REPORT
CODE: BIOULA-CYTOREX

TITLE: Experimental evaluation of the effect of the Cytoreg® formulation on fertility and reproductive parameters in C57BL/6//BIOU mice.

PLACE OF DEVELOPMENT: VIVARIUM OF THE UNIVERSITY OF LOS ANDES - MÉRIDA - VENEZUELA.

Issued by:

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EVIDENCE OF TERATOGENIC EFFECT.

The procedures applied were endorsed by the Ethics Committee of the Vivarium - CEBIOULA - according to AVAL N ° CEBIOULA/072.

1. PART I. DETERMINATION OF THE EFFECT OF THE CYTOREG® FORMULATION ON THE PHYSIOLOGICAL CONDITIONS OF THE FEMALES

1.1. Monitor the estrous cycle under the administration of the Cytoreg® formulation in female mice C57BL/6//BIOU

1.2. Determine the weight of each of the ovaries of the female mice C57BL/6//BIOU

1.3. Determine the weight of the uterus of female mice C57BL/ 6//BIOU

2. PART II. DETERMINATION OF THE EFFECT OF THE CYTOREG® FORMULATION ON THE PHYSIOLOGICAL CONDITIONS OF THE MALES

2.1. Determine the weight of each testicle of male mice C57BL/6//BIOU

2.2. Determine the weight of each of the epididymis of male mice C57BL/6//BIOU

2.3. Evaluate the sperm motility of male mice C57BL/6//BIOU

2.4. Determine sperm concentration of male mice C57BL/6//BIOU

2.5. Identify each of the morphologies of the sperm head of male mice C57BL/6//BIOU

3. PART III. DETERMINATION OF THE EFFECT OF THE CYTOREG® FORMULATION ON REPRODUCTION

3.1. Determine the number of corpora lutea in each of the ovaries of the female mice C57BL/6//BIOU

3.2. Quantify the number of live embryos in each of the uterine horns of female mice C57BL/6//BIOU

3.3. Quantify the number of dead embryos in each of the uterine horns of the female mice C57BL/6//BIOU.

3.4. Quantify the total number of embryos in female mice C57BL/6//BIOU

Statistical Analysis

The statistical test of the Wilcoxon signed ranges was used, as this is a non-parametric test which allows comparing the average range of two related samples and determining if there are differences between them. The statistical program used was the Statistix10.

Biological Model

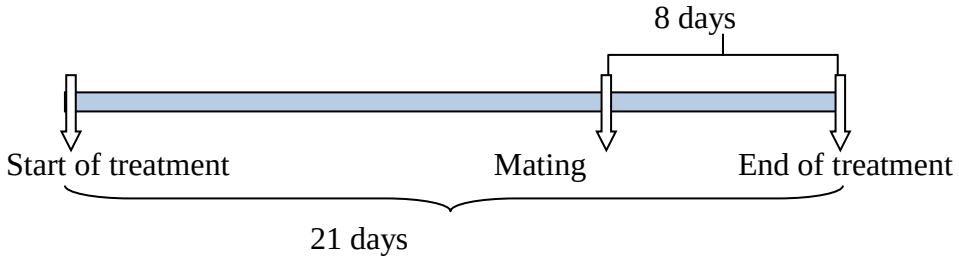
A total of 80 mice of the inbred line C57BL/6//BIOU were used, produced and maintained in the Vivarium of the Universidad de Los Andes. The total number of mice used was 40 female mice and 40 males, 8 to 10 weeks of age (25-30 g), distributed as follows: 20 male mice and 20 female mice of the experimental group consuming the Cytoreg® formulation and the control group by 20 male mice and 20 female mice that do not consume the formulation.

The animals were individually housed in T2 boxes, for monogamous mating (1 female x 1 male). They were housed in an environment with 12h light/12h dark, with heat treated food (1min/121 ° C) and sterilized water (10min / 121 ° C), both for consumption at will of the animals (Cuervo, 2012).

