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Estrogen-iron axis in cyclic mares: Effect of age

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ABSTRACT

In woman and in animal models, estrogens are involved in iron (Fe) homeostasis supporting the hypothesis of the existence of an "estrogen-iron axis". Since advancing age leads to a decrease in estrogen levels, the mechanisms of Fe regulation could be compromised. In cyclic and pregnant mares, to date, there is evidence linking the iron state with estrogens pattern. Then, the objective of this study was to determine the relationship among Fe, ferritin (Ferr), hepcidin (Hepc) and estradiol-17β (E_2) in cyclic mares with advancing age. A total of 40 Spanish Purebred mares of different ranges of age was analyzed: 4–6 years (n = 10), 7–9 years (n = 10), 10–12 years (n = 10), and >12 years (n = 10). Blood samples were obtained on days −5, 0, +5 and + 16 of the cycle. Compared to mares of 4–6 years, serum Ferr was significantly higher ($P < 0.01$) and Fe significantly lower ($P < 0.01$) in mares >12 years of age. Hepc was significantly higher in mares >12 years ($P < 0.01$) than in those 7–9 years of age. E_2 levels were higher in mares of 7–9 years ($P < 0.01$) than in 4–6 and >12 years of age. Fe and Ferr were negatively correlated with Hepc ($r = -0.71$ and $r = -0.02$, respectively). E_2 was negatively correlated with Ferr and Hepc ($r = -0.28$ and $r = -0.50$, respectively), and positively with Fe ($r = 0.31$). There is a direct relationship between E_2 and Fe metabolism, mediated by the inhibition of Hepc in Spanish Purebred mares. The reduction of E_2 decreases the inhibitory effects on Hepc, increasing the levels of stored Fe and mobilizing less the free Fe in circulation. Based on the fact that ovarian estrogens participate in changes in the parameters indicative of iron status with age, the existence of an "estrogen-iron axis" in the mares'estrous cycle could be considered. Future studies are required to clarify these hormonal and metabolic interrelationships in the mare.

1. Introduction

In humans and experimental animals, changes in iron (Fe) status are estrogen-related, supporting the hypothesis of the existence of an "estrogen-iron axis". Estrogens are related to Fe metabolism through their ability to suppress hepcidin (Hepc) synthesis, maintain ferroportin (Fpn) integrity, increase Fe release from hepatocytes, duodenal enterocytes and macrophages. In general, the pattern of 17-β estradiol (E_2) during the different phases of the menstrual cycle, and pregnancy is positively correlated with Fe demand [1].

Specifically, high estrogen concentrations are supposed to downregulate Hepc synthesis [1–4] and reduce the Fpn expression [5]. Then, it is reasonable to assume that the estrogens'changes during the estrous cycle influence the metabolism both of Hepc and Fe. Therefore, the interpretation of the regulation of Fe metabolism and Hepc modifications would depend on the different phases of the estrous cycle [6,7]. In fact, in premenopausal women, E_2 was positively correlated with Hepc during the early follicular phase, although the elevation of this hormone in the mid-luteal phase exerted a negative feedback mechanism on the synthesis of Hepc [4]. The latter it stimulates the synthesis of Hepc as a counterweight mechanism to limit intestinal Fe absorption [8].

This potential "estrogen-iron axis" directs iron metabolism in response to hematologic (erythropoiesis) and non-hematologic (uterine growth, gestation, etc.) needs. In women, alterations in this axis have been shown to exacerbate iron overload and lead to disease processes such as cancer, osteoporosis, cardiovascular complications, and neurodegenerative symptoms [1]. Only recently, the existence of an "estrogen-iron axis" also in cyclic and pregnant mares has been demonstrated [9,10].

Moreover, in equines, the effect of age on parameters of iron

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status is poorly understood and sometimes fragmentary. The study of the effect of age on Fe and ferritin (Ferr) levels in foals has been deepened, finding a negative correlation of both parameters with age [11]. Therefore, Fe and Ferr are lower in newborn foals than in adult horses, followed by a rapid increase in the first 24 h as a result of Fe uptake from colostrum [12,13]. During the first 3 weeks of life, its concentrations drop below those of adults [14], when hemoglobin breakdown leads to storage of Fe for the future production of red blood cells. Serum Ferr concentrations decline by 12 months of age but return to adult levels by 15 months [15,16]. In Arabian horses, classified as young (3 years) or older (>10 years), Nielsen et al. [17] found a positive correlation between age and Ferr levels. Advancing age leads to an elevation in ferretinemia, as previously suggested [18,19], accounting for Fe accumulation in tissues over time. In fact, aging is associated with Fe accumulation or increased Ferr in the equine liver [19]. Therefore, the non-heme Fe concentration in the liver is much lower in young growing animals, including horses less than 2 years of age than in adults [20]. What is more, it is well known that Hepc regulates the systemic bioavailability of Fe, is modified by estrogens and declines with age. On this scientific basis it is important to evaluate the possible interactions among Fe, Hepc and E_2 status in healthy mares to provide a few new insights into the physiology of Fe homeostasis with advancing age also in the equine species.

