ASSOCIATION OF ONCOTHERAD® IMMUNOTHERAPY AND PLATELET-RICH PLASMA IN THE TREATMENT OF INDUCED OVARIAN CANCER IN RATS: EVALUATION OF ESTROUS CYCLE, WEIGHT PARAMETERS AND MACROSCOPY

Keywords: Ovarian Cancer, OncoTherad, Platelet Rich Plasma

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INTRODUCTION

Ovarian Cancer (OC) ranks fifth among the causes of death from cancer, being the second type of cancer that most affects women in Brazil (American Cancer Society, 2021; INCA, 2020). Many OC conventional treatments can cause cytotoxic effects by attacking healthy and tumors cells (Cortez et al., 2018). Regarding immunotherapy strategies, Toll like receptors (TLRs) agonists are strong immunostimulating agents that can be used in cancer treatment (Husseinzadeh; Davenport, 2014). Our research team developed a nanopharmaceutical called Inorganic Phosphate Complex 1 (MRB-CFI-1) or OncoTherad®. In non-muscle invasive bladder cancer (NMIBC) induced in rats and mice, OncoTherad® led to a distinct activation of the innate immune system mediated by TLRs 2 and 4 which resulted in an increase in signaling pathways for interferon production (Fávaro; Durán, 2017; Durán et al., 2019; Sasaki et al., 2021). Additionally, OncoTherad® showed immunomodulatory and antitumor effects for bladder cancer treatment in veterinary (Böckelmann et al., 2019) and human clinical trials (Fávaro et al., 2019; Alonso et al., 2020)

Platelet-rich plasma (PRP) is a platelet lysate concentrated in a small volume of plasma with the presence of growth factors (GF), which are released when the platelets are activated. Previous studies have shown that PRP is able to promote immune activation and trigger antitumor effects in bladder cancer model (Dias et al., 2018; Sasaki et al., 2021). In our pilot study, there was an interaction between PRP proteins and the phosphates present in OncoTherad® and a higher concentration of proteins after this interaction, possibly due to the capacity of the inorganic component (CFI-1) to induce the release of proteins from platelets. Considering the need to develop new effective treatment approaches for OC, for which there has been no substantial reduction in the mortality rate in recent decades (Muccioli; Benencia, 2014), the promising effects of PRP and its potential association with OncoTherad® might constitute a new therapeutic option.

This study aimed to evaluate the effects of this association on estrous cycle, ovarian and body weight, feed and water consumption and reproductive tract macroscopic features of OC induced rats.

METHODOLOGY

We used thirty-five Fischer 344 rats that were ramdomly divided into five groups as shown in **Figure 1**. The OC chemical induction consisted of a single injection with 7,12-dimethylbenzoanthracene – DMBA (Sigma Chemical Co, St Louis, Mo) into the ovarian bursa (Chuffa et al., 2018) and the tumor development were monitored using X-ray computed microtomography (micro-CT). PRP used came from the peripheral blood of 4 human volunteers, following the protocol

based on Dias et al. (2018). This project is associated with the protocol approved by the Ethics Committee for Research with Human Beings – CEP/UNICAMP (**CAAE number: 51774515.0.0000.5404**). OncoTherad® was synthesized, purified and characterized according to Fávaro & Durán (2017). All applied methodologies were authorized by the Ethics Committee in the Use of Animals – CEUA/UNICAMP (**Registration 5475-1/2020**).

The estrous cycles were monitored daily by cytological examination of vaginal smear. The body weight of animals was evaluated throughout the experimental period, as well as the feed and water consumption. The absolute and relatives weights of the ovaries and uterus were recorded. Macroscopic changes and their respective frequencies were quantified. Quantitative data were represented as mean ± standard deviation and evaluated using the parametric analysis of variance ANOVA, complemented by the Tukey test, when they presented normality. In cases of absence of normality, the Kruskal-Wallis non-parametric analysis of variance was used, complemented with the Student-Newman-Keuls test. Statistical significance was 5% ($p<0.05$).