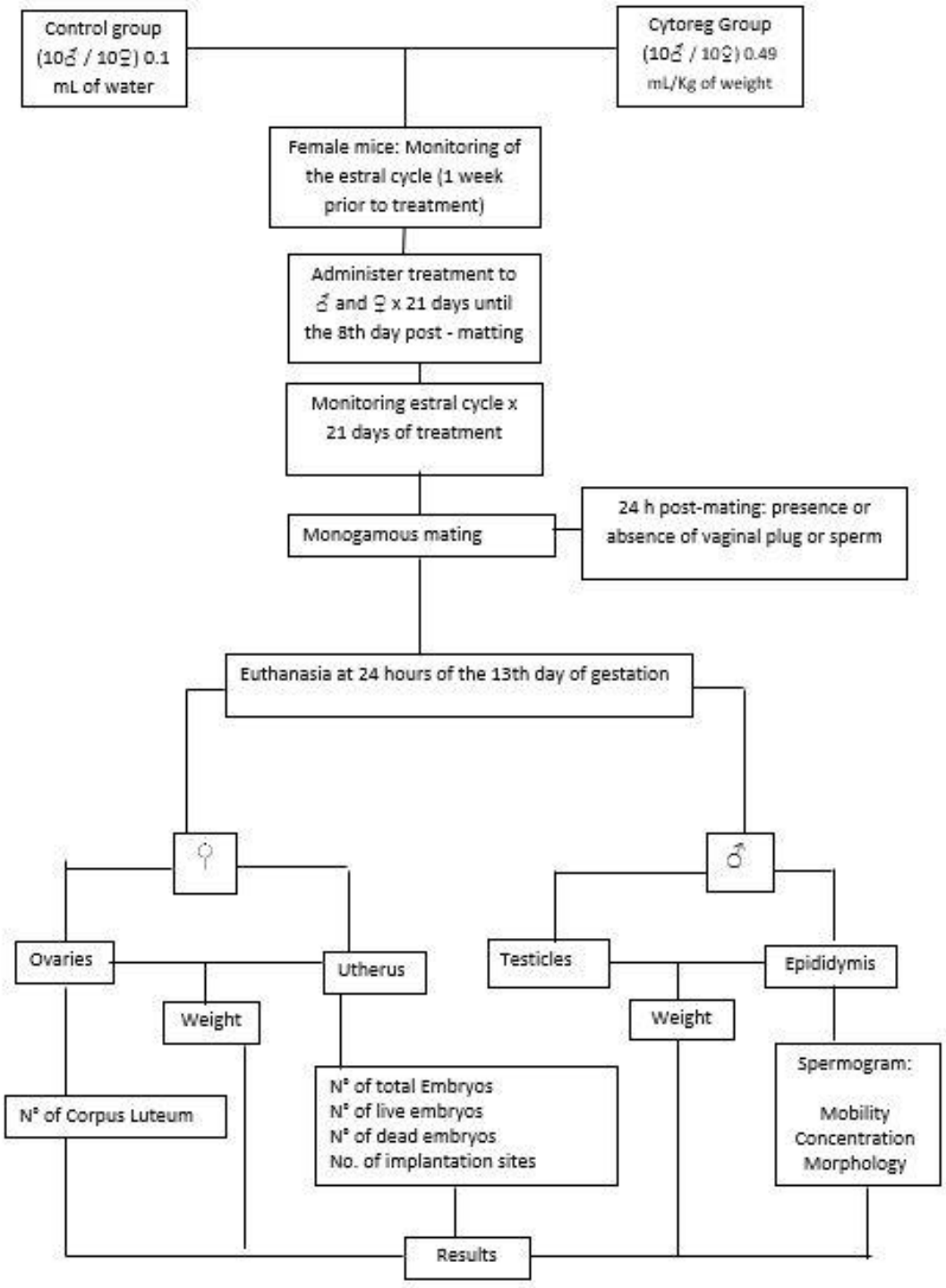
Experimental design / TREATMENT SCHEMES

I. PROOF OF FERTILITY

Cytoreg® administration for 21 days until the 8th post mating day.



Scheme of developed procedure.



The mating was verified by the presence of the vaginal plug and/or presence or absence of sperm. The estrous cycle was monitored during the 21 days of treatment. The females are sacrificed 13 days post mating.

For the monitoring of the estrous cycle, vaginal washes were performed. For the taking of the samples, the female mice were held and placed approximately 0.15 mL of physiological solution with a plastic pipette in the vaginal canal; the vaginal wash was performed and the sample was extracted by suction and placed on a slide, placed an object cover, and was observed under a microscope with a 10X and 40X objective to identify the cells present. Monitoring was always carried out at the same time during the time of the trial.

To achieve the objectives of weighing ovaries, uterus, corpus luteum, implanted embryos, live and dead embryos, the females were necropsied at the corresponding times; and for weighing testicles, epididymis, sperm concentration, mobilization sperm and morphology of the spermatozoa, necropsy was performed on the males. For the sacrifice, a method of excess inhalation anesthesia, eufluoran, was used.

