FULL ARTICLE

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Disease characterization in cloned foals in Colombia, the effect of placental pathologies on the success and survival of cloned foals from two different cell lines

Caracterização de doenças em potros clonados na Colômbia, o efeito das patologias da placenta no sucesso e sobrevivência de potros clonados de duas linhagens celulares diferentes

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ABSTRACT

Cloned foals born in the different cloning programs in the USA, Argentina, and recently in Colombia have experienced several pathological entities during their pregnancy and neonatal period. These entities include abortions, stillbirths, hypoxic-ischemic encephalopathy, large umbilical remanent, and angular deformity on the fore limb. The objectives of this study were to determine whether cloned foals suffer similar diseases and have the same mortality rate as non-cloned ones and the differences between cloned foals from two different cell lines (fibroblastic and bone marrow cells) and, to establish whether mares carrying cloned foals from different cell lines have ultrasonographic, paraclinical, and hormonal profile parameters equal to traditional pregnancies and to determine whether there are differences between the cloned foals from the two different cell lines. In the analysis of the continuous variables, significant mean differences were established in the three groups of mares in PCV, hemoglobin, erythrocyte count, MCV, MCH, serum proteins and globulins, and in lymphocyte counts $p<0.05$. A risk analysis was made for morbidity and mortality associated with the cell origin of the clone and its pregnancy using chi-square sequential tests and logistic regression, and it was considered statistically significant at $p< 0.05$. A principal component analysis of the paraclinical findings of the mares with the outcome of the foal and the paraclinical parameters of the foals divided by the origin of gestation was performed to evaluate the components of the response variable and their dynamics. Thirty-four pregnancies of clones of fibroblastic origin, 26 of clones of bone marrow origin, and 32 of non-clones were studied. The presentation of placental diseases compatible with placentitis was significantly more frequent ($p= 0.026$) in mares with cloned foals from fibroblastic cell lines. Neonatal sepsis was determined to be the most frequent disease and was diagnosed more commonly in foals of fibroblastic cell origin—an increase in the risk (OR 37) ($p=0.0006$). The risk of death was positive and significant with the fibroblast cell line foals (OR: 9.1. $P<0.01$) compared to the cloned foals of marrow cell origin and non-cloned foals. The risk of death can be predicted in the neonatal period, and some indicators found in this study, such as signs related to placentitis, are associated with increased neonatal mortality and are more frequent in foals of fibroblastic cell origin.

Keywords: Clones. Morbidity. Placentitis. Mares. Mortality.

RESUMO

Potros clonados nascidos em diferentes programas de clonagem nos EUA, na Argentina e, recentemente, na Colômbia sofreram diversas entidades patológicas durante a gravidez e o período neonatal. Essas entidades incluem abortos, natimortos, encefalopatia hipóxica-isquêmica e grandes remanescentes umbilicais e deformidade angular no membro anterior. Os objetivos deste estudo foram determinar que potros clonados sofrem de doenças semelhantes e apresentam a mesma taxa de mortalidade que potros não clonados e se existem diferenças entre potros clonados de duas linhagens celulares diferentes (células fibroblásticas e de medula óssea); e também, estabelecer se éguas portadoras de potros clonados de diferentes linhagens celulares apresentam parâmetros ultrassonográficos, paraclínicos e de perfil hormonal iguais aos de gestações tradicionais, além de determinar se há diferenças entre os potros clonados das duas diferentes linhagens celulares. Na análise das variáveis contínuas foram estabelecidas diferenças médias significativas nos três grupos de éguas no PCV, hemoglobina, contagem de eritrócitos, VCM, MCH, proteínas e globulinas séricas e na contagem de linfócitos

p<0,05. Foi feita uma análise de risco para morbimortalidade associada à origem celular da linhagem celular do clone e sua gestação quando utilizados testes qui-quadrado sequencial e regressão logística e foi considerado estatisticamente significativo p< 0,05. Uma análise de componentes principais dos achados paraclínicos das éguas com o desfecho do potro e os parâmetros paraclínicos dos potros divididos por origem da gestação foi realizada para avaliar os componentes da variável resposta e sua dinâmica. Foram estudadas 34 gestações de clones de origem fibroblástica, 26 gestações de clones de origem medular óssea e 32 gestações de não clones. Verificou-se que a apresentação de doenças placentárias compatíveis com placentite foi significativamente mais frequente (p= 0,026) em éguas com potros clonados de linhagens celulares fibroblásticas. A sepse neonatal foi considerada a doença mais frequente e foi diagnosticada mais comumente em potros de origem fibroblástica – aumento do risco (OR 37) (p=0,0006). O risco de morte foi positivo e significativo com os potros da linha celular de fibroblastos (OR: 9,1. P<0,01) em comparação com os potros clonados de origem celular medular e potros não clonados. O risco de morte pode ser previsto no período neonatal e alguns indicadores encontrados neste estudo, como sinais relacionados à placentite, estão associados ao aumento da mortalidade neonatal e são mais frequentes em potros de origem fibroblástica.

Palavras-chave: Clones. Morbidade. Placentite. Éguas. Mortalidade.

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Introduction

The era of equine cloning started with a mule that resulted from using fetal tissue in Italy (Woods et al., 2003). Since its beginnings, several equine cloning enterprises have been undertaken worldwide (Johnson et al., 2010). However, Argentina has recently led this market, producing more cloned foals with high viability rates, especially those obtained from bone marrow cells (Olivera et al., 2018b). Using nuclear transfer (TN) in cloning horses has resulted in an estimated production of 200 viable specimens worldwide, as Johnson & Hinrichs (2015) reported. Since 2016, cloned foals have been produced for commercial purposes in Colombia. This has been done to genetically conserve high-value specimens in the equine industry and produce athletic horses for sports competitions, mainly polo and jumping (Rojas et al., 2019).