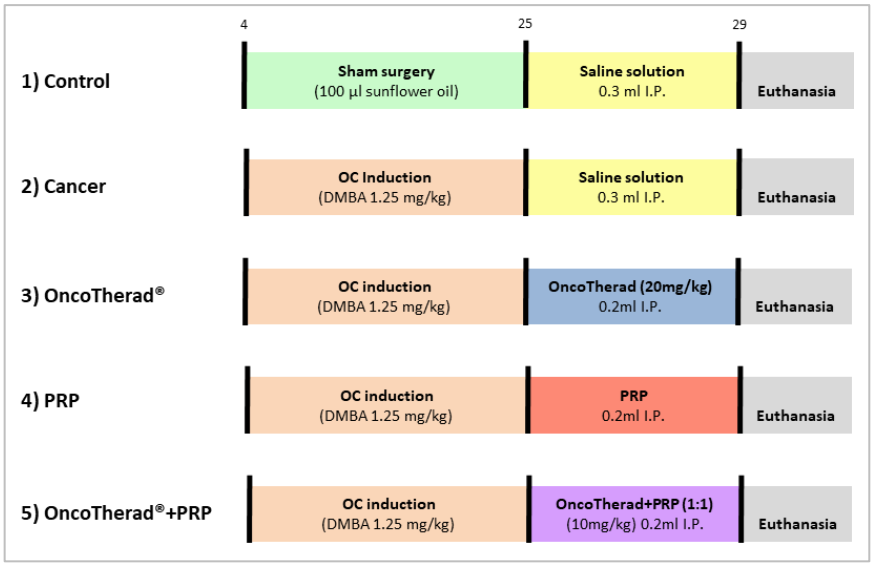

Figure 1. Experimental protocol. In the 3rd and 4th weeks, chemical induction surgeries of OC (single injection of DMBA in the ovarian bursa, dose of 1.25 mg/kg, diluted in sunflower oil) and Sham surgery (single injection in the ovarian bursa of 100 µl sunflower oil) were performed. The period between 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 (20mg/kg) - intraperitoneal application (I.P.) of 0.2ml; PRP - IP application 0.2ml and OncoTherad associated with PRP (1:1) - I.P. of 0.2 ml at the same concentrations as the treatments alone. Thus, the association in a 1:1 ratio contains 0.1 ml of OncoTherad (20mg/ml) and 0.01 ml of PRP, reaching the final concentration of the drug suspended in PRP of 10mg/ml. Treatments were carried out twice a week for 4 weeks. The 29th week corresponds to the euthanasia.

RESULTS AND DISCUSSION

The surgical intervention (Sham), OC induction, and treatments with OncoTherad and PRP did not prevent the cyclicity, which was confirmed by the occurrence of the estrus phase (**Figure 2**). The maintenance of cyclicity could be explained by the presence of the right ovary that did not undergo surgical intervention. However, it was possible to observe changes in the rhythm of the estrous cycle during the experiment. At the end of the treatment period, the duration of the estrous cycle was greater in the induced groups compared to healthy animals, except for PRP group (**Table 1**). There was a probable dragging of the cycle with an increase of days in the estrus phase (**Table 1**), possibly related to the damage caused to the left gonad during the tumor development. It might be possible that PRP had some kind of effect in decreasing the duration of the estrous cycle. Regarding diestrus, there were no statistically significant differences between the groups.

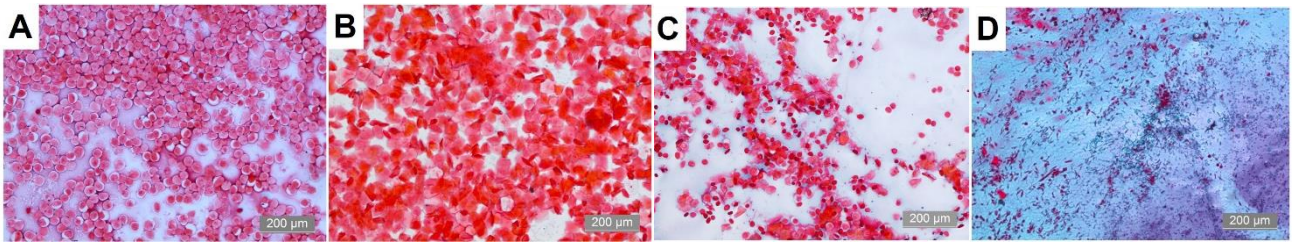

Figure 2. Stages of the estrous cycle. Images obtained after the vaginal lavage technique to identify the phases of the cycle, viewing the proportion between the different cell types. (A) Proestrus, consisting mostly of nucleated epithelial cells. (B) Estrus, with predominance of anucleated keratinized cells. (C) Metaestrus, consisting of leukocytes in combination with keratinized cells, nucleated epithelial cells, and mucus. (C) Diestrus, with predominance of leukocytes and mucus. Shorr and Hematoxylin staining. Bars = 200 µm.