2. Material and methods

2.1. Animals

All procedures and methods applied in this study were in compliance with the guidelines of the Spanish law (RD 37/2014) that regulated the animals' protection used for scientific purposes (BOE, 2023).

Forty cyclic non-pregnant mares, wormed and vaccinated, classified in 4 groups of age as follow: 4–6 years (n = 10), 7–9 years (n = 10), 10–12 years (n = 10) and >12 years (n = 10) ranging from 13 to 25 years old were used in the present study. The criteria adopted to justify this age grouping of mares was suggested by the statistical survey. When no statistically significant differences were found among mares belonging to each individual group of 4–6, or 7–9, or 10–12 or greater than 12 years old, we defined the four groups studied. We obviously started with a larger number of subjects, so that we could form small groups of mares of at least 10 subjects each. The inclusion criteria of selected mares were as following: 1) the presence of physiological cyclicity along the previous breeding seasons, and the absence of reproductive pathologies; 2) absence of hospitalized case occurred one month before the start of blood sampling and no treatment with antibiotics or anti-inflammatory. The same management and feeding treatment were applied to all mares. The amounts of concentrate were 2–3 kg twice daily, fiber needs were covered with 2–3 kg of alfa-alfa hay and wheat straw; the water was *ad libitum*.

The mares belonged to private stud farms located in the Valencian Community (Valencia, Spain). Mares were kept in their stables and close to stallions in nearby pits. Signs of estrus including urination, clitoral winking, lateral tail shift, and hindlimb parting were identified by direct observation. We consider that the appearance of these signs of heat indicated the first day of estrus. Each mare was examined for 21 days during the estrous cycle beginning on the first day of heat until ovulation (day of cycle 0) and then 5 days post-ovulation (cycle day +5) and the last day, before the next estrus (cycle + 16). Transrectal ultrasonography with a 5 MHz transducer (Sonosite 180 Plus), was used to determine the size of preovulatory follicles and the presence of endometrial edema. Ultrasound examination was performed on the first, second, third, fourth, and fifth days of heat until ovulation was confirmed (day 0). The days before ovulation were numbered −5, −4, −3, −2 and −1, while those after ovulation were numbered sequentially from day +1 to day +16. After ovulation, the presence of the CL in the ovary was confirmed, remaining at 5 days and detecting a regressing CL at 16 days.

2.2. Blood samples

Blood samples were obtained by jugular venipuncture at −5 pre ovulation day, at day 0 when preovulatory follicle reached the maximum diameter and ovulation occurred, at +5 and + 16 post ovulation days, observed by ultrasound evaluation.

Blood collections were performed between 8:00 and 11:00 a.m., using 20 mL disposable syringes, with luer cone (Becton Dickinson Discardit® II) attached to 40 mm. 18–20 G needles (Sterican®, Braun Melsungen AG). A total of 20 mL was collected and each blood sample was added to glass tubes with clotactivators and polystyrene granules (PS) to collect serum (Tapval®). Samples were refrigerated at 4 °C for transport, then were centrifuged at 3500×*g* for 10 min (P Selecta® Centrifuge); then, the serum obtained was stored at −20 °C until analyzed.

2.3. Determination of circulating iron (Fe), ferritin (Ferr), hepcidin (Hepc) and 17β-estradiol- (E_2) concentrations

Serum Fe and Ferr (μg/dL) concentrations were analyzed by a Spin 200E spectrophotometer using commercial house reagents based on colorimetry for Fe (FerroZine) and turbidimetry for Ferr (Latex) (Spinreact®, Barcelona, Spain). The sample detection limits for Fe and Ferr were 0.850 μg/dL to linearity limit of 1000 μg/dL, and 5.04 μg/L, respectively. The intra- and interassay coefficients of variation (CVs) were of 0.79% and 3.17%, and of 5.1% and 6.3%, for Fe and Ferr, respectively.

