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Hematology and clinical chemistry in mule foals from birth to two months of age: A preliminary study

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ABSTRACT

In horses and donkeys, age-related changes in hematological and biochemical parameters preclude the use of normal values of adults in the evaluation of foals. This study aimed to obtain data on hematological and biochemical parameters of mule foals from birth up to the second month of life and to assess age-related changes in order to determine if dedicated reference ranges are required in younger animals. Blood samples from seven healthy mule foals were obtained at birth before colostrum consumption, 24 h, 48 h of life, and then weekly until the second month of life. Results were expressed as mean and standard deviation or median, minimum, and maximum values if showing non-gaussian distribution. Kruskal-Walls and Dunn tests were used to verify the differences among sampling times. Significance was set at $P < 0.05$. Red blood cell count, packed cell volume and hemoglobin decreased from 24 h to one week of age. Mean corpuscular volume and mean corpuscular hemoglobin decreased over the first month. White blood cells increased from birth to seven days of life. Aspartate amino transferase increased while alkaline phosphatase decreased in the first week of life. Urea, creatinine, and lactate decreased, while glucose concentrations increased at 24 h. Ionized calcium and magnesium and total sodium and potassium showed no changes. In mule foals, several laboratory parameters may be the same or intermediate, lower or higher than in equine or donkey foals, but also compared to all other adult species. The preliminary results suggest that for mule foals, age influences hematological and biochemical parameters.

1. Introduction

Mules are horse-donkey hybrids (*Equus asinus* x *Equus caballus*) that have existed since ancient times and have been used in agriculture and transport because of their strength as work animals, and also for competitive sports [1,2]. The mule is a healthy but sterile hybrid offspring with unique chromosomal (63) and physiological characteristics. Intraspecific and interspecific differences are of considerable importance and may include changes in phenotypic, behavioral, or metabolic patterns, leading to variations in the most commonly used hematological and biochemical clinical reference parameters [1]. Therefore, data generated specifically for the horse, such as the reference range of laboratory parameters and drug doses, may not be directly applicable to non-horse equids [2].

In newborn animals, crucial adaptations to the extrauterine environment occur; including the transition from fetal to adult circulation, the onset of pulmonary respiration, and enteral nutrition. For the entire perinatal period, foals are physiologically unstable and more susceptible to infections and metabolic diseases. In fact, successful transition from the fetal to the neonatal state involves physiological adaptations on the part of both the newborn foal and the mare.

The interpretation of clinical pathology data requires knowledge or evaluation of what is considered normal, or associated with health, in the animal of interest [3–5]. In mules, the low numbers of the population and their demographic spread [1] pose challenges to the generation of appropriate reference intervals (RIs) as well as the interpretation of the clinical pathology findings.

Several recent papers have aimed to establish benchmarks for hematological and biochemical data for adult mules [3–7]. However, the RIs for adults cannot be used in foals, because hematological and biochemical parameters vary depending on age [6,8,9].

To the best of our knowledge, no studies have been carried out on the hematological and biochemical parameters in mule foals, except for lactate and blood glucose values [8]. This preliminary study thus aimed to evaluate the hematological and biochemical parameters in a cohort of mule foals from birth to two months of life.

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2. Material and methods

2.1. Animals

Twelve Italian Trotter mares were artificially inseminated (AI) with fresh Amiata donkey semen and became pregnant. The mares were housed in collective paddocks (6–8 animals/each) during pregnancy. Close to parturition (between 10 and 15 days before the presumptive date), mares were stabled in individual 6 × 6m boxes until two-week post-partum, when the dams and foals then returned to smaller paddocks (6 × 6 m). The dams were fed during pregnancy and post-partum period in accordance with NRC energy recommendations [45 NRC], [10] and underwent regular deworming and a vaccination program against common equine pathogens in accordance with the guidelines of the AAEP Infectious Disease Committee. Finally, mares were submitted to periodic clinical examinations during the pregnancy and postpartum periods.

Foals were housed with their dams in boxes until the second week of age and included in the study if they met the follow criteria: (1) unassisted delivery; (2) Apgar score ≥7, 5 min after birth [11]; (3) IgG ≥800 mg/dL at 24 h of age (Snap Foal IgG test Kit, Idexx, USA) [12]; (4) clinically healthy before every blood sample based on clinical examinations performed at birth and weekly.

Approval for this study was obtained from the Ethical Committee of the University of Pisa (22 and 26/19).