Table 1. Length of estrous cycle, number of estrus and diestrus at different stages of the experimental period.

	Experimental groups (n=7/group)				
Parameters	**Control**	**Cancer**	**OncoTherad**	**PRP**	**OncoTherad+PRP**
Duration of cycles (days)					
Before surgery	6.6 ± 0.8 a	6.3 ± 0.7 a	7.1 ± 1.1 a	6.6 ± 1.2 a	6.9 ± 0.6 a
Tumor development	6.9 ± 0.9 a	8.2 ± 3.1 a	10.5 ± 3.8 ab	12.7 ± 3.8 b	11.5 ± 1.6 b
During treatments	6.5 ± 0.5 a	12.6 ± 2.8 b	12.3 ± 3.4 c	9.0 ± 2.8 ac	12.4 ± 2.4 c
Number of estrous (in 15 days)					
Before surgery	3.2 ± 0.5 a	2.9 ± 0.6 a	3.4 ± 0.5 a	3.0 ± 0.8 a	3.9 ± 0.8 a
Tumor development*	3.4 ± 1.1 a	5.7 ± 2.5 ab	4.3 ± 1.6 ab	7.1 ± 1.7 b	5.5 ± 2.1 ab
During treatments	3.4 ± 0.6 a	8.1 ± 3.3 b	8.1 ± 3.9 b	6.4 ± 1.7 b	6.3 ± 3.5 ab
Number of diestrus (in 15 days)					
Before surgery	5.2 ± 1.1 a	5.3 ± 1.3 a	5.9 ± 1.3 a	5.0 ± 0.9 a	6.6 ± 1.2 a
Tumor development	5.4 ± 0.6 a	6.0 ± 2.5 a	6.8 ± 1.4 a	4.9 ± 1.6 a	5.5 ± 1.9 a
During treatments	6.4 ± 0.6 a	4.5 ± 2.1 a	4.1 ± 2.8 a	4.3 ± 2.0 a	3.8 ± 2.3 a

Values expressed as mean ± standard deviation. Kruskal-Wallis, Student-Newman-Keuls test. *ANOVA, Tukey Test. In the same line, values followed by different letters indicate a statistically significant difference between groups (p<0.05).

The variation in body weight over the period is showed in **Figure 3**. Tumor development and OC induction did not affect the body weight, as OncoTherad and PRP also did not change the final weight compared to Control and Cancer groups (**Table 2**). In agreement with this, the weekly weight gain rate also did not show statistically differences between groups (**Table 2**).

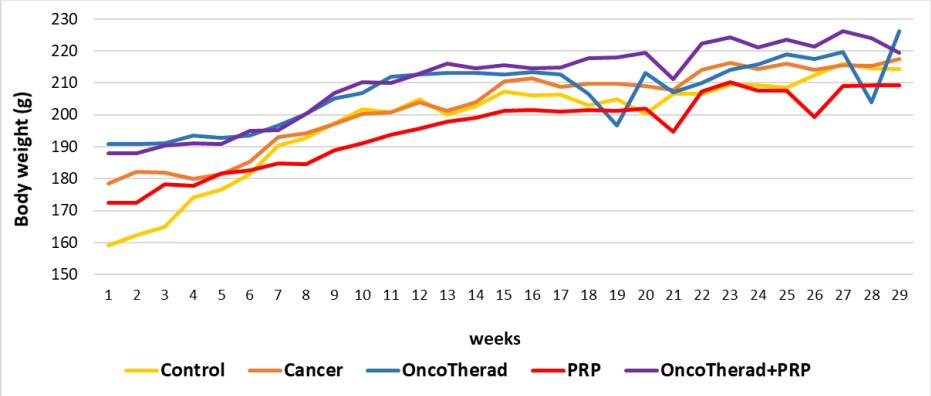

Figure 3. Body weight variation graph from 1st to 29th week. 3rd and 4th weeks: OC chemical induction and Sham surgery. From 4th to 25th: tumor development (around 140 days). From 25th to 28th: 4 weeks of treatments with OncoTherad, PRP, and OncoTherad associated to PRP.