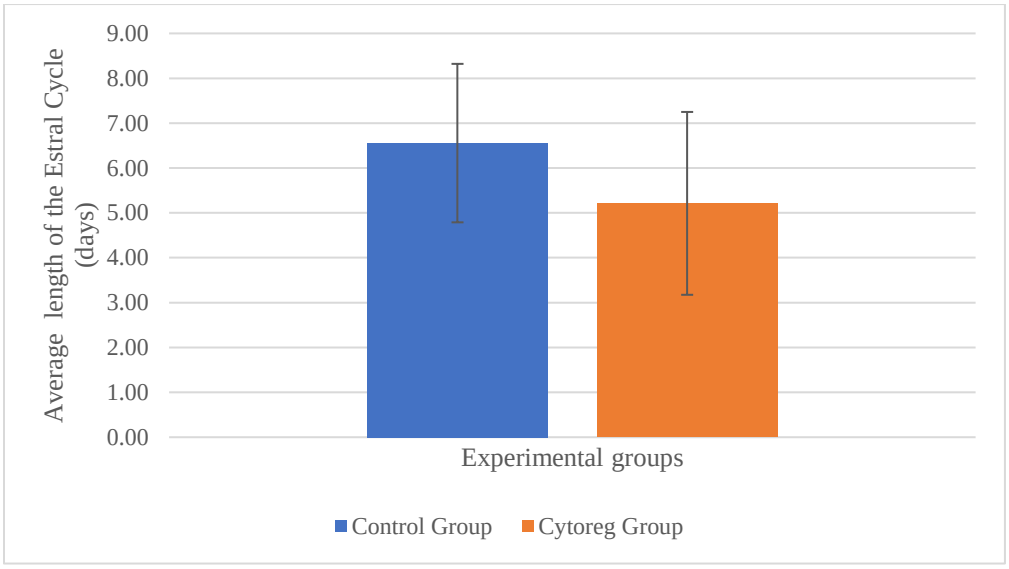
Results

1. PART I. DETERMINATION OF THE EFFECT OF THE CYTOREG® FORMULATION ON THE PHYSIOLOGICAL CONDITIONS OF THE FEMALES.

1.1. Monitoring of the estrous cycle under the administration of the Cytoreg® formulation in female mice C57BL/6//BIOU

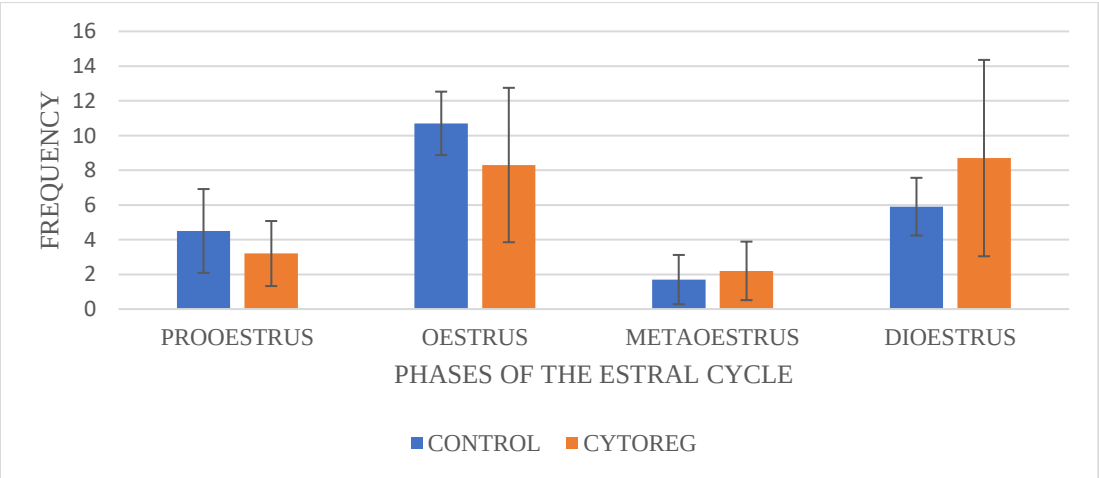
The monitoring consisted of determining the stage of the estrous cycle in which the female was, which allowed to record the time of complete duration of the different stages as well as the frequency of the same. The complete duration of the estrous cycle is estimated in 4 to 5 days, consisting of 4 stages: right-handed, proestrus, estrus and metestrus, each with an estimate of: two to three days for the right-handed, twelve hours to eighteen hours for the proestrus, twelve hours for the estrus, from 10 to 14 hours for the metamaster.

Graph 1 shows the average estrus cycle duration of the female mice for each group of mice for both the treated and the control. It can be observed that the estrous cycle for the control group lasted 6.56 days, while the estrus cycle of the Cytoreg® experimental group lasted 5.21, that is, approximately 1.35 days of difference. The statistical analysis through the Wilcox sum test showed no statistically significant differences between the obtained values ($p = 0.4148$, $p > 0.05$).