2.2. Sampling procedures

Blood samples were obtained from the jugular vein at birth (T0) before colostrum intake and weekly for two months, from 1 to 8 weeks of life. To prevent alterations related to diurnal variations, blood samples were collected at the same time each day (8:00–9:00 am) in EDTA and serum tubes per mule foals. The EDTA samples were kept refrigerated and analyzed within 4 h using an automated cell counter (ProCyte Dx®, Idexx Laboratories, USA) to determine a complete blood count (CBC). The serum tubes were centrifuged at 4032 g force for 8 min, then serum was collected in Eppendorf tubes, stored at -20 °C and analyzed in a single batch.

The biochemical parameters obtained using an automatic analyzer (Sat450®, Assel S.r.l., Italy) and according to the manufacturer's guidelines were aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatine phosphokinase (CK), gamma-glutamyl-transpeptidase (GGT), glutamate dehydrogenase (GLDH), triglyceride (triglycerides), albumin (ALB), total protein (TP), creatinine (CREA), blood urea (BUN), total, direct, and indirect bilirubin.

Lastly, an aliquot of blood was also drawn with a blood gas syringe (Monovette®, Sarstedt, Italy), and immediately processed for electrolytes (total chlorine, potassium and sodium, and ionized calcium and magnesium), glucose and lactate concentrations using a hemogas analyzer (Stat Profile Primevet® analyzer, Nova Biochemicals, USA).

2.3. Statistical analysis

Distribution was assessed using the Shapiro-Wilk test. On the basis of data distribution, the results were expressed as the average and standard deviation if showing a gaussian distribution; or median, minimum, and maximum values if showing a non-gaussian distribution.

Since data showed both gaussian and non-gaussian distributions, a non-parametric test was performed. Post hoc Kruskal-Walls and Dunn tests were thus used to verify the differences between sampling times for each parameter analyzed.

Significance was set at $P < 0.05$. Statistical analysis was performed using commercial software (GraphPad Prism 9, USA).

3. Results

Of the 12 pregnant mares, four aborted at different pregnancy stages, while eight mule foals (4 fillies and colts, respectively) were born at term with non-induced and not assisted eutocic delivery. At birth, one mule foal did not meet the inclusion criteria (Agar score 6/8, exhibited neurological signs, mandibular prognathism, failure of transfer of passive immunity), and thus was excluded from the study.

Hematological and biochemical parameter results are reported in Tables 1 and 2, respectively. RBC, HGB and PCV values decreased significantly after one week of life and remained so up to two weeks of life, then increased at four weeks and remained constant throughout the study period. MCV and MCH decreased from birth to 8 weeks of age. RDW increased starting from two weeks of life until the end of the study period. WBC increased from birth to seven days of life, then remained constant.

AST values increased and ALP values decreased until one week of life, then remained constant until the end of the study period. Creatinine decreased from birth to one week of life, and then remained constant until the end of the study period. Urea concentration decreased starting from one week of life and then remained constant until seven weeks, when the concentration then increased to reach the values observed at one week of life. A significant increase in blood glucose was observed from birth until one week of life, when it then decreased and remained constant, approaching the values measured in adults.

Ionized calcium and magnesium and total sodium and potassium concentrations showed no statistical differences over time.

4. Discussion

Interpreting hematology and clinical biochemistry values in foals in the first months of life is critical, and should not be based on the reference ranges of adult parameters. Physiological differences in foals determine the deviation of values from normal adult reference ranges [13].

This study aimed to assess the hematological and biochemical parameters in a cohort of seven mule foals from birth to two months of life. To the best of our knowledge, this is the first report on the hematological and biochemical parameters in mule foals during the first two months of life.

In mule foals, the decrease in RBC, HGB and PCV until four weeks agrees with findings reported in equine [14–17] and donkey foals [18, 19] of the same age. The increase immediately after birth could be due to the effect of placental blood transfusion and the physiological stress of birth which induces splenic contraction [20]. Otherwise, the subsequent decreases may be related to the hemodilution due to the blood volume expansion after colostrum intake, the physiological destruction of erythrocytes in the spleen, and the decrease in erythropoietin production secondary to the increase in blood oxygenation from the lungs after birth [21]. The decrease in PCV after birth may also be related to fluid displacement, as observed in human infants, and proposed in equine foals [22]. Starting from three weeks of age, the RBC, PCV and HGB values were within the reference range reported for adult mules, which is more similar to the reference range of horses rather than donkeys and hinnies [11,23,24].