The cloned foals born in the different programs of cloning in the USA, Argentina, and recently in Colombia have had several pathological entities during pregnancy, peripartum, and neonatal period. The entities include early embryo mortality, abortions, stillbirths, and the birth of weak foals (Johnson et al., 2010; Johnson & Hinrichs, 2015). These diseases have been less frequently observed in pregnancies of cloned foals and neonates from bone marrow cells when compared with the ones from fibroblast cells (Olivera et al., 2018a). Several cloning processes have resulted in 65 live cloned foals between two months and three years old in Colombia. These foals were the product of nuclear transfer from fibroblasts or bone marrow cells (Rojas et al., 2019). This previous study also indicated higher viability in pregnancies and cloned foals from bone marrow origin. Similar results have also been reported in Argentina (Olivera et al., 2018a, 2018b). These latest authors argue that the efficiency of nuclear transfer is due to the capacity of the donor cell to be in a totipotent stage commanded by the oocyte recipient. Therefore, the plasticity of the cell is crucial to guarantee the progression of the embryo development and the result in a viable cloned offspring.

Clones' pregnancies have been associated with placental abnormalities in species such as cows, sows, sheep, and mice (Hill et al., 1999). In cattle, cloning is associated with embryo losses and placental abnormalities between days 30 and 90 of pregnancy. The most common placental abnormalities are poor development of placentomes, reduction of the vascular villi, hypoplasia of epithelial trophoblastic cells, and reduction of the number of binucleate cells (Kohan-Ghadr et al., 2008). The study by Hashizume et al. (2002) reports that 25% of pregnancies show placental edema and lengthening of the umbilical cord between days 12 and 120 of gestation. In contrast, in horses, the abnormalities reported include placental edema, increase in size of the uteroplacental unit (UUP), umbilical cord thickening associated with dilatation of the umbilical vessels, umbilical edema, urachus dilatation, deficient lung maturation, among others problems (Johnson & Hinrichs, 2015).

One study in Texas with eight clones from the same individual found clinical and ultrasonographic signs of placentitis during pregnancy. At the time of birth, the inspection of the placenta indicated a wide variety of pathological findings compatible with this pathology, too, manifested by a significant increase in the weight of the placenta, color change and appearance, and streptococcus alfa hemolytic was isolated from the placenta in one case. Seven of the eight foals died or were aborted, showing signs compatible with placentitis, like edema, hemorrhages, and thrombi in the chorioallantoic vessels (Pozor et al., 2016). As indicated previously, all the species that have undergone cloning processes show abnormalities in the placenta. However, the pathophysiology of these abnormalities has not been fully understood. It appears to be associated with aberrant gene reprogramming in the cytoplasm of the recipient cell, resulting in inappropriate epigenetic modification of genes that regulate placental development (Palmieri et al., 2008).

The objectives of this study were to determine whether cloned foals suffer similar diseases and have the same mortality rate as non-cloned ones, and the differences between cloned foals from two different cell lines (fibroblastic and bone marrow cells) and to establish whether mares carrying cloned foals from different cell lines have ultrasonographic, paraclinical, and hormonal profile parameters equal to pregnancies of no-cloned foals and to determine differences between the cloned foals from the two different cell lines.

Materials and Methods

A retrospective study was carried out between April and December 2019 and a prospective one between 2019 and 2022 of mares with pregnancies of 9.5 months forward, their neonatal foals, and the neonatal foals that arrived at a reference center in Cajicá Cundinamarca, Colombia, 2650 meters above sea level. They were classified as illustrated in Graph 1.

Inclusion criteria of mares, their management, and monitoring

The study included recipient mares with pregnancies resulting from somatic cell nuclear transfer that maintained pregnancy until month 9 of pregnancy and started to be monitored at 9.5 months of gestation and mares from traditional pregnancies that arrived for monitoring of parturition at the equine perinatology center in the sampling period.

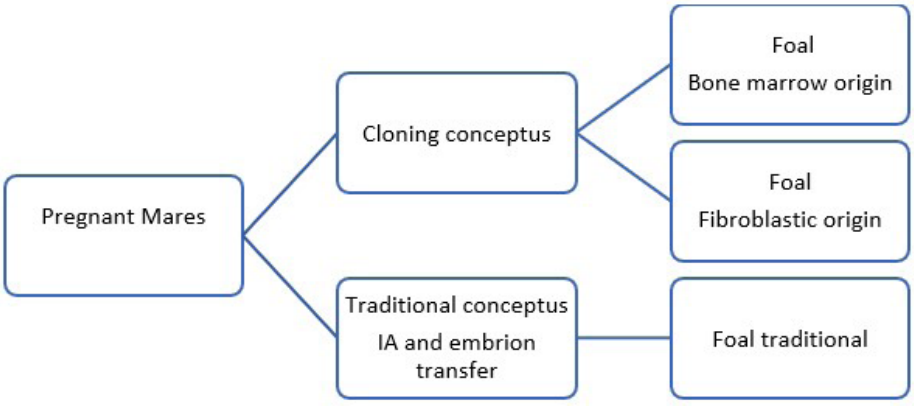
Management and evaluation of mares

The mares underwent a complete, thorough physical, transrectal, and transabdominal ultrasound examination to determine parameters of the viability of the offspring and the state of the placenta where the thickness of the uteroplacental junction, presence of folds in the placenta, areas of edema, detachment placenta and amniotic fluid wedge measurements were evaluated. (Hendriks et al., 2009). Fetal heart rate and activity were checked to determine well-being. Two qualified professionals conducted these tests and developed a unified concept of the parameters (Adams-Brendemuehl & Pipers, 1987). A complete cell blood count and measurement of total plasma protein, albumin, globulin, and fibrinogen levels were done on each mare, and serum progesterone and estrogen levels were also determined (Douglas, 2004).

The evaluation of each pregnancy determined whether the pregnancy was at risk or not.

Classification of high-risk pregnancy: A high-risk pregnancy was defined as one that had at least 3 of the 4 following altered parameters:

- Mares with uteroplacental union greater than 1 mm per month of gestation are evaluated by ultrasound and performed an average in 5 anatomical points of the placenta (Bucca et al., 2005);



Graph 1 – Animal distribution in the study.

