of the reproductive tract, the presence of a yellowish nodular cystic lesion in the left ovary (LO). **(F)** Detail of the right and left ovaries (black arrowhead) and fallopian tubes (red arrowhead), highlighting the difference between the ovary undergoing induction surgery compared to the right ovary. **(G)** Exposure of the reproductive tract. **(H)** Reproductive tract detail, left ovary (LO). **(I)** Detail of the ovaries (black arrowhead), the left one shows moderate atrophy and the right one shows a functional cyst; and fallopian tubes (red arrowhead). **(J)** Exposure of the reproductive tract showing the presence of heterogeneous or cystic nodular peritoneal implants (red arrows). **(K)** Detail of the reproductive tract, left ovary (LO). **(L)** Detail of the right and left ovaries (black arrowhead) and fallopian tubes (red arrowhead), the most edematous aspect of the left ovary stands out. **(M)** Exposure of the reproductive tract. **(N)** Detail of the reproductive tract, note the presence of a yellowish cystic nodule in the left ovary (LO). **(O)** Detail of the right and left ovaries (black arrowhead) and fallopian tubes (yellow arrowhead), the cystic nodular lesion in the ovary stands out.

The occurrence of atrophy may be related to the mechanism of stimulation of the immune system and the antitumor response triggered by treatment with OncoTherad, associated or not to EPO. A careful investigation will be carried out to determine whether this event is due to antitumor activity, considering the context of the OC-induced ovary

From the interpretation of the Kaplan-Meier curve (**Figure 7**), the lowest survival rate is found in the Cancer group, which from the 22nd to the end of 29 weeks averaged 83.33%. The OncoTherad group had a survival rate of 87.5% from the 28th week, while in the OncoTherad +EPO group this rate was 90% from the 23rd week. The Control and EPO group exhibited 100% survival throughout the period. Possibly, the occurrence of deaths was related to tumor development, since, for example, the incidence of death in the OncoTherad+EPO group occurred in the 23rd week, that is, before treatment with the drugs. However, these differences in survival rates were not statistically significant (p>0.05) by the log-rank test (Mantel-Cox) (**Figure 7**). In this way, it is not possible to state that the OC induction performed promoted a reduction of survival compared to healthy animals. In addition, treatments with OncoTherad and Erythropoietin, alone or in association, did not change the animals' survival.

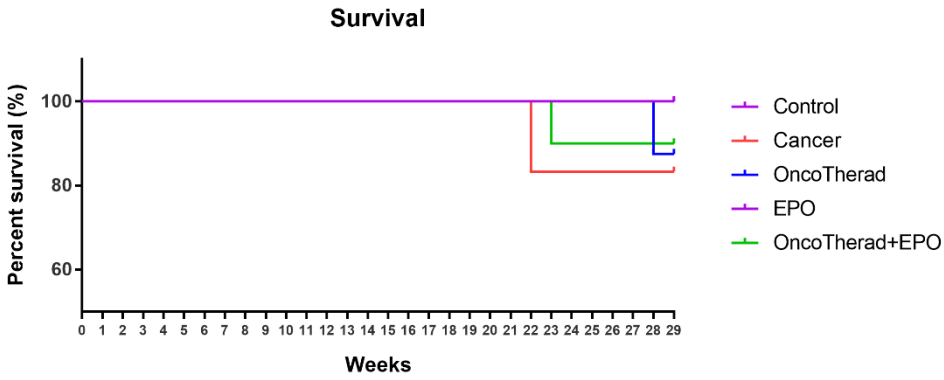

Figure 7. Kaplan-Meier curve showing the survival rates (%) of the groups throughout the experimental period. From 1st to 2nd week: period before OC induction. In the 3rd and 4th weeks, OC chemical induction surgeries and Sham surgery were performed. The period between the 4th and 25th week corresponds to the period of tumor development (around 140 days). The 25th and 28th week corresponds to the 4 weeks of treatments with OncoTherad, Erythropoietin and OncoTherad plus EPO. The 29th week corresponds to the euthanasia of the animals. Log-rank test (Mantel-Cox) p=0.639.