A progressive reduction in MCV and MCH was observed over two months, while MCHC remained constant after birth. These results agree with the trend found in equine foals [14–17], while no changes over time have been reported in donkey foals [18,19]. MCV reduction could be associated with the destruction of fetal erythrocytes from the bloodstream and the increasing production of microcytes, resulting in microcytosis and mild anisocytosis. The MCH trend tends to follow findings observed for MCV [25]. Moreover, values in mule foals are lower than in adult mules [22,23].

In mule foals, as in donkey foals [19], the RDW values were higher than in adults of the same age [24,26], supporting the hypothesis of the

Table 1
Hematological parameters from birth to eight weeks of life in the cohort of mule foals ($n = 7$). Values are expressed as mean (X) ± standard deviation (SD) or median (minimum-maximum) values.

	T0 ($n = 7$)	T1w ($n = 7$)	T2w ($n = 7$)	T3w ($n = 7$)	T4w ($n = 7$)	T5w ($n = 7$)	T6w ($n = 7$)	T7w ($n = 7$)	T8w ($n = 7$)	*p*
RBC (M/μL)	9.7 ± 0.86^{a,c}	7.1 ± 1.2^{b}	7.8 ± 0.7^{a,c}	8.3 ± 1.2^{a,b,c}	9 ± 0.9^{a,b,c}	9.6 ± 0.7^{a,c}	9.2 ± 1.1^{a,b,c}	9.7 ± 0.8^{a,c}	9.6 ± 0.8^{a,c}	*P = 0.0001*
PCV (%)	44.6 ± 3.5^{a}	28.9 ± 4.3^{b}	30.3 ± 4.4^{b}	31.9 ± 5.9^{b}	34.1 ± 4.2^{a,b}	36.3 ± 3.5^{a,b}	34.7 ± 3.6^{a,b}	35 ± 1.6^{a,b}	34.9 ± 2.7^{a,b}	*P = 0.0002*
HGB (g/dL)	14.8 ± 0.8^{a}	10.5 ± 1.4^{b}	11 ± 1.4^{b}	11.4 ± 1.8^{b}	12.3 ± 1.2^{a,b}	13.2 ± 1^{a,b}	12.4 ± 1.2^{a,b}	12.8 ± 0.9^{a,b}	12.7 ± 0.8^{a,b}	*P = 0.0001*
MCV (fL)	47.1 ± 2.7^{a}	41.4 ± 3.4^{a,b}	38.8 ± 3.1^{a,b}	36.5 ± 5.7^{b}	38.1 ± 3.2^{a,b}	37.9 ± 4^{a,b}	37.7 ± 3.3^{a,b}	36.3 ± 2.7^{b}	36.3 ± 3.2^{b}	*P = 0.0010*
MCH (pg)	15.2 ± 0.8^{a}	15.0 ± 1.1^{a}	14.2 ± 1^{a,b}	13.8 ± 0.8^{a,b}	13.7 ± 0.7^{a,b}	13.7 ± 0.9^{a,b}	13.5 ± 0.8^{a,b}	13.2 ± 0.6^{b}	13.2 ± 0.7^{b}	*P = 0.0008*
MCHC (g/dL)	32.2 ± 0.8^{a}	36.3 ± 0.9^{b}	37.1^{b} (35-37.6)	36.4 ± 1.6^{a,b}	36 ± 1.4^{a,b}	36.4 ± 1.7^{b}	35.7 ± 1.2^{a,b}	36.4 ± 1.4^{b}	36.9^{a,b} (33.1-37.2)	*P = 0.0091*
RDW (%)	30 ± 2.5^{a}	28.4 ± 3.2^{a,b}	32.2 ± 3.8^{a,b}	34.8 ± 3.9^{a,b}	35.3 ± 4.1^{a,b,c}	35.7 ± 4.8^{b,c}	35.2 ± 4.7^{a,b,c}	36.5 ± 3.2^{b,c}	36.2 ± 3.4^{b,c}	*P = 0.0018*
WBC (K/μL)	6.2 ± 1.9	8.1 ± 1.9	8.9 ± 2.3	10.2 ± 3.2	9.8 (7.8-17)	8.1 ± 2.4	9.4 ± 1.2	8.2 ± 2.4	8.9 ± 2.5	*P = 0.1331*
NEU (K/μL)	4.1 ± 1.7	5.2 ± 1.3	4.3 ± 1.3	4.4 (4.3-12.2)	5 (4.4-12.2)	5.8 (4-13.1)	5 ± 0.9	5.2 ± 1.3	5 ± 0.9	*P = 0.2982*
LYM (K/μL)	1.8 ± 0.4^{a}	2.2 ± 0.7^{a}	2.9 ± 0.6^{a,b}	3.3 ± 1^{a,b}	3.6 ± 1^{a,b}	3.5 ± 1.1^{a,b}	3.5 ± 0.9^{a,b}	4.1 ± 1^{b}	4.1(2-5)^{b}	*P = 0.0003*
MONO (K/μL)	0.2 ± 0.1^{a}	0.6 ± 0.2^{b}	0.7 ± 0.5^{a,b}	0.5 ± 0.1^{a,b}	0.6 ± 0.2^{b}	0.5^{a,b} (0.3-1.2)	0.6^{b} (0.4-4.9)	0.6 ± 0.2^{b}	0.5 ± 0.2^{a,b}	*P = 0.0185*
EOS (K/μL)	0^{a,c} (0-0.02)	0.1 ± 0.03^{a,c}	0.1^{a,b,c} (0-0.2)	0.1 ± 0^{a,b,c}	0.1 ± 0^{a,b,c}	0.2 ± 0.1^{b,c}	0.1^{b,c} (0-2.8)	0.3 ± 0.2^{b}	0.3 ± 0.2^{b}	*P<0.0001*
BASO (K/μL)	0 (0-0.5)	0 (0-0.2)	0(0-0.2)	0.1 ± 0	0.1 ± 0.1	0.1 ± 0	0 (0-1.8)	0 ± 0	0 ± 0	*P = 0.3898*
PLT (K/μL)	256.7 ± 62.3	211.3 ± 92.8	286.3 ± 166	276.1 ± 110.9	299.4 ± 96.9	326 ± 71.9	293.4 ± 29.4	225.1 ± 55	257.3 ± 57.1	*P = 0.1905*