- Mares with progesterone levels above the normal ranges for gestational age >10ng/ml (Müller et al., 2019);
- Mares with estrogen levels below the range for gestational age. <800pg/ml (Curcio et al., 2018);
- Mares with hematological alterations compatible with acute (leukocytosis with neutrophilia and hyperfibrinogenemia) or chronic infection (marked increase in serum globulins and albumin-globulin ratio with alterations in leukocyte count or morphology) (Hendriks et al., 2009). These mares were placed in a specific therapeutic management regimen for treating placentitis according to protocols described by Pozor et al. (2016).

Mares in no high-risk pregnancy

Mares with normal ultrasonographic, hormonal, and hematological parameters were considered no high-risk pregnancies. These mares continued to be evaluated by ultrasound every two weeks until the time of foaling to verify that the parameters remained within normal ranges. If signs of abnormality were detected, the corresponding treatment was performed (Pozor et al., 2016).

Monitoring of parturition

The mares were admitted to the perinatology center at 9.5 months pregnant. A routine clinical examination was performed upon admission, including the color of mucous membranes, capillary refill time, pulse quality, heart rate, respiratory rate, intestinal motility, rectal temperature, and fetal movements were taken, and an udder score was used to determine its development. Ultrasounds of each subject were performed with a 7-12 MHz rectal linear transducer and a 2-6 MHz convex transducer to evaluate the uteroplacental junction at the star and in the body and uterine horns, taking the Bucca et al. (2005). During the monitoring time, a routine clinical examination was performed twice daily (morning and afternoon). In addition, fetal movements, fetocardia, and udder development were constantly evaluated to determine the fetus's state.

Foaling care

When the mares showed imminent signs of parturition, phase II assisted parturition was performed, and the extraction was carried out following the rhythm of the mare's contractions.

Expulsion of fetal membranes

After foaling, the mares were expected to expel the fetal membranes naturally. When the membranes were not expelled within 3 h, the retained placenta was diagnosed, and Oxytocin was administered, 20 IU total dose, and maneuvers such as the Burns technique that included que es vessel infusion or uterine lavage as indicated (Meijer et al., 2015).

Evaluation of the newborn, diagnostic tests, and definition of diseases

An APGAR score was performed at one minute and 5 min after birth (Panzani et al., 2012) to determine the risk of perinatal asphyxia and determine the neurological status of each foal. Each foal was dried as best as possible, the navel disinfected with a tincture of iodine, and its ligation performed in cases of extensive bleeding and opening were too great. Help to stand up was given if needed, and assistance to suckle by the foal. In cases where the foals could not suckle, the colostrum was administered to them using a bottle at will or passage of a nasogastric tube.

Intravenous Catheter placement: After standing up and suckling for the first time, all neonate foals were placed in a 14 G intravenous catheter (Nipro) in the jugular vein from which blood samples were taken to evaluate blood gases and acid-base parameters, clinical chemistry tests, and blood cell counts. The clinical diagnosis of the foal diseases was done based on the clinical examination of each foal and the diagnostic tests.

All foals had the following tests performed within the first 3 h after birth: Complete blood count with hematology in the Mindrai BC2800Vet to impedance hematology, with visual hemocytology verification by cell morphology microscopy; AST, GGT, and creatinine plasma levels for spectrophotometry method Mindrai VA77AVET. The venous blood gas evaluation was done using Siemens EPOC® equipment, which includes Ph, PCO_2, PO_2, HCO_3, EB, Na, K, Cl, Ca, anion gap, creatinine, lactate, and glucose. Evaluation of IgG levels was made through the IDEXX Snap test.

Definition of diseases of neonates included in the study

The foals were diagnosed with different entities based on the following definitions according to the scientific literature:

Failure of passive immunity transfer: foals with a history of low colostrum intake in the first hours, with serum IgG levels less than 400mg/dl after 24 h of life (Hofsaess, 2001; Giguere & Polkes, 2005; Franco Ayala & Oliver Espinosa, 2015);

Neonatal sepsis: A clinical diagnosis of acute sepsis or evidence of localization of infection in various organs (gastrointestinal tract, lung, joints, brain), with a sepsis score

greater than 11 required (Brewer et al., 1988); hematology and mandatory serum glucose (Koterba et al., 1984);

Diarrhea: Presence of unformed feces with increased water content and frequency of defecation (Magdesian, 2005). It is classified between severe and mild/moderate where: Severe, classified as an infectious disease, are foals with diarrhea and signs of systemic disease, alteration of body temperature, depression, and loss of the sucking reflex, and mild/moderate include foals with diarrhea, but without systemic involvement where therapeutic intervention is required mandatory Hematology (Wohlfender et al., 2009);

Neonatal maladjustment: Foals that in the first 72 h show neurological signs such as depression, difficulty or inability to gain sternal recumbency, seizures, indifference to the mother, and absent sucking reflex (Katz, 2006); no evidence of infectious compromise, hematology mandatory (Mackay, 2005);

Hypoxic ischemic syndrome: Foals with a low APGAR score <8 with serum lactate levels >5.5mol/Lt, with or without neurological signs and with alterations in the function associated with hypoxia of other systems or organs such as kidney, liver, or intestine (Mackay, 2005; Toribio, 2019);

Prematurity: Foals born before 320 days that present signs such as low birth weight, plush coat, bulging forehead, drooping ears and lips, orthopedic disorders such as tendon and ligament laxity, and respiratory distress with sudden onset between 24 and 48 h after birth (Lester, 2005);

Dysmaturity: Foals born after 320 days or considered at term that present signs such as low birth weight, plush coat, bulging forehead, drooping ears and lips, and orthopedic disorders such as tendon and ligament laxity (Lester, 2005);

Respiratory disease: Foals with clinical signs such as cough, tachypnea, nasal discharge, enlarged submandibular nodes, rales, and/or wheezing. Hematology and response to therapy, if undertaken (Wilkins, 2003);

Respiratory distress syndrome of the newborn: respiratory distress of sudden onset between 24-72 h after birth with restricted lung expansion, wheezing, and symptoms of respiratory distress (Stoneham, 1998);

Omphalitis: Enlargement of the navel with pain, heat, and discharge. Optional Ultrasound and hematology (Lavan et al., 1997);

Persistent urachus: Urine outlet through the umbilicus and ultrasonographic confirmation of connection of the umbilical remnant with the bladder (Lavan et al., 1997);

Angular alterations: Foals with alterations in the normal vertical line of aplomb in any of the joints. Mandatory radiographic study (Trumble, 2005).