Graph 1. Arithmetic measures of the length of the estrous cycle by research groups

In Graph 2, the average frequency of each one of the stages of the estrous cycle of the female mice for each experimental group is shown. Differences in frequency were observed for each stage of the cycle between the groups of treated and control animals. The stage of Proestrus and Oestrus presented greater frequency in the control than the group treated with Cytoreg®, and the stages of the Metestrus and the Diestrus presented greater frequency in the Cytoreg® treated group. Despite these observations, the statistical analysis using the Wilcoxon sum test did not present statistically significant differences between the average frequencies between the experimental groups and the controls, for each stage of the estrous cycle (Proestrus: p = 0, 3627, Oestrus: p = 0.2084, Metestrus: p = 0.5076, Diestrus: p = 0.4446 p> 0.05).



Graph 2. Frequencies of each one of the stages of the estrous cycle in the female mice of the test groups.

Conclusion: Neither the duration of the estrous cycle, nor the frequency of the estrous cycle stages of the female mice treated with the Cytoreg® formulation, showed statistically significant differences when the same parameters were compared to the mice that were not treated with the formulation.

1.2. Weight of each of the ovaries of the female mice C57BL/6//BIOU

The weight of the right ovary of the control group was 0.0064g, while that of the group treated with Cytoreg® was 0.00425g, with the ovary of the control group being of greater weight. The statistic did not present significant differences, with a value of $p = 0.121$ for a $p > 0.05$ (Table 1) and a confidence level of 95%. In the case of the weight of the left ovary, the ovary of the group treated with Cytoreg®, the weight was slightly greater than 0.00435g, in relation to the control group, 0.0041g; statistically there were no significant differences when comparing the weights, obtaining a value of $p > 0.05$ ($p = 0.940$), (See Table 1).

1.3. Weight of uterus of female mice C57BL/6//BIOU

In relation to the weight of the uterus of the control group, an average equal to 1.4156g was obtained, being greater than the uterus of the experimental group Cytoreg®, which was 1.24199 g. The statistical test showed that there is no statistically significant difference between both weights with $p = 0.496$ (Table 1).

Table 1. Mann-Whitney U test for some fertility parameters considered for control female mice vs group treated with Cytoreg®

	U de Mann-Whitney	p-value
Weight Ovary D	29.500	.121
Weight Ovary I	49.000	.940
Weight Uterus	41.000	.496

Note: no statistically significant differences were found at a Confidence Level of 95%.

2. PART II. DETERMINATION OF THE EFFECT OF THE CYTOREG® FORMULATION ON THE PHYSIOLOGICAL CONDITIONS OF THE MALES

2.1. Weight of each testicle of male C57BL/6//BIOU mice

In the case of the males, the weight of the right testicle of the control group was 0.085g while the weight of the testicle of the group treated with Cytoreg® was 0.0891g, the latter presenting a greater weight. In the same way, when performing the analysis, no statistically significant differences were found between the weights of both testicles (p = 0.762), (Table 2). For the left testicle, the weights obtained for each group were 0.0793g and 0.08288g, for the control group and the group treated with Cytoreg®, respectively, observing that the left testicle of the experimental group Cytoreg® has a greater weight than the control; however, statistically, there are no significant differences between both results (p> 0.05, p = 1), (Table 2).

2.2. Weight of each of the epididymis of male mice C57BL/6//BIOU

Extracted the right and left epididymis (only head and body) for each of the groups of test mice, and having weighed them, finding were that, for the right epididymis of the control group, a weight equal to 0.0199g was obtained, while the group treated with Cytoreg® was 0.01885g, observing in the right epididymis of the control group a slightly higher weight; however, the statistic did not present significant differences between both values (p = 0.880). For the weight of the left epididymis, for the mice of the group treated with Cytoreg®, the weight was 0.018260g and that of the control group was 0.0187g, with the latter being greater; no statistically significant differences were observed between them (p = 0.650)., (Table 2).

2.3. Evaluation of sperm motility of male mice C57BL / 6 // BIOU

Another of the fertility parameters evaluated for male mice was the type of mobility presented by the spermatozoa of each of the groups. The criteria used to determine the type of mobility were: Progressive Mobility (PM), Non-Progressive Mobility (NPM) and immobile (I).