Legend - T0, birth; T1w, 1 week; T2w, 2 weeks; T3w, 3 weeks; T4w, 4 weeks; T5w, 5 weeks; T6w, 6 weeks; T7w, 7 weeks; T8w, 8 weeks of age.
RBC, total red blood cell count; PCV, packed cell volume; HGB, hemoglobin concentration; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cells distribution width; WBC, total leucocyte count; NEU, neutrophils; LYM, lymphocytes; MONO, monocytes; EOS, eosinophils; BASO, basophils; PLT, total platelet counter; PDW, platelet volume distribution width; MPV, mean platelet volume; PCT, plateletcrit.
Different superscripts denote a significant difference (a≠b≠c) within the same row.

presence of smaller red blood cells.

The PLT value at birth was higher in mule foals than donkey and equine foals [7,15,18,19,26,27]because PLT may be less reactive to aggregation induced by adenosine diphosphate (ADP) and collagen during the first week of life [28].

We found that the WBC count at birth in mule foals was similar to equine [14], but higher compared with donkey foals [18,19]. The WBC increased during the first week of life when values were comparable to adult mules [24]. The increase might be related to the cortisol surge immediately after birth within 30 min and the subsequent higher bone marrow production related to environmental exposure [18]. NEU showed an increase over time in mule foals, similar to equine foals [15], although showing lower values; this is in contrast with donkey foals in which no increase has been reported [19]. The LYM value in mule foals increased after birth, in line with donkey foals [19] but higher than equine foals [15], in which LYM has shown lower values after birth [15,25]. At two months of life, LYM values were similar to adult mules [24]. MONO, EOS and BASO values were higher in mules and donkeys than in equine foals of the same age [19], throughout the study period, while in line with values in adults of the same species [24,27].

Regarding biochemical parameters, some differences related to the age were found for AST and ALP activities, and creatinine, urea, glucose, and lactate concentrations. AST values were lower in mule foals than in adult mules at birth [7], reaching similar values at one week of life [24], similar to findings already reported in horses [29]. This might be related to the physiological increase in muscle activity in foals [25]. The AST activity found in our study was higher than that reported in Amiata donkey foals [18], but lower than the Martina Franca foals [19]. The higher ALP activity in mule foals compared to adults [7,18,19,27] was in line with equine [20] and donkey foal findings [18,19], owing to the osteoblastic activity in growing bones and intestinal development associated with pinocytosis activity.