Statistical analysis

A descriptive analysis of the numerical variables was made with measures of central tendency and dispersion to the quantitative variables. Frequency and percentage of presentation were used in the descriptive variables. The non-linear variables were categorical for analysis. ANOVA or Kruskal Wallis analysis by cell line and type of pregnancy was considered significant with $p< 0.05$. All factors that were found to be significant would be included in the binomial regression analyses with the dependent variable death or live divided by cell line and non-clone pregnancies to assess the risk of each type of foal becoming ill or dying as dependent variables are considered significant with $p< 0.05$. A principal component analysis of the paraclinical findings of the mares with the outcome of the foal and the paraclinical parameters with the foals divided by the origin of gestation was performed to evaluate the components of the response variable and their dynamics between them. Data analysis was performed using SAS software edition 9.4 and by R foundation for statistical version 4.1.2 and Jamovi 4.3.

Results

Mares

A total of 92 pregnant mares met the inclusion criteria. The study subjects had a mean age of 10±3 years, body weight of 435±97Kg, and body condition of 7±1 /9. The pregnancies were distributed as follows: 34 of fibroblastic origin clones, 26 of bone marrow origin clones, and 32 of non-clones. Significant differences ($p<0.05$) were found in erythrocyte counts, CV, hemoglobin levels, MCCM, MCV, total lymphocyte, monocyte, and eosinophil counts, total plasma protein levels, and globulins among the mares that gestated foals of the different cell origins to the non-cloned foals (Table 1). When performing the logistic regression, a protective relationship was found with the plasma proteins of the mares associated with the bone marrow cell line (OR: 0.020 p 0.026) (0.023-0.78). Hemoglobin, on the other hand, was found to have a positive relationship with the increase of the mortality of the foals born to these mares with an (OR2.27 CI 1.11-4.63 p 0.024). In the analysis of the categorical variables, we found that in mares

with pregnancies of clones of fibroblastic cell origin, the frequency of presentation of placentitis was significantly higher (p= 0.026) (13.68%) than the ones that carried clones of bone marrow origin (5.68%) and the non-clone pregnancies (3.41%) Table 2.

In the principal component analysis, it is evident that the pregnancies from cell lines show different patterns. The fibroblastic cell origin is the most distant from the non-clones. In contrast, those of bone marrow cell origin have points like those of fibroblastic cell origin and non-clones, indicating important differences (Graph 2). Regarding the variables, estrogens have a behavior opposite to leukocytes, as leukocytes were higher, estrogen levels were lower in these animals, serum globulins were also found related to mares that gestated clones of fibroblastic origin along with lymphocytes, hematocrit, and platelets (Graph 2). It can also be seen that the ellipses of the three different origins are opposite, and only the clones of fibroblast cell origin and those of bone marrow cell origin intersect in some factors. However, they are opposite to the non-clones, which only have some factors in common with clones of bone marrow origin.

In the principal component analysis of the variables of the mares in relation to the outcome of the foals (to live or die). A greater distribution of deaths towards the fibroblastic cell origin is observed when compared to those of marrow cell origin and non-clones. It can also be observed that live animals are related to high estrogen values in mares and dead animals with high levels of globulins and progesterone in mares (Graph 3).

Foals

No significant differences in adaptation parameters were found among the three groups of foals regarding time to stand and suckle colostrum, and the average APGAR score was 12.6 ± 1.58, with no significant difference among groups.

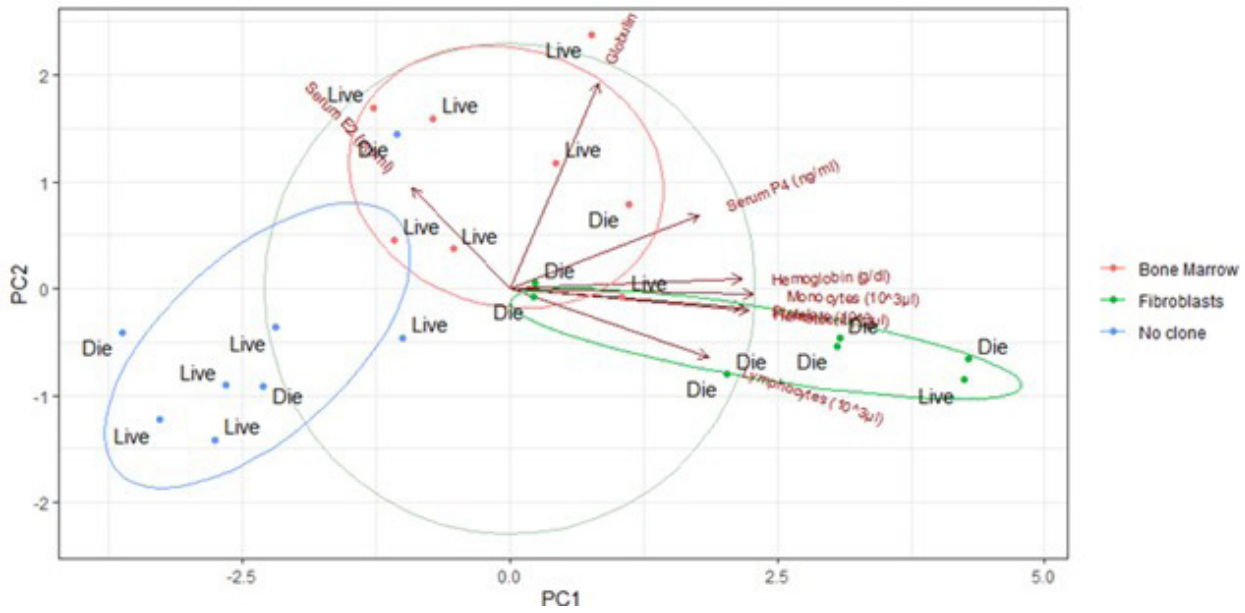
Table 1 – Analysis of the mean difference of the continuous variables of the mares that gestated foals from different origins