Serum Hepc (ng/mL) concentrations were analyzed according to the manufacture's guideline of horse hepcidin (HPC) enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource.com, San Diego, CA, USA). The detection range was of 15.6 ng/mL-500 ng/mL, the sensivity of 2.0 ng/mL and intra- and interassay CVs rate was pared to <15%.

Serum E_2 (pg/mL) concentrations were determined by a competitive enzyme-linked immunosorbent assay (E_2 Sensitive, Demeditec ELISA DE4399) specifically validated in the equine species. The limit of detection was 1.4 ng/mL. The percentage of recovery in plasma was 98.72%. The CVs intra - and inter -analyses at low and high concentrations were 7.87% and 5.52%, respectively.

2.3.1. Statistical analyses

Descriptive statistics including mean, maximum and minimum values and standard deviation (SD) of all parameters were obtained. The normality and homoscedasticity of the data were verified using the Kolmogorov-Smirnov and Levene test. Logarithmic transformations of the data were performed to achieve homogeneity of variance. A one-way analysis of variance (ANOVA) was performed to compare the Fe, Ferr, Hepc, and E_2 among 4 groups of age, and the comparisons of means were made using the Tukey HSD test. The interrelationships among all parameters were examined by linear regression analysis and the correlation was expressed by Pearson's correlation coefficients. The level of significance was set at $P < 0.05$.

3. Results

Descriptive statistics of the Fe, Ferr, Hepc and E_2 concentrations according to the age and the estrous cycle's days in Spanish

Purebred mares were shown in Table 1.

At −5 days: Ferr of mares 4–6 years old (y) was lower ($P < 0.01$) than all other age groups; Fe of mares 4–6 and 7–9 y was higher ($P < 0.01$) than those of >12 y; Hepc of mares 7–9 y was lower ($P < 0.01$) than 10–12 and > 12 y; E_2 of mares >12 y was lower ($P < 0.01$) than all other age groups.

At 0 day: Ferr of mares 4–6 and 7–9 y was lower ($P < 0.01$) than >12 y; Fe of mares 4–6 and 7–9 y was higher ($P < 0.01$) than those of >12 y; Hepc of mares 4–6 y and of 7–9 y was lower ($P < 0.01$) than 10–12 and > 12 y; E_2 of mares 4–6 y was higher ($P < 0.01$) than >12 y, and of 7–9 y was higher ($P < 0.01$) than 10–12 and > 12 y.

At +5 days: Ferr of mares 4–6 and 7–9 y was lower ($P < 0.01$) than 10–12 and > 12 y; Fe of mares 4–6 y was higher ($P < 0.01$) than all other age groups; Hepc of mares 4–6 y was lower ($P < 0.01$) than >12 y, and of 7–9 y was lower ($P < 0.01$) than 10–12 and > 12 y; E_2 of mares 4–6 and 7–9 y was higher ($P < 0.01$) than >12 y.

At +16 days: Ferr of mares 4–6 y was lower ($P < 0.01$) than 7–9 and >12 y; Fe of mares 4–6 y old was higher ($P < 0.01$) than all other age groups, and of 7–9 was higher ($P < 0.01$) than >12 y; Hepc of mares 4–6 and 10–12 y was lower ($P < 0.01$) than >12 y, and of 7–9 y was lower ($P < 0.01$) than 10–12 and > 12 y; E_2 of mares 4–6 and 10–12 y was higher ($P < 0.01$) than >12 y, and of 7–9 y was lower ($P < 0.01$) than 10–12 y and higher ($P < 0.01$) than>12 y. Circulating concentrations of iron, ferritin, hepcidin and estradiol-17β (mean ± SD) in the four age groups in cyclic Spanish Purebred mares were shown in Fig. 1.

The concentrations of Ferr and Fe were higher and lower ($P < 0.01$), respectively, in mares >12 years than in mares 4–6 years. Mares >12 years had higher Hepc concentrations ($P < 0.01$) than mares of 7–9 years old (Fig. 1). E_2 concentrations in mares of 7–9 years were higher ($P < 0.01$) than in those of 4–6 and >12 years. In mares of 4–6 and 10–12 years, E_2 concentrations were higher ($P < 0.01$) than mares of >12 years.