Creatinine and urea decreased over time in line with equine [20] and donkey foals [18], probably due to milk intake and diuresis [13,30]. After the first 24–48 h of life, the creatinine concentration fell within the adult reference range of adult mules [7], horses [24], donkeys [27] and hinnies [24]. Urea values were higher than adult mules and horses [7,24] for the study period, but in line with donkeys [24,27]. This might be due to the more efficient recycling of urea in mules [24], as in donkeys [31], due to a better digestive efficiency [3,5].

The glucose concentration increased from birth to one week of life when values were similar to adults of all equid species [24], in line with findings reported in donkeys [18,19] and equine foals [21,30]. The glucose increment has been reported during the first 24 h of life due to milk feeding [8,19,23,25,32]. In this study, the same hypothesis might support our results, although the sampling was performed at a one-week interval and not close to birth.

Lactate concentration constantly decreased throughout the study period, as already reported for equine [8,33,34] and donkey [19,35] foals. Moreover, lactatemia decreased more rapidly in mules than in equine foals [3,19,35] This could be linked to a more efficient adaptability to the external environment, with greater oxygen uptake decreasing anaerobic metabolism and consequently resulting in a decrease in lactatemia [11,18,19,25]. Further studies are needed to confirm this hypothesis.

CK, GGT and GLDH activities were constant over the study period. The mule foals examined, as newborns, showed lower CK values that recovered within one week, as in equine foals [25]. Other studies, however, have reported that it is common to observe a higher CK activity at birth in donkey foals during the first day of life, likely due to the muscle compression that occurs during parturition and the physical exertion that the foal makes to stand up and feed [21]. Starting from one week of life, the CK values were within the reference range of adult mules [24], but higher than donkeys [21] and hinnies [7,31]. In mule

Table 2
Biochemical parameters from birth to eight weeks of life in the cohort of mule foals ($n = 7$). Values are expressed as mean (X) ± standard deviation (SD) or median (minimum-maximum) values.