	Fibroblastic		Bone marrow		No clon		ANOVA or KW
	Media	DS	Media	DS	Media	DS	P
Erythrocytes (10^6µl)	9.14	1.27	8.46	1.11	7.79[b]	1.10	0.002
Hematocrit (%)	43.07[a]	5.89	38.86[a]	4.52	40.13[a]	5.42	0.011
Hemoglobin (g/dl)	14.67	1.87	13.80[b]	1.48	15.15[b]	1.90	0.021
MCV (fl)	47.33	2.21	46.18[b]	4.51	51.78[b]	4.71	0.001
MCH (pg)	16.17[a]	1.72	16.55[b]	2.42	19.61[ab]	2.23	<0.001
MCHC (g/dl)	34.18[a]	3.37	35.84	3.74	37.91[a]	2.72	0.02
Platelets (10^3µl)	2.41	0.84	14.73	58.27	171.75	54.36	0.32
Leukocytes (10^3µl)	11.61	2.68	12.24	2.25	10.63	3.93	0.134
Neutrophiles (10^3µl)	6.39	2.43	6.45	2.09	6.43	3.46	0.074
Lymphocytes (10^3µl)	4.65	1.91	4.62[b]	2.03	3.55[b]	1.79	0.012
Monocytes (10^3µl)	0.13	0.13	0.09[b]	0.12	0.26[b]	0.21	<0.001
Eosinophils (10^3µl)	0.35[a]	0.26	0.79[ab]	0.58	0.31[b]	0.34	<0.001
Basophiles (10^3µl)	0.04	0.10	0.08	0.10	0.07	0.16	0.237
Bands (10^3µl)	0.01	0.02	0.00	0.00	0.00	0.02	0.51
Total Protein (g/dl)	7.52[a]	0.40	7.89[a]	0.51	7.68	0.66	0.012
Albumin (g/dl)	2.94	0.33	2.73	0.51	3.26	.	0.185
Globulin (g/dl)	4.49[a]	0.44	5.19[a]	0.68	3.19	.	0.002
Fibrinogen(mg/dl)	460[a]	226	643[a]	241	200.00	.	0.044
Seric Estrogens (pg/ml)	651.57	659.70	878.50	901.12	664.33	779.51	0.68
Seric Progesterone (ng/ml)	36.44	21.13	34.87	23.53	45.73	18.29	0.45

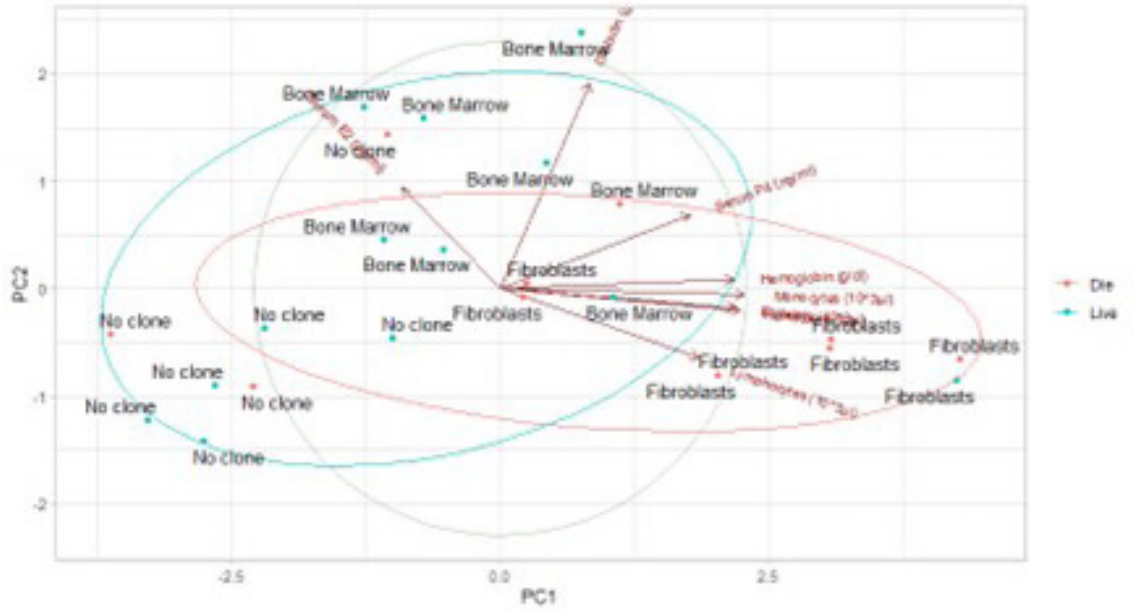
The same letters represent the significant mean difference between the columns p<0.05. DS: Standar desviation, MVC: Medium Corpuscular Volumen, MCH: mean hemoglobin corpuscular, MCHC: mean corpuscular hemoglobin concentration.