CONCLUSIONS

Based on the results obtained with this ongoing study, it was possible to preliminarily ensure the chemical induction model of ovarian cancer used and to characterize part of its effects on animals, such as: alterations in the estrous cycle; characterization of ovarian lesions with milder characteristics, especially in terms of dimensions, which provide more possible possibilities of response to treatments; and absence of significant changes in the survival analysis. Furthermore, it was possible to observe that treatments with OncoTherad and EPO had an effect in the treatment of induced ovarian cancer, mainly regarding the macroscopic characteristics of the reproductive tract. The effects of this association will be better understood and investigated with analyzes that are being done.

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BIBLIOGRAPHY

ALONSO, J.C.C. et al. Oncotherad immunotherapy elicits promising responses in Bacillus Calmette-Guérin-unresponsive non–muscle invasive bladder cancer: Results from phase I/II study. **J Clin Oncol**, v. 37, 15 Suppl, 2020.

AMERICAN CANCER SOCIETY. **Key Statistics for Ovarian Cancer**, 2020. Available from:<https://www.cancer.org/cancer/ovarian-cancer/about/key-statistics.html> (acessed in August 12, 2021).

BÖCKELMANN, P. K.; TIZZIANI, S. H. S.; DURÁN, N.; FÁVARO, W. J. New Therapeutic Perspective for Bladder Cancer in Dogs: Toxicological and Clinical Effects of OncoTherad Nanostructured Immunotherapy. **J Phys Conf Ser**, v. 1323, n. 1, p. 012022, 2019.

CHUFFA, L. G. A. et al. P-MAPA immunotherapy potentiates the effect of cisplatin on serous ovarian carcinoma through targeting TLR4 signaling. **J Ovarian Res**, v. 11, n. 1, p. 8, 2018.

DURAN, N.; DIAS, Q. C.; FAVARO, W. J. OncoTherad: A New Nanobiological Response Modifier, its Toxicological and Anticancer Activities. **J Phys Conf Ser**, v. 1323, p. 012018, 2019.

FÁVARO, W. et al. Single-arm phase I/II study of the safety and efficacy of OncoTherad immunomodulator in patients BCG-refractory or relapsed non-muscle invasive bladder cancer. **J Clin Oncol**. 37, suppl. 15, e16000, 2019.

FÁVARO, W.; DURAN, N. Process of obtaining a nanostructured complex (CFI-1), associated to nanostructured CFI-1 with a protein (MRB-CFI-1) and its use. **Brazil Patent** No. PIBR 10.2017.012768.0. PCT/BR2018/000031. 2017.

HARDEE, M. E. et al. Human recombinant erythropoietin (rEpo) has no effect on tumour growth or angiogenesis. **Br J Cancer**, v. 93, n. 12, p. 1350-1355, 2005.

LIFSHITZ, L. et al. Macrophages as novel target cells for erythropoietin. **Haematologica**, v. 95, n. 11, p. 1823-1831, 2010.

LOMBARDERO, M.; KOVACS, K.; SCHEITHAUER, B. W. Erythropoietin: a hormone with multiple functions. **Pathobiology**, v. 78, n. 1, p. 41-53, 2011.

LUVERO, D. et al. Treatment options in recurrent ovarian cancer: latest evidence and clinical potential. **Ther Adv Med Oncol**, v. 6, n. 5, p. 229-239, 2014.

MAHMOODI, M. et al. Effects of erythropoietin on ischemia, follicular survival, and ovarian function in ovarian grafts. **Reproduction**, v. 147, n. 5, p. 733-741, 2014.

SASAKI, B.R. et al. A potential new therapeutic option for the treatment of nonmuscle invasive bladder cancer: Combination of intravesical Oncotherad immunotherapy and platelet rich plasma (PRP). **J Clin Oncol**, v. 39, n.6_suppl, p. 461, 2021.

SAYAN, D. C. et al. Could erythropoietin reduce the ovarian damage of cisplatin in female rats? **Gynecol Endocrinol**, v. 34, n.4, p. 309-313, 2018.

ZHANG, S. et al. Analysis of CD8+ Treg cells in patients with ovarian cancer: a possible mechanism for immune impairment. **Cel Mol Immunol**, v. 12, n. 5, p. 580-591, 2015.