The correlation coefficients among all parameters considered in this study along the estrous cycle's days of mares and according to their different age were shown in Table 2. Specifically, Fe and Ferr were negatively correlated with Hepc ($r = -0.71$ and $r = -0.02$, respectively). E_2 was negatively correlated with Ferr and Hepc ($r = -0.28$ and $r = -0.50$, respectively), and positively with Fe ($r = 0.31$).

4. Discussion

The most relevant results of this study were the following: 1) the existence of a clear and direct relationship between estradiol and the systemic metabolism of Fe, suggesting an "estrogen-iron axis" also in cyclic Spanish Purebred mares, according to different age; 2) an increase of Ferr and Hepc concentrations, and a decrease of Fe concentrations with age; 3) the decrease in E_2 in older mares seems to reduce the inhibitory effect on Hepc, increasing presumably the iron stores and mobilizing a greater amount of Fe to the plasma. These results were maintained for Fe, Hepc and E_2 in mares 4–6, 7–9 and >12 years old, when the effect of age, along the days of the cycle were analyzed, with the exception of Ferr, finding no significant differences among the days of the cycle.

The increase in Ferr with advancing age is a proven fact in the horse. In fact, compared to young animals (3 years of age) in Arabian horses older than 10 years, a positive association between age and levels of Fe stored has been found [17]. Hence, aging has been shown to be associated with the increased iron accumulation in the liver in horses over time [18,19]. Although serum Ferr increases with age, this finding should be interpreted with caution because its elevated levels may be secondary to age-associated pathologies, such as acute or chronic infections or inflammation, regardless of the individual's iron status. Ferr is considered a moderately positive acute phase protein [17,21], and has recently received increasing attention as a possible marker of prognosis in intensive care patients [11]. Elevated Ferr concentrations during iron overload related with insulin resistance, hemolysis, liver disease and inflammation have been found [17,22]. Indeed, Dondi et al. [22] demonstrated that non-surviving foals had significant higher Ferr concentrations than survivors, suggesting a more severe acute phase response.

In addition, previous equine medicine reports have suggested that plasma Fe concentrations in adult horses represent an acute and sensitive indicator of systemic inflammation [11,23–25,26], and low Fe concentration is an reliable marker of inflammation in horses [23,27]. What is more, the inflammatory hypoferremia occurs due to the upregulation of Hepc by inflammatory cytokines, restricting enteric iron absorption and, by limiting iron availability, and iron sequestration deficiency [28].

Some studies carried out in humans have indicated that age is associated with a low-grade pro-inflammatory state [29], causing a

Table 1
Mean ± SD of circulating iron (Fe), ferritin (Ferr), hepcidin (Hepc) and estradiol-17-β (E_2) concentrations in the four groups of age along the estrous cycle's days of Spanish Purebred mares.

Mean ± SD					
Days of cycle	Age group	Ferr (μg/dL)	Fe (μg/dL)	Hepc (ng/mL)	E_2 (pg/mL)
−5	4–6	171.1 ± 20.0[b]	166.0 ± 23.4[a]	132.1 ± 25.2	27.9 ± 4.05
	7–9	175.2 ± 17.8	172.5 ± 19.9[a]	112.3 ± 17.8[ac]	40.2 ± 6.70
	10–12	182.3 ± 19.1	148.3 ± 18.5	156.2 ± 22.3	35.6 ± 9.30
	>12	183.5 ± 18.0	140.2 ± 18.3	168.2 ± 17.5	20.2 ± 4.01[b]
0	4–6	168.3 ± 219.1[a]	168.3 ± 19.1[a]	102.0 ± 37.4[ac]	38.5 ± 6.29[a]
	7–9	179.5 ± 23.4[a]	176.1 ± 23.3[a]	99.5 ± 18.2[ac]	40.2 ± 6.70[ac]
	10–12	187.8 ± 23.1	157.3 ± 23.3	156.2 ± 22.3	31.6 ± 8.20
	>12	192.2 ± 20.1	145.3 ± 21.1	156.3 ± 10.3	24.5 ± 5.20
+5	4–6	178.0 ± 22.0[ac]	178.0 ± 22.0[b]	124.6 ± 18.9[a]	35.9 ± 5.21[a]
	7–9	182.3 ± 19.1[ac]	148.3 ± 18.5	113.3 ± 22.3[ac]	40.2 ± 6.70[a]
	10–12	190.5 ± 25.6	135.6 ± 21.5	136.7 ± 21.3	31.5 ± 8.30
	>12	199.1 ± 22.0	133.2 ± 23.4	158.1 ± 23.5	23.3 ± 5.07
+16	4–6	182.1 ± 20.7[ad]	171.2 ± 20.7[b]	147.0 ± 27.2[a]	25.8 ± 3.94[a]
	7–9	190.2 ± 21.5	145.6 ± 23.4[a]	150.3 ± 25.6[ac]	33.5 ± 7.59[ac]
	10–12	179.3 ± 22.3	130.2 ± 21.3	156.2 ± 22.3[a]	35.6 ± 9.30[a]
	>12	188.8 ± 21.3	120.2 ± 17.8	170.3 ± 20.2	17.3 ± 5.67