Table 2 – Binomial logistic regression with the dependent variable LIVES or DIES the foal in the analysis of the mares' risk factors, considering the cell line of origin of the pregnancy

Predictor	Estimator	Standar error	Z	P Value	Odds Ratio	Confidence interval	
						lower	Superior
Celular LIne:							
Bone marrow – Fibroblastic	3.609	1.4403	2.51	0.012	36.921	2.1942	621.262
Total protein (g/dl)	-2.006	0.9003	2.23	0.026	0.135	0.0230	0.786
Hematocrit(%)	-0.167	0.0975	1.71	0.087	0.846	0.6992	1.025



Graph 2 – Main components analysis of the variables evaluated in the mares concerning the cellular origin of the pregnant foals.



Graph 3 – The main components analyze the variables evaluated in the mares concerning the outcome of living or dying in the foals separated by cellular origin.

A total of 11 foals presented total failure of transfer of passive immunity, and 21 had partial failure of transfer of passive immunity. However, no statistically significant differences were found between groups, possibly indicating no greater risk due to the cellular origin of the foals. Table 3 summarizes the factors that were evaluated and those that found statistically significant differences when comparing the cellular origins of the foals. The factors that found differences were total erythrocyte counts, MCV, MCH and MCH, albumin, and anion gap. In the qualitative analysis, neonatal sepsis was determined to be the most frequent disease, and it was diagnosed more frequently in foals of fibroblastic cell origin (20 of 34) compared to those of medullary cell origin (7 of 26) and non-clone (4 of 32). An increase in the risk (OR:37) (p=0.0006) of septicemia was determined in foals of fibroblast origin. There is evidence of a highly significant association between the primary diagnoses and the groups, those of fibroblastic cell origin, the ones with the highest incidence of septicemia vs the other two groups.

When carrying out the risk of death analysis, it was positive and significant with the fibroblast cell line (OR: 9.1. P<0.01) in comparison with the cloned foals of marrow cell origin and non-cloned foals. According to the estimators (the non-clone animals are compared against the absent

Table 3 – Analysis of the difference of means of the continuous variables of the foals of fibroblast cell origin, bone marrow, and non-cloned foals

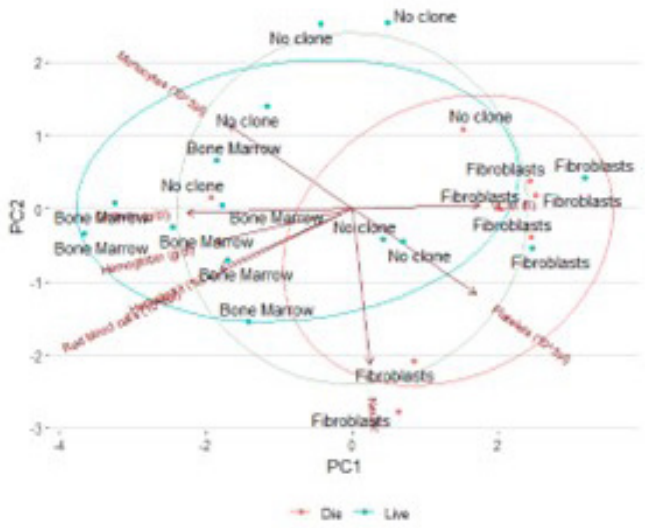
	Fibroblastic		Bone Marrow		No clon		ANOVA or KW
	Media	DS	Media	DS	Media	DS	p
Weigth Foal (kg)	36.84	8.47	36.52	8.90	35.50	12.60	0.884
Heart Rate	125.31	25.99	125.68	31.63	128.03	28.59	0.97
Breath Rate	44.07	10.78	40.64	16.28	44.69	16.93	0.708
Temperature (°C)	37.12	1.53	37.82	0.52	37.61	0.62	0.065
Erythrocyte (10^6µl)	10.36[a]	1.50	9.92[b]	1.44	10.35	10.10[ab]	0.001
Hematocrit (%)	39.93	7.64	39.36	5.69	37.89	4.46	0.55
Hemoglobin (g/dl)	13.11	2.34	13.02	3.07	13.86	2.32	0.26
MCV (fl)	38.51[a]	3.50	39.71[b]	3.10	42.19[ab]	11.84	<0.001
HCM (pg)	12.66[a]	1.06	13.41[b]	2.63	21.24[ab]	28.75	<0.001
MCHC (g/dl)	33.02[a]	3.04	33.73	5.70	36.80[a]	6.60	0.004
Platelets (10^3µl)	241	110.59	201	83.8	247.17	53.94	0.507
Leukocytes (10^3µl)	8.22	4.23	8.09	3.44	8.09	3.48	0.85
Neutrophiles (10^3µl)	6.78	3.95	6.27	2.93	6.35	3.40	0.97
Lymphocytes (10^3µl)	1.34	0.61	1.77	1.05	1.56	0.63	0.159
Monocytes (10^3µl)	0.06	0.11	0.09	0.12	0.13	0.13	0.103
Eosinophils (10^3µl)	0.04	0.15	0.02	0.04	0.02	0.05	0.674
Basophiles (10^3µl)	0.00	0.00	0.00	0.00	0.00	0.00	0.367
Bands (10^3µl)	0.04	0.16	0.02	0.06	0.07	0.19	0.36
Total Protein (g/dl)	5.19	0.72	5.24	0.80	5.22	0.75	0.716
Albumin (g/dl)	2.06[a]	0.52	2.44	0.60	2.54[a]	0.22	0.028
Globulin (g/dl)	3.04	0.86	2.81	1.18	2.05	0.68	0.059
Fibrinogen(mg/dl)	521.05	364.51	643.75	242.42	420.00	109.54	0.283
AST (u/l)	233.02	130.88	167.25	100.09	102.40	.	0.161
GGTP (u/l)	56.36	65.70	41.26	31.06	16.15	7.71	0.343
Na (mmol/L)	137.10	5.00	135.47	5.70	138.63	6.79	0.29
K (mmol/L)	3.83	0.66	4.21	2.08	4.19	1.62	0.93
Ca (mmol/L)	1.24	0.25	1.16	0.41	1.20	0.32	0.993
Cl (mmol/L)	102.80	4.13	101.89	4.93	103.43	5.48	0.649
AGap (mmol/L)	10.90	5.63	8.44[b]	2.57	12.86[b]	6.75	0.014
AGapk (mmol/L)	14.90	5.45	12.65	2.18	15.37	4.4	0.05
Glucose (mg/dL)	146.70	65.59	126.83	61.44	114.46	36.5	0.254
Lactate (mmol/L)	6.41	4.32	4.20	2.20	5.67	3.21	0.076
Creatinine (mg/dL)	2.23	0.58	2.18	0.98	2.79	1.39	0.209
pH Blood	7.34	3.46	7.35	0.06	7.342	0.06	0.85
pCO_2 Blood (mmHg)	41.53	5.12	44.21	7.18	12.62	1.78	0.519
pO_2 Blood (mmHg)	32.05	5.14	34.34	12.18	-1.02	3.22	0.228
BE(ecf) Blood (mmol/L)	-2.30	4.01	-0.39	2.09	-0.85	3.56	0.803