Table 2. Body weight (g) in different periods and rate of weight gain (g/week) of animals.

	Experimental groups (n=7 animals/group)				
Parameters	**Control**	**Cancer**	**OncoTherad**	**PRP**	**OncoTherad +PRP**
Initial body weight (g)*	192.8 ± 4.7 a	183.4 ± 9.2 a	192.8 ± 10.7 a	184.7 ± 4.5 a	190.9 ± 17.1 a
Body weight before treatment	208.4 ± 7.0 a	218.6 ± 5.4 a	210.0 ± 14.1 a	207.3 ± 5.2 a	214.0 ± 3.2 a
Final body weight (g)	214.4 ± 6.6 a	219.4 ± 8.4 a	226.1 ± 15.7 a	209.3 ± 6.4 a	219.5 ± 13.9 a
Weight gain/week (g/week)	0.98 ± 0.11 a	1.41 ± 0.37 a	1.39 ± 0.42 a	1.12 ± 0.34 a	1.19 ± 0.64 a

Values expressed as mean ± standard deviation. ANOVA. *Kruskal-Wallis test. In the same line, values followed by equal letters indicate the absence of a statistically significant difference between the groups (p>0.05).

Treatment with OncoTherad or PRP, alone or in combination, performed in the context of induced OC did not change the feed intake (**Table 3**), as well as the weight gain or final weight of the animals, which shows indications of absence of acute toxicity promoted by the treatments. The water consumption of the treated groups (OncoTherad, PRP and OncoTherad+PRP), in the complete experimental period, was lower in comparison with the Control and Cancer groups (**Table 3**). Because this result, the analysis was performed in different periods, which showed that the change occurred during tumor development and not due to the influence of treatments. There was no difference in water consumption between Control and Cancer groups, then it is not possible to state that this variation is due to OC development. Furthermore, there are few reports in the literature on murine consumption patterns in the OC context.

In the PRP and OncoTherad+PRP groups, the absolute and relative weight of the left ovary was lower (p<0.05) when compared to the Cancer group. Also, OncoTherad+PRP group had a lower (p<0.05) relative weight of the left ovary compared to the Control group (**Figure 4A and B**).

Table 3. Feed (g/animal/week) and water (ml/animal/week) consumption in the different periods.

	Experimental groups (n=7/group)				
Parameters	**Control**	**Cancer**	**OncoTherad**	**PRP**	**OncoTherad+PRP**
Feed consumption/week (g)					
General (all period)	79.98 ± 16.6 a	79.01 ± 9.0 a	77.49 ± 18.2 a	71.35 ± 13.7 a	75.19 ± 11.4 a
Before surgery	90.50 ± 30.9 a	76.22 ± 3.7 a	68.25 ± 20.7 a	68.17 ± 13.8 a	68.85 ± 13.7 a
Tumor development	78.37 ± 13.0 a	81.12 ± 9.7 a	76.36 ± 6.5 a	74.73 ± 8.3 a	78.03 ± 10.1 a
During treatments	75.25 ± 3.2 a	73.36 ± 3.7 a	91.98 ± 31.4 a	63.70 ± 24.3 a	71.85 ± 4.9 a
Water consumption/week (ml)					
General (all period)	101.8 ± 22.1 a	103.0 ± 18.3 a	85.3 ± 22.3 b	89.1 ± 20.9 b	87.1 ± 17.2 b
Before surgery*	114.0 ± 19.8 a	115.0 ± 17.1 a	92.1 ± 27.9 a	98.7 ± 20.5 a	93.5 ± 15.7 a
Tumor development	104.1 ± 22.6 ac	102.8 ± 17.2 a	80.1 ± 11.76 b	89.5 ± 15.5 bc	87.6 ± 18.7 b
During treatments	81.3 ± 4.2 a	84.2 ± 3.9 a	92.9 ± 37.7 a	78.3 ± 33.8 a	77.3 ± 6.8 a

Values expressed as mean ± standard deviation. Kruskal-Wallis, Student-Newman-Keuls test. *ANOVA. In the same line, values followed by different letters indicate a statistically significant difference between groups (p<0.05).