In the control group, 24.4 ± 5% of spermatozoa characterized with an PM movement were found, while the Cytoreg® group presented 20.2%; for the control group, 55.3 ± 6% presented an NPM movement and the group treated with Cytoreg® 54.7 ± 8% of sperm with this mobility. The control group presented 31.4 ± 6% of immobile sperm, while the Cytoreg® group obtained 26.1 ± 4%, being lower than the control group. The results obtained for each mobility did not show statistically significant differences, presenting, for p> 0.05: MP, p = 0.2799; MNP, p = 0.1232 and for I, p = 0.7578, (See Table 2).

2.4. Sperm concentration of male mice C57BL/6//BIOU

In relation to the sperm concentration parameter (Table 2), the control group had a concentration equal to 9.9 x 106 cell/mL, while the group treated with Cytoreg® obtained a concentration of 10.14 x 106 cell/mL, the Cytoreg® group having a higher concentration of sperm. Once the

statistical analysis was performed, the result obtained was that there is no statistically significant difference between the groups (p = 0.970).

Table 2. Mann-Whitney U test for some fertility parameters considered for male control mice vs. Cytoreg® experimental group.

	U de Mann-Whitney	p-valor
Peso Testicle D	46.000	.762
Peso Testicle I	50.000	1.000
Peso Epid D	48.000	.880
Peso Epid I	44.000	.650
MP	25.500	.064
MNP	40.500	.472
I	49.000	.940
Nº sperm	49.000	.940
Concentration	49.500	.970

Note: no statistically significant differences were found at a confidence level of 95%.

2.5. Evaluation of the morphology of the sperm head of male mice C57BL/6//BIOU.

For the evaluation, 200 spermatozoa per mouse are detailed, after performing a coloration with Papanicolaou and observing them under a microscope at 100X with immersion oil. In the evaluation of the morphology of the head of the spermatozoa of the mice of each of the groups, the following criteria were taken into consideration: Normal, amorphous, banana, without hook and others (Table 3). For normal morphology, the average in the control group obtained a value equal to 165.9 spermatozoa, while the group treated with Cytoreg® with 151.48 cells, with the highest number of spermatozoa with this morphology for the control group; for amorphous morphology, the average found was 26.6 cells for the control group, while for the treated group it was 37.22. The average in the control group was 3.8 spermatozoa whose head had the shape of a banana, while in the treated group with Cytoreg® the average was 5.55 sperm. For headless hook morphology, the control had an average of 3.2 cells, while for the mice of the treated group it was 4.66 sperm. For other morphologies, the average was 0.5 for the control group and 1.11 spermatozoa for the group of mice treated with Cytoreg®. Statistically, no significant differences

were observed between groups for the normal morphologies, banana, without hook and others (p = 0.07, p = 0.430, p = 0.484 and p = 0.152 respectively). However, in the case of Amorphous morphology, there was a significant difference between the control groups and the groups treated with Cytoreg®, obtaining a value of p = 0.038

Table 3. Statistics of independent samples of sperm morphology.

	Group	N	Average	Typical Deviation	Typical Error of the Average	p-value
Normal	Control	10	215.900	15.81455	5.00100	.077
	Experimental	9	201.555	17.42922	5.80974	
Amorphous	Control	10	26.6000	10.75174	3.40000	.038 (**)
	Experimental	9	37.2222	9.75676	3.25225	
Banana	Control	10	3.8000	5.05085	1.59722	.430
	Experimental	9	5.5556	4.33333	1.44444	
Sin Gancho	Control	10	3.2000	2.04396	.64636	.484
	Experimental	9	4.6667	6.12372	2.04124	
Other	Control	10	.5000	.52705	.16667	.152
	Experimental	9	1.1111	1.16667	.38889	

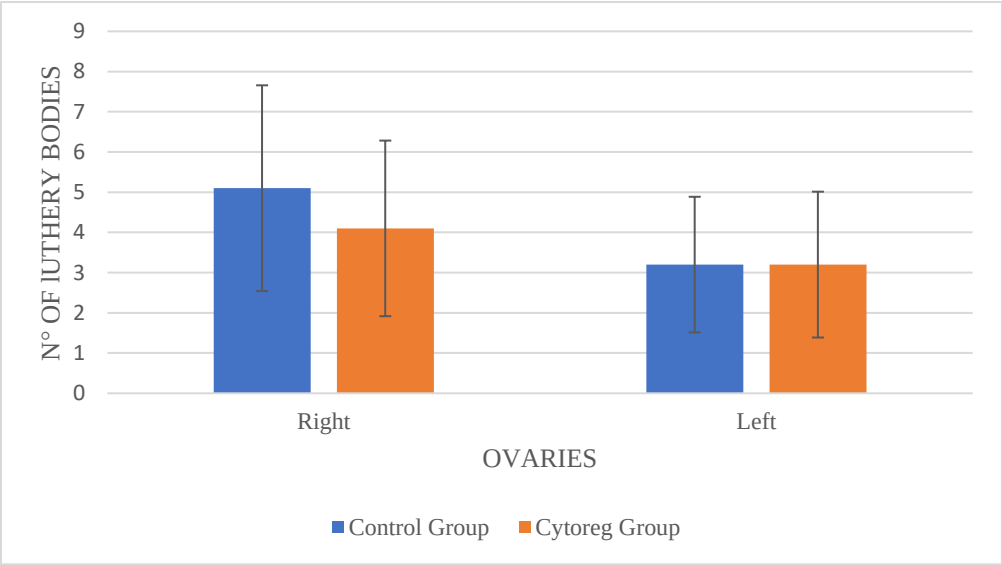
(**) There are statistically significant differences at a confidence level of 95% (p <0.05).