	T0 ($n = 7$)	T1w ($n = 7$)	T2w ($n = 7$)	T3w ($n = 7$)	T4w ($n = 7$)	T5w ($n = 7$)	T6w ($n = 7$)	T7w ($n = 7$)	T8w ($n = 7$)	*P*
AST (U/L)	48[a] (35.8-149)	181.9 ± 44.7[a,b]	188.1 ± 12.9[a,b]	181.1 ± 20.3[a]	189 ± 40.1[a,b]	199.2[a,b] (124-379)	218.9 ± 123.1[b]	203.6 ± 23[b]	221.3 ± 42.9[b]	*P = 0.0052*
ALP (U/L)	3375 ± 1279[a]	829.6 ± 317.4[b]	720.7 ± 261.1[b]	760[b] (403-3690)	879.6 ± 312.1[a,b]	880.7 ± 179.5[a,b]	814.8 ± 96.5[b]	930.8 ± 78.3[a,b]	1179 ± 594.7[a,b]	*P = 0.0002*
CK (U/L)	150.9 ± 72.5	167.7 ± 81.1	253.3 ± 154	155.8 ± 55.7	227 ± 86.2	201.3 ± 45.5	252.4 ± 127.4	222.8 ± 92	175 (95.8-510.7)	*P = 0.1065*
GGT (U/L)	10.6 ± 5.1	9.5 (6.4-29.5)	8.5 (6.9-30.8)	12.5 ± 9.7	10.1 ± 4.3	9.9 ± 5.7	7.5 ± 4.1	8.1 ± 4	10.3 ± 7.2	*P = 0.5775*
GLDH (U/L)	1 (1-12)	3(1-15)	4.9 ± 4.1	3.3 ± 2.1	3.6 ± 2.1	2(1-11)	3.5(2-17)	3.3 ± 1	4.5 ± 1.9	*P = 0.4996*
TRIG (mg/dL)	32.9 ± 13.9	43 (22-220)	62 (29-159)	68.4 ± 33.6	50.7 ± 17.3	52.3 ± 9	48.3 ± 10.1	63.8 ± 24.5	40 (35-230)	*P = 0.1401*
ALB (g/L)	4.2 ± 0.5	3.6 ± 0.4	3.5 ± 0.5	3.5 ± 0.5	3.5 ± 0.6	3.6 ± 0.6	3.3 ± 0.7	3.6 ± 0.5	3.4 ± 0.6	*P = 0.3890*
TP (g/L)	4.7 ± 0.6	5.9 ± 0.9	5.9 ± 0.7	5.8 ± 0.8	5.8 ± 0.8	5.6 ± 0.6	5.4 ± 0.4	5.3 ± 0.7	5.3 ± 0.4	*P = 0.0648*
CREA (mg/dL)	1.8[a] (1.1-4.6)	1.1 ± 0.2[a,b]	1.1 ± 0.3[a,b]	1.1 ± 0.4[a,b]	1[a,b] (0.9-1.5)	1.1 ± 0.2[a,b]	1.1 ± 0.3[a,b]	1.1 ± 0.3[a,b]	1.1 ± 0.2[b]	*P = 0.0173*
BUN (mg/dL)	34 ± 4.6[a]	22.3 ± 8.1[a]	19.6 ± 6.1[b]	20.9 ± 7.5[b]	17 (16-30)[b]	19.4 ± 6.9[b]	20.6 ± 6.1[b]	19.1 ± 9.4[b]	23 ± 7.3[a]	*P = 0.0001*
Tot bil (mg/dL)	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	*P = 0.5639*
Direct bil (mg/dL)	1.3 (1.1-2.9)	1.7 ± 0.9	1.2 ± 0.4	1.1 ± 0.3	1.2 ± 0.3	1.1 ± 0.3	0.9 ± 0.5	1.2 ± 0.3	1.2 ± 0.3	*P = 0.5639*
Indirect bil (mg/dL)	1.3 (1.1-1.8)	1.7 ± 0.9	1.2 ± 0.4	1.1 ± 0.3	1.2 ± 0.2	1.1 ± 0.3	0.9 ± 0.5	1.2 ± 0.3	1.2 ± 0.3	*P = 0.1180*
Glucose (mg/dL)	81.5 ± 12.1[a,c]	147.9 ± 13.3[b]	128[a,b] (123-164)	140[a,b] (105-153)	142.3 ± 17.8[a,b]	125.7 ± 15.2[a,b,c]	122.3 ± 8.2[a,b,c]	122.9 ± 11.4[a,c]	122.1 ± 12.3[a,c]	*P < 0.0001*
Lactate (mg/dL)	3.8 ± 0.6[a]	1.5 ± 0.3[a,c]	1.2 ± 0.3[a,b,c]	1.3 ± 0.4[a,b,c]	1.3 ± 0.1[a,b,c]	1.2 ± 0.2[b,c]	1[b,c] (0.8-2.8)	0.9[b,c] (0.9-1.3)	1 ± 0.1[b]	*P < 0.0001*
K (mmol/L)	4.2 (4.2-33.4)	4.2 ± 0.2	4.2 ± 0.3	4.3 ± 0.3	4 ± 0.4	4.4 (4.1-7.8)	4.5 (4.1-5.6)	4.4 ± 0.1	4.5 ± 0.3	*P = 0.2461*
Na (mmol/L)	133.1 ± 4.1	130.7 ± 5.5	132.9 ± 1.5	132.8 ± 2.6	131.7 ± 2.2	133.4 ± 1	133.4 ± 1.2	134.6 ± 1.6	134.5 ± 1.4	*P = 0.2265*
Ca (mmol/L)	1.5 ± 0.2	1.6 (1.2-1.6)	1.6 ± 0.1	1.6 ± 0	1.6 ± 0	1.5 ± 0	1.6 ± 0	1.6 ± 0	1.5 ± 0	*P = 0.8751*
Cl (mmol/L)	107.1 ± 2.4[a]	105.7 ± 2.8[a,b]	103.5 ± 2.9[a,b]	103.6 ± 2.6[a,b]	103.1 ± 1.7[a,b]	104 ± 2.2[a,b]	104.1 ± 1[a,b]	103 ± 1.3[b]	103.4 ± 1.1[a,b]	*P = 0.0268*
Mg (mmol/L)	0.5 ± 0.1	0.5 ± 0.1	0.6 (0.4-0.6)	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.6 (0.4-0.7)	*P = 0.3392*