There were no significant differences (p>0.05) in the absolute weight of the uterus of the rats in the experimental groups. However, when the relative weight was analyzed, the rats in the Cancer group had higher (p<0.05) uterine weight compared to the OncoTherad and OncoTherad+PRP groups.

The female rats in the Control group did not show any macroscopically visible structural changes in the reproductive tract (**Table 4, Figure 5A**). On the other hand, in the Cancer group several alterations were observed in the left ovaries, such as: presence of cystic nodular lesions (**Figure 5B**) in most animals (71.4%) and a more vascularized appearance (**Table 4**). The fact that most lesions on the left ovaries were small nodular cysts (diameters not greater than 5 mm) explains the absence of statistical difference in gonad weight between the Control and Cancer groups. Other abnormalities observed in some of the animals in the group include a more swollen appearance of the right uterus and ovaries and peritoneal implants (28.6%) (**Table 4**). The used induction protocol did not lead to large lesions or cachexia, being a valid model for therapeutic evaluation with better chances of response to treatments. However, confirmation of the occurrence of neoplasms and diagnoses can only be described after histopathological analysis.

In the OncoTherad group, most rats had left ovaries with normal appearance and presence of corpora lutea without apparent lesions (85.7%) and, in 57.1%, the left gonad was more atrophied in a discrete or more apparent way (**Table 4**, **Figure 5C**). In the PRP group, it was possible to identify, in several female rats, left ovaries with atrophy (42.9%), as well as some without apparent structural alterations (57.1%) (**Table 4**, **Figure 5D**). In the OncoTherad+PRP group, left ovaries with atrophied appearance (57.1%) (**Figure 5E**) and others with normal appearance and presence of corpora lutea (42.9%) were identified (**Table 4**).

The effect of reducing the weight of the left ovary linked to atrophy might be related to the stimulation of the immune system and antitumor response promoted by OncoTherad associated or not to PRP. This effect might have been enhanced by this association, since atrophy was more frequently observed in the macroscopic analysis for OcoTherad+PRP group.

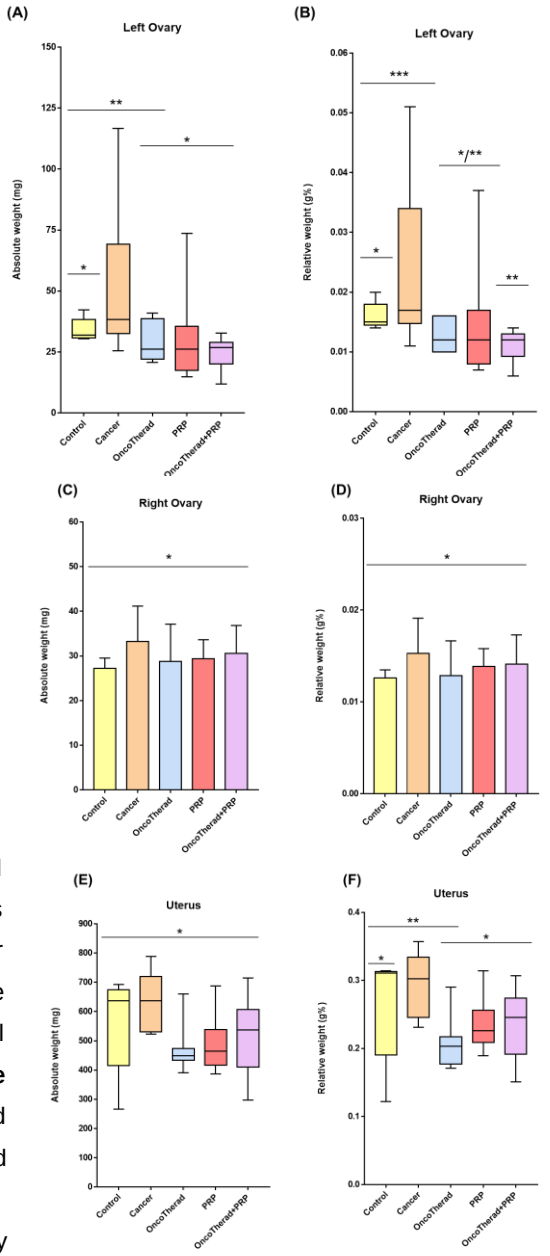

Figure 4. Absolute and relative weight of the left ovary, right ovary and uterus of rats. (A, B) Left ovary. Kruskal-Wallis test, Student-Newman-Keuls test. (C, D) Right ovary. ANOVA. (E, F) Uterus. Kruskal-Wallis test, Student-Newman-Keuls test. */**/*** Different symbols indicate a significant difference between groups (p<0.05).