3. PART III. DETERMINATION OF THE EFFECT OF THE CYTOREG® FORMULATION ON REPRODUCTIVE CONDITIONS

3.1. Number of corpora lutea in each of the ovaries of the female mice C57BL/6//BIOU

Differences could be observed between the number of corpora lutea of the right ovary of the control group and the group treated with Cytoreg®; the control group had a higher mean than the experimental group: 5.1 ± 2.5 and 4.1 ± 2 respectively. These means did not present statistically significant differences (p = 0.3394, p> 0.05). In relation to the averages of corpora lutea

observed in the left horns for both experimental groups, an average of 3.2 ± 4 was obtained; no statistically significant differences were observed (p = 0.9693, p> 0.05), (Graph 3)



Graph 3. Arithmetic means of the corpus luteum number of each of the experimental groups

3.2. Quantification of the number of live embryos in the groups of female mice C57BL/6//BIOU

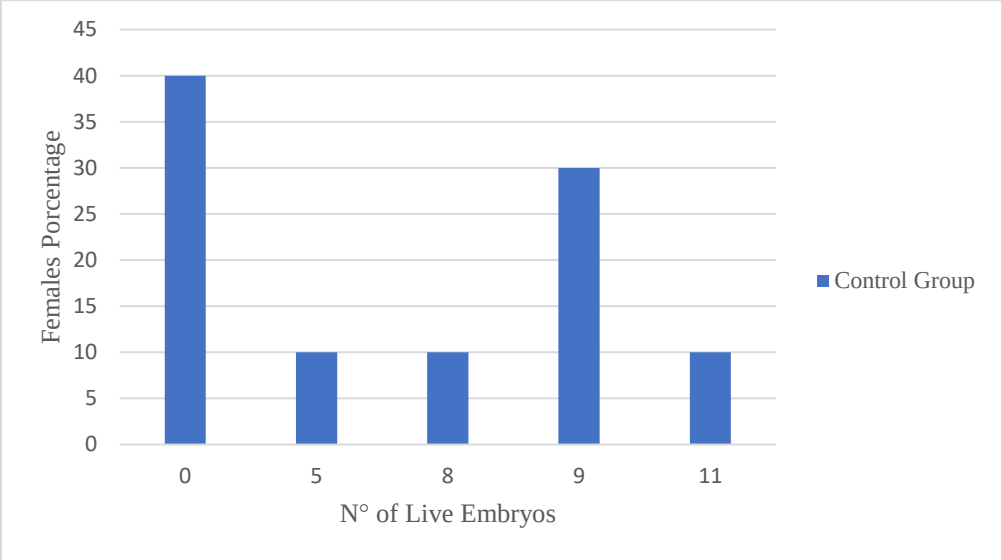
The gestation time was interrupted for the female mice of each experimental group, applying their sacrifice through an appropriate method of euthanasia, in order to determine the number of live and dead embryos, as well as the total number of embryos that developed.

It was observed that the number of live embryos for the control group was higher than for the experimental group treated with Cytoreg® (with an average equal to 5.10 and 3.30), showing a difference of 1.8 units; however, when performing the test, these results did not show statistically significant differences (p = 0.4012, p> 0.05).