Legend - T0, birth; T24h, 24 h; T48 h, 48 h of life; T1w, 1 week; T2w, 2 weeks; T3w, 3 weeks; T4w, 4 weeks; T5w, 5 weeks;T6w, 6 weeks; T7w, 7 weeks; T8w, 8 weeks of age. AST, aspartate aminotransferase; ALP, alkaline phosphatase; CK, creatine phosphokinase; GGT, gamma-glutamyl-transpeptidase; GLDH, glutamate dehydrogenase; TRIG, triglycerides, ALB, albumins; TP, total protein; CREA, creatinine; BUN, blood urea; Tot bil, total bilirubin concentration; Direct bi., direct bilirubin concentration; Indirect bil, indirect bilirubin concentration; Glucose, blood glucose concentration; Lactate, blood lactate concentration. Different superscripts denote a significant difference (a≠b≠c) within the same row.

foals, GGT activity is much lower than in equine foals [20] and Martina Franca donkey foals [19], but closer to, though still lower than, the values observed in Amiata donkey foals [18].

It has been reported that GGT values in equine foals are between 1.5 and 3 times higher than normal adult values for the first 3, 4 weeks of life [13], in contrast to what we found. In the same study [13], the GGT activity was lower in adult horses than in foals, unlike in adult mules [21]. The higher values showed in equine foals could be related to the induction of liver enzymes observed at the same time as hepatocellular maturation after birth [13,25] and not by the transfer of GGT through colostrum as occurs in newborn ruminants [20]. GLDH activity was similar to that reported for adult donkeys and horses [21,29].

Triglycerides, albumin, total proteins, and total, direct, and indirect bilirubin concentrations were constant over the study period. Triglycerides were comparable to adult mules and horses [24], while lower than in donkeys [6,27] and higher than in hinnies [24]. Compared to equine foals, the values obtained in mules were lower [18]. This is probably due to the slight transient hypertriglyceridemia associated with hepatic dysmaturity at birth reported in equine foals [14,25] or transient postprandial lipemia. Thus, equine foals show a high variability in triglyceride concentration [20].

The albumin concentration has been found to be constant in mule foals of the present study as reported in equine [36] and donkey foals [18,19], but total proteins in mule foals did not show an increase due to immunoglobulin absorption from colostrum ingestion, as it happens in other foals species [18–20,25]. This outcome might be related to the low number of mule foals. Anyway, the values obtained in mule foals for albumin and total proteins were found to be similar to adult horses [20,36], mules [7], hinnies [24] and donkeys [27].

Total bilirubin was higher in mule foals than in equine and donkey foals [18,19]. Our results may be explained with the neonatal hyperbilirubinemia, which is well documented in equine foals and characterized by an increase in total bilirubin concentration. This is mainly attributable to a moderate increase in unconjugated bilirubin attributed to accelerated neonatal red blood cell breakdown or immature liver function [14,20,25]. It has also been observed that in foals less than five days old, there is less hepatic glucuronyl transferase activity than in adults and a lower bilirubin conjugation rate [13].

Regarding electrolytes, only the total chlorine concentration showed a decrease over time, while total potassium and sodium concentration and ionized calcium (Ca^{2+}) and magnesium (Mg^{2+}) remained constant throughout the study period. Total potassium concentration matched those reported for adults [7,37,38] and foal species [18,19,25], while sodium in mule foals was found to be in line with adult horses and donkeys [37,38] and donkey foals [18,19], but lower than in equine foals [20,25]. In mule foals, Ca^{2+} was lower than in donkeys [18,19] and equine foals [20,25], as well as adult donkeys [7,38], but similar to adult horses [37]. Mg^{2+} was lower than in donkey foals [19] but similar to equine foals [25]. Total chlorine concentration decreased over time, and values were similar to those in equine foals [25] and adults [37], but higher than in donkey foals [19].

Some limitations of the study need highlighting. Firstly, only a small sample of mule foals was enrolled, thus type II errors in the statistical analysis may have occurred. The results need to be considered as

preliminary data on neonatal mule assessments, and a larger study population is needed to support our findings. A second limitation may be the intra-specific genetic variability of mules which may have affected the results. Further studies should therefore also consider controlling for genetic variation to limit the influence on the conclusions.

5. Conclusions

The data collected in this study could be used as preliminary data to support the reference database for hematological and biochemical values in clinically normal mule foals, in the sequential phases of their lives. Knowledge of the physiological parameter values of the newborn mule is essential to classify the animal as healthy or affected by a pathological condition. Further studies are needed to obtain even more precise data, including a larger population of mule foals, in order to establish a valid reference range for the breed.

The preliminary results suggest that also for mule foals, age influences various hematological and biochemical parameters and should be considered in order to obtain a correct clinical evaluation of the animal.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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