The different letters represent significant differences between the columns $p<0.05$. DS: Standar desviation, MVC: Medium Corpuscular Volumen, MCH: mean hemoglobin corpuscular, MCHC: mean corpuscular hemoglobin concentration, AST: aspartate aminotransferase, GGTP: gamma glutamyl transpeptidase.

group), it was shown that there is a greater probability that the foals will die if they are from the fibroblast cell line OR 4.4 (CI 1.03-17) vs. if they are not cloning. However, for the bone marrow line, the difference between the probabilities was not significant OR: 0.38 (CI: 0.038-3.95).

In the analysis of the principal components of the variables evaluated in the foals, it is evident that the cell lines show different patterns; the fibroblastic cell origin is entirely different from bone marrow and not clones. The monocyte count variable has a behavior opposite to the platelet count; the VCM has an opposite direction to hemoglobin and hematocrit, and the differential variables for the fibroblast line, for its part, globulins, hemoglobin, hematocrit, and red blood cells are associated with the bone marrow cellular origin (Graph 4).

In the analysis of the principal components of the variables evaluated in the foals with the response variable live or die, an association with the group of fibroblastic cell origin is evidenced, and in turn, the main components are the vectors of high platelets, high MCV, and increased neutrophils and in the ellipse of the foals product of cloning of fibroblast cell origin, the living ones are in the ellipses of bone marrow cell origin and the non-clones and with the vectors of high globulins, hematocrit and hemoglobin.



Graph 4 – Main components analysis of the foal variables with their cellular origin.

Discussion

This study included many clones. Sixty were followed from 9.5 months of gestation and were allocated based on their cellular origin. The clones were obtained from fibroblasts and bone marrow cells. This study describes the findings during the last month and a half of gestation and the neonatal adaptation processes, performing a detailed analysis of the clinical and paraclinical findings of these animals from two different cell origins and compared with non-clone foals. Previous reports have been limited to describing foal diseases (Johnson & Hinrichs, 2015) or gestational diseases (Pozor et al., 2016). However, a more recent study by Olivera et al. (2018b) described the abnormalities of foals according to their cell line of origin and previously to it without describing the abnormalities of the mares. On the other hand, Lagutina et al. (2005) described some viability characteristics according to the origin of the donor cell in a study that included a small number of cloned animals.

A convenience sample of cohorts of mares with gestations was used in the current study. They were divided into traditional pregnancies (n=32), pregnancies with cloned foals of fibroblastic cell origin (n=34), and pregnancies of cloned foals of bone marrow cell origin (n=26). An increase in gestational abnormalities was found in mares that gestated clones of fibroblastic cell origin. A statistically significant difference ($p<0.05$) was shown in the presentation of gestational abnormalities compatible with placentitis compared to those that gestated clones of bone marrow origin and traditional pregnancies. These findings agree with the ones Olivera et al. (2018b) reported, who observed greater viability in foals produced from bone marrow. However, they did not describe the behavior of pregnancies that resulted in the clone foals. In our study, we also report the risk factors that can serve as predictors of viability or as indicators of the severity of the gestational pathology of the foals produced by cloning, depending on the cellular origin of fibroblasts or bone marrow. A report by Pozor et al. (2016) described the gestational abnormalities of 8 mares that gestated cloned foals of fibroblast cell origin with changes compatible with placentitis from which only one viable foal was obtained. However, it does not evaluate the factors associated with pregnancy in the mare and is limited to animals from a single fibroblast cell line.

In this study, significant differences are reported between the values of the erythrocyte indices (MCV, Hb, MCH, MCHC), erythrocyte count, and hematocrit of the mares that gestated clones compared to non-clones, which have higher erythrocyte indices. In the white cell line, the lymphocyte count was increased in the mares that gestated foals with a bone marrow cell origin compared to the non-clones ($p = 0.012$). When performing the regression analysis, it was found that the increases in hemoglobin values were associated with an increase in the mortality of the foals of these mares (OR2.27 IC 1.11-4.63 p 0.024), possibly associated with anemic chronic processes related to placentitis that be described in mares that gestated foals. A protective relationship was also found for the levels of globulins and total proteins (OR: 0.020 p 0.026 (0.023-0.78), which are associated with an increase in serum globulin levels in these mares, possibly related to inflammatory responses directed to the uterus in these mares. This is the first report analyzing risk factors in mares with clone pregnancies. When the principal component analysis was carried out, it was observed that pregnancies of fibroblastic cell origin manifested by an increase in serum globulins, lymphocyte counts, and platelet counts. These alterations might be associated with chronic inflammatory responses related to the type of concept these mares carried, implicating that it could be associated with an immune response of the mare to the concept, thought to be mediated by lymphocytes. This type of response has been described in bovine clones. It has even been associated with an exaggerated response of the major histocompatibility complex type 1 and with embryonic loss and abortions in bovines as a result of nuclear transfer (Hill et al., 2002). In horses, exacerbated inflammatory responses have been characterized in gestations when the expression of MHC-1 proteins is increased, and these responses generate increased retention of fetal membranes compared to animals with reduced expression of the MHC-1 2βM protein (Rapacz-Leonard et al., 2018).