The number of implantations observed in the uterine horns indicates the number of ovules that were fertilized during copulation of the animals. In relation to this parameter, the implantations observed for the control group in the right horn was 3.4 implantations, while for the group treated with Cytoreg®, was of 3 implantations. In the left horn, the number of implantations for the control was 2.2 and for the Cytoreg® a value of 2.50 was obtained. When comparing the results obtained for the control and the experimental Cytoreg® group, it can be seen that the differences are not so pronounced between the means. When performing the statistical analysis, it was observed that there are no statistically significant differences between the number of

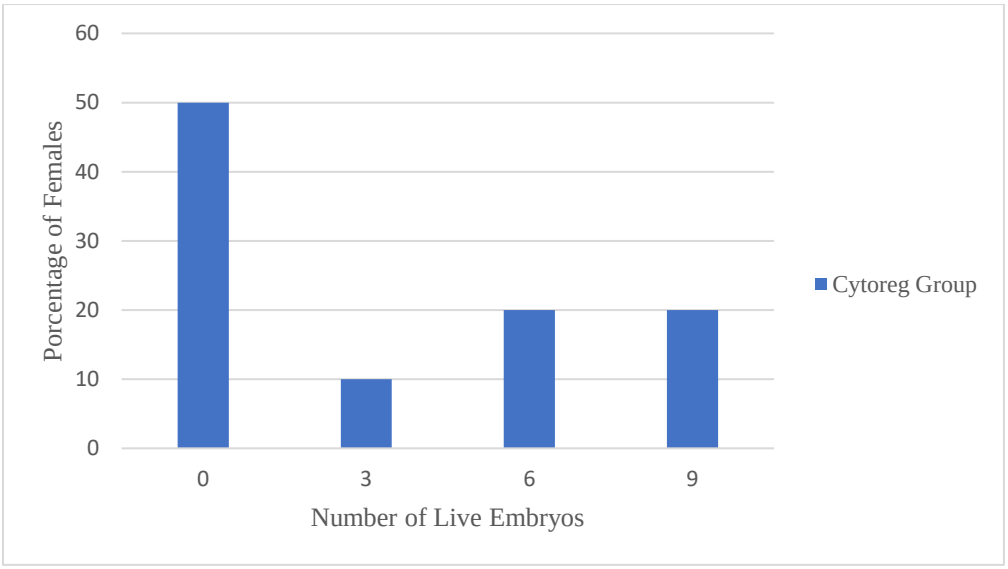
implantation sites in the right horn of the control groups and the group treated with Cytoreg®, as well as the number of implantations in the left horn between both experimental groups (p = 0.7841 and p = 0.9059, respectively, where p> 0.05).

In Graph No. 4, the results of the control group are presented. In this graph, it can be seen that 40% of animals were not gestated, that is, no embryos were found, they did not have gestation. Of the total of 60% of the pregnant females, in 10% a pregnancy of 5 embryos was observed, another 10% presented a total of 8 embryos and another 10% presented 11 embryos, and 30% presented 9 live embryos.



Graph 4. Percentage of female mice of the control group with a determined number of live embryos

In relation to what was observed in the experimental group treated with Cytoreg® (Graph No. 5), it was observed that 50% of animals that were not gestated; of the 50% that were gestated, 20% of the female mice presented nine live embryos, 20% of the animals produced six embryos and 10% produced 3 embryos.

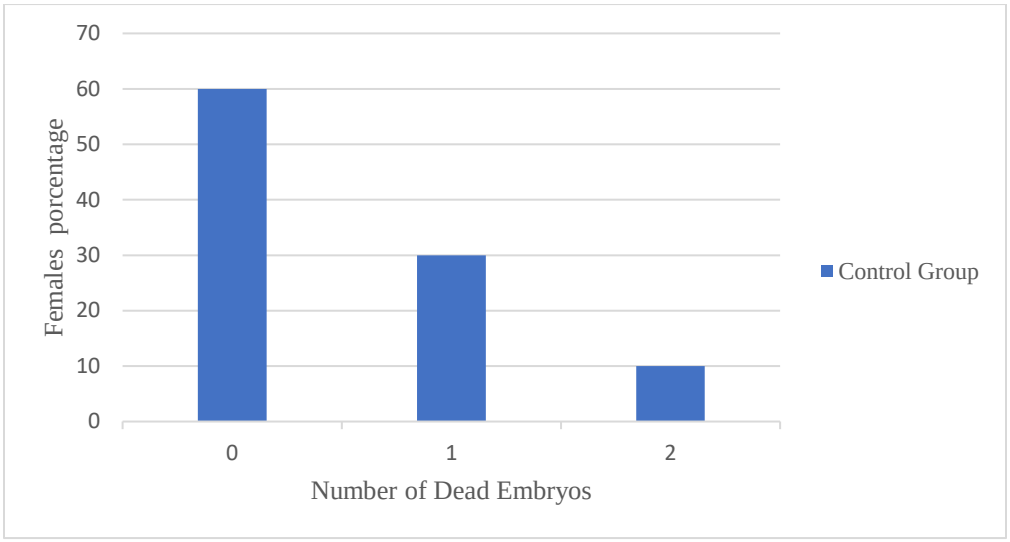


Graph 5. Percentage of female mice of the Cytoreg® group with a determined number of live embryos.