Table 4. Frequency of macroscopic changes in different experimental groups.

	Experimental groups (n=7 animals/group)				
Alterations	**Control**	**Cancer**	**OncoTherad**	**PRP**	**OncoTherad+PRP**
Left ovary lesion					
Absent	7/7 (100%)	2/7 (28.6%)	6/7 (85.7%)	6/7 (85.7%)	7/7 (100%)
Nodular cystic	-	5/7 (71.4%)	-	-	-
Pure cystic	-	-	-	1/7 (14.3%)	-
Solid mass	-	-	1/7 (14.3%)	-	-
Atrophy					
Absent	7/7 (100%)	5/7 (71.4%)	2/7 (28.6%)	4/7 (57.1%)	3/7 (42.9%)
Present on left ovary	-	2/7 (28.6%)	4/7 (57.1%)	3/7 (42.9%)	4/7 (57.1%)
Present on right ovary	-	-	1/7 (14.3%)	-	1/7 (14.3%)
Edema					
Absent	7/7 (100%)	3/7 (42.9%)	7/7 (100%)	6/7 (85.7%)	6/7 (85.7%)
Present on left ovary	-	-	-	-	-
Present on right ovary	-	2/7 (28.6%)	-	-	1/7 (14.3%)
Present in the uterus	-	1/7 (14.3%)	-	1/7 (14.3%)	-
Peritoneal implant					
Absent	7/7 (100%)	5/7 (71.4%)	5/7 (71.4%)	6/7 (85.7%)	6/7 (85.7%)
Present	-	2/7 (28.%)	2/7 (28.6%)	1/7 (14.3%)	1/7 (14.3%)

Incidence: number of animals that presented a certain characteristic/total number of animals in the experimental group

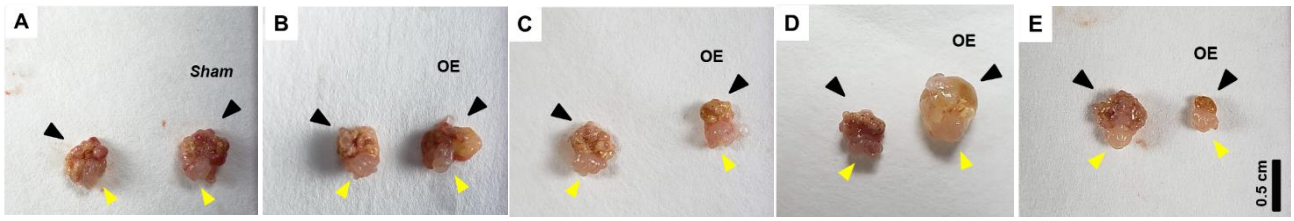

Figure 5. Mascroscopic evaluation of the ovaries of females from the Control (A), Cancer (B), OncoTherad (C), PRP (D), and OncoTherad+PRP (E) groups. (A) Detail of the right and left ovaries (black arrowhead) of normal appearance, with visible corpora lutea and fallopian tubes (yellow arrowhead). (B) Presence of a nodule in the left ovary. (C) The left ovary has moderate atrophy. (D) Cystic lesion in the atrophied left ovary. (E) Atrophied left ovary with absence of visible corpora lutea.

CONCLUSIONS

This study is ongoing, however it has already shown that the model of ovarian cancer induction with DBMA can be preliminarily assured and characterized. The OC induction did not cause cachexia in the rats, nor significant changes in weight or consumption, while it promoted a drag on the cycle, persistent estrus and lesions in the ovaries with a milder dimensional aspect, which provides better possibilities of response to treatments. In addition, it was observed that treatments with OncoTherad and PRP mainly altered the macroscopic aspects of the reproductive tract in comparison with animals induced to the OC without treatments. Further, OncoTherad and PRP did not significantly alter the estrous cycle or showed signs of systemic toxicity by weight and consumption assessments.

ACKNOWLEDGMENTS

Process number: 2020/07898-6

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