Serum estrogens were observed to be in the opposite direction to globulin levels and lymphocyte counts when the significant component analysis was done. Lower levels in these animals and low estrogen levels are associated with underdevelopment of the fetal gonads. This reduction in estrogen levels is associated with fetal stress (Canisso et al., 2015), intrauterine growth restriction, and foals with less viability in the postnatal period and has been associated with low levels of blood estrogens (Canisso et al., 2015) in foals that are dependent on the levels of these hormones in the mare and high levels of progestogens have been associated with critically ill foals in recent studies (Dembek et al., 2023),

It was also possible to determine a greater risk of presentation of placentitis associated with the cell line, specifically with those from fibroblasts. This is the first report to make this risk relationship. An increase in the risk of death OR 4.4 (CI 1.03-17) for foals with fibroblast cell origin was possibly associated with placentitis symptoms in the mares that were pregnant with these foals.

Several of the factors evaluated in the foals' adaptation processes were insignificant. However, an increase in the risk of neonatal septicemia could be seen in foals from the fibroblast cell line. This coincides with the findings found in the mother mares of these foals, who have a higher risk of presenting placentitis. In the study by Olivera et al. (2018b), where they reported the differences between the foals of the two cell lines show a higher score of poor neonatal adjustment, septicemia, and days of hospitalization, significant differences were found in the erythrocyte indices and erythrocyte count in the cloned foals in relation to the non-clones regardless of their cell line ($p<0.05$), with the non-clone foals having the highest values, which could suggest a hypothesis where the clones have a deficiency in the production of erythrocytes and their maturation processes secondary to the processes that they suffered in the peripartum.

The main components analysis associated with the mortality of foals related to their cellular origin, a strong trend towards vectors with the ellipse of pregnancies of fibroblastic cellular origin was evident as observed in Graph 3. A paired risk analysis indicated an increase in the risk of death OR 4.4 (CI 1.03-17) in the fibroblast cell line when compared with foals of bone marrow cell origin OR: 0.38 (CI: 0.038-3.95), confirming different behavior of this cell line. This study generates information on the increased risk of death in foals depending on their cellular origin. In the study by Olivera et al. (2018b), they found a lower viability rate in foals of fibroblastic origin of 52.9% for a total of 22 viable foals compared to those with bone marrow with 54 viable foals, which corresponded to 95.2% of the foals. Regarding implanted embryos, this latest research group argues that the efficiency of nuclear transfer is based on the ability of the donor cell to be in a totipotent state commanded by the recipient oocyte. Therefore, the cell's plasticity is crucial to guarantee the progression of embryo development and viable cloned offspring (Olivera et al., 2018a).

This study evaluated neonatal adaptation parameters such as postpartum time to stand up, time to first colostrum intake, and APGAR test score of cloned foals. However, no significant difference was found among the groups. The values observed are important information for standardizing the adaptation parameters of cloned foals. It was also possible to estimate the presentation of failure of immunoglobulin passive transfer. Regarding partial failure of passive transfer according to the cell line, no significant differences were observed compared to traditional gestation foals. This could have been because all foals were born in a neonatal clinic facility, and close monitoring and care were provided, guaranteeing the administration of colostrum once they were born. However, these data might be valuable in generating animal preventive measures. In the study by Johnson & Hinrichs (2015), all 20 foals were administered hyperimmune plasma as a preventive protocol for neonatal sepsis, and IgG levels were not evaluated in these animals. The study by Olivera et al. (2016) did not analyze this important parameter either.

Regarding the most prevalent diseases in the groups of foals studied, the one with the greatest presentation was neonatal sepsis, which was overrepresented in the foals of fibroblastic cellular origin. This finding contrasts with the findings by Olivera et al. (2018a) in a population of 38 cloned foals of bone marrow cell origin and fibroblasts that found significantly high numbers of neonatal maladjustment and angular deformities in cloned foals of fibroblastic cell origin versus those of bone marrow. In the study of 14 cloned foals by Johnson et al. (2010), the most prevalent diseases were the thickening of the umbilicus, neonatal maladjustment, and angular deformities.

The analysis of the principal components in the foals showed that the foals' fibroblastic cell origin is completely different from that of marrow and no clones. The foals of fibroblastic cell origin had an increase in platelet counts, VCM, and other variables strongly associated with neonatal sepsis (Corley et al., 2005).

The main limitations of this study included the purposive sample, given that it was limited to a single reference medical center where cloned foals were received from a company dedicated to commercial cloning, and the lack of information on variables such as embryo handling that can influence

the final results. Another limitation was some individuals' lack of diagnostic tests due to their unavailability at birth. This did not allow statistical analysis or inference due to missing data in the sample.

Conclusions

The presentation of abnormalities in pregnancies, perinatal disease, and foal death is related to the embryo's original cell line. The cloned foals from fibroblastic cell origin had the highest risk for mortality, perinatal disease, and pregnancy abnormalities. In contrast, the pregnancies of foals of bone marrow origin and the no-cloned pregnancies had similar risks.

Foals of fibroblastic cell origin were at high risk of developing neonatal septicemia, and this was associated with a previous presentation of placentitis in the mare.

The risk of suffering neonatal septicemia is more associated with foals of fibroblastic cell origin and is associated with an increase in the presentation of placentitis in mares.

Analyzing these pregnancies' histopathological and molecular characteristics is necessary to establish a pathophysiology that identifies the processes by which these individuals exhibit this behavior.

Limitation

Limitations of the study: The study was done only in one perinatal center, there was no control of the process of cloning and the selection of the mares, and there was a limited number of animals in the study.

The positive aspect of the study: This is the first time a comparative study has been done; the risk of disease was evaluated, and more common diseases of the clones were reported.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethics Statement

It was not necessary to submit it to the ethics committee as it was a case seen in the routine of a veterinary service.

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