3.3. Quantification of the number of dead embryos in each of the uterine horns of female mice C57BL/6//BIOU

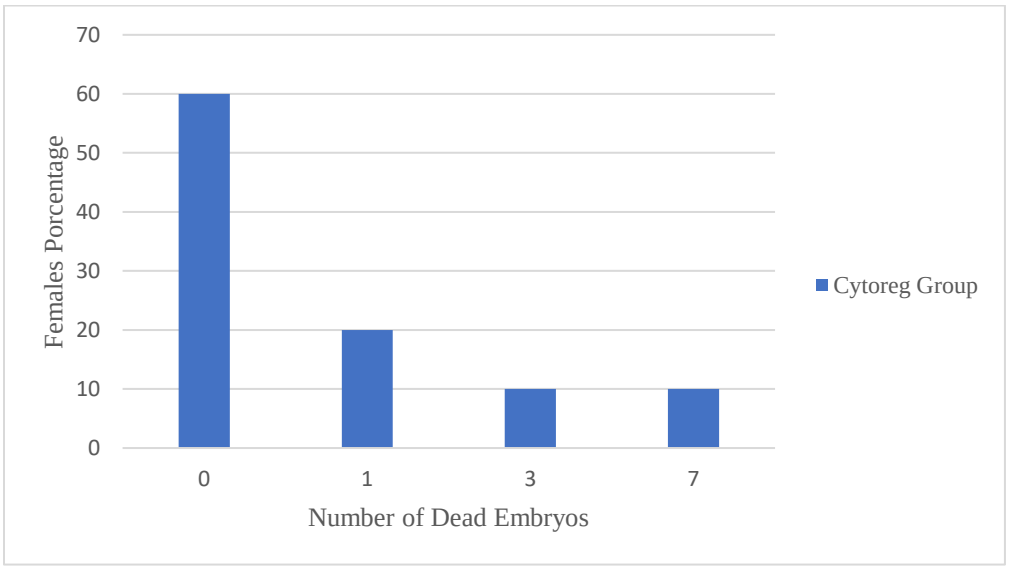
In the results obtained, it was observed that the mice treated with Cytoreg® presented a higher mean of the number of dead embryos compared to the female mice of the control group (1.20 and 0.5, respectively). The statistical analysis did not present, statistically, significant differences ($p = 0.8295$, $p > 0.05$).

In relation to the frequency of dead embryos, in Figure 6 it can be observed that 30% of the females in the control group had a dead embryo and only 10% of the females had two dead embryos. 60% of the females did not present dead embryos.



Graph 6. Percentage of female mice of the control group with a determined number of dead embryos.

In relation to the group treated with Cytoreg® (Graph No. 7) 10% of the females presented a total of seven dead embryos, 20% of the animals had a dead embryo, and only 10% lost three embryos. Both groups had in common that 60% of the animals did not have dead embryos.



Graph 7. Percentage of female mice of the Cytoreg® group with a determined number of dead embryos.

3.4. Quantification of the total number of embryos in the groups of female mice C57BL/6// BIOU

In general, the total embryos for each group (alive and dead) was 56 embryos for control and 45 embryos for the experimental group Cytoreg®, where for both groups four animals were not gestated. Being the total average per female of 5.6 ± 1 and 5.5 ± 4 for the control group and for the group treated with Cytoreg®, respectively.

Table 4 shows a summary of the results related to the statistics of the group of fertility parameters of the female mice of the control group and experimental group or treated with Cytoreg®.

Table 4. Average number of corpora lutea, number of live embryos, number of dead embryos, total number of embryos and number of implantation sites of the experimental groups.

	Group							
	Control				Experimental			
	Average	Typical mean error	Median	Typical Deviation	Average	Typical mean error	Median	Typical Deviation
N_Bodies LCD	5.10	.809	5.50	2.558	4.10	.690	4.50	2.183
N_Bodies LCI	3.20	.533	4.00	1.687	3.20	.573	3.00	1.814
N_Live Embryos	5.10	1.464	6.50	4.630	3.30	1.221	1.50	3.860
N_Dead Embryos	.50	.224	.00	.707	1.20	.712	.00	2.251
N_Total Embryos	5.60	1.579	7.50	4.993	5.50	1.544	7.50	4.882
N_Implatation Sites D	3.40	1.166	2.50	3.688	3.00	.931	3.00	2.944
N_Implatation Sites I	2.40	.702	3.00	2.221	2.50	.764	2.50	2.415

General conclusion.

In the trial conducted, no significant differences were observed between the results of the control groups and the experimental groups treated with Cytoreg®.