Letters indicate significant differences vs > 12 years: a = $P < 0.01$; vs all other age groups: b = $P < 0.01$; vs 10–12 years: c = $P < 0.01$; vs 7–9 years: d = $P < 0.01$.

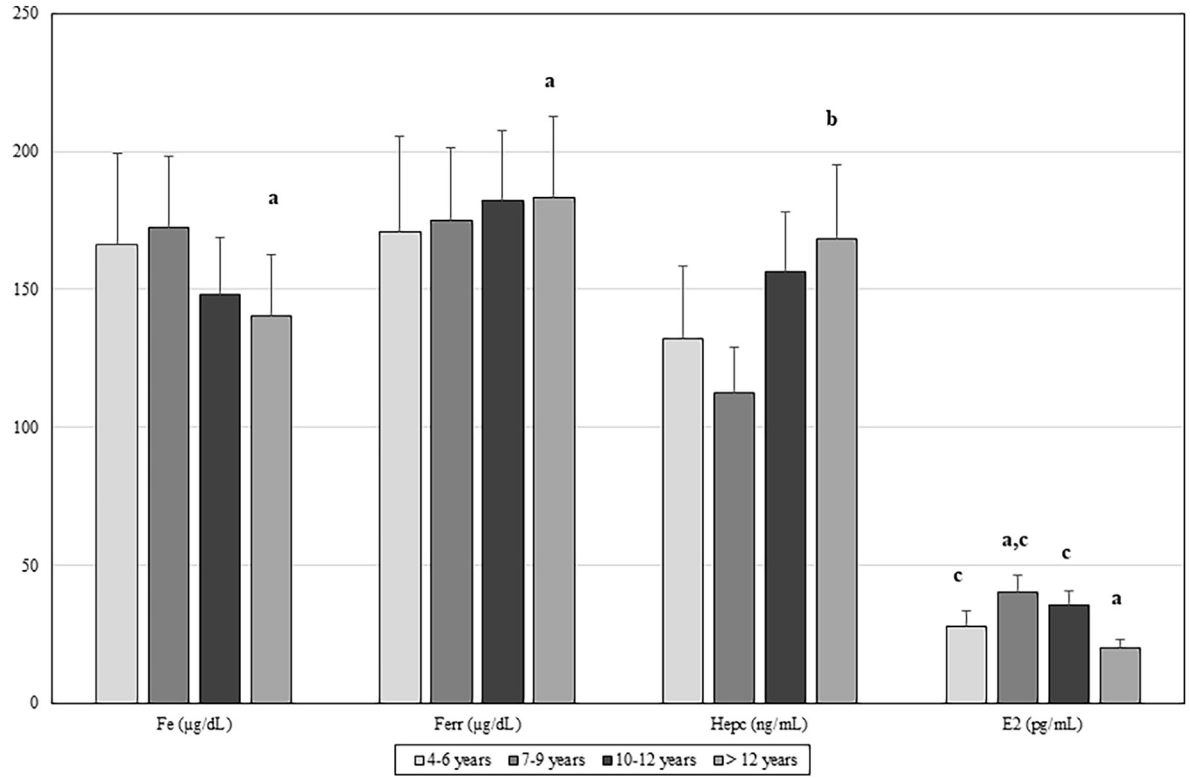

Fig. 1. Circulating concentration of iron, ferritin and hepcidin (mean ± SD) in the four age groups in cyclic Spanish Purebred mares.
Letters indicate significant differences vs 4–6 years: a = P < 0.01; vs 7–9 years: b = P < 0.01; vs > 12 years: c = P < 0.01.

Table 2
The correlation coefficients among all parameters considered in the study.

	Ferr (µg/dL)	Hepc (µg/dL)	E_2 (pg/mL) P<
Fe (µg/dL)	0.12	−0.71	0.31 0.01
Ferr (µg/dL)		−0.02	−0.28 0.01
Hepc (ng/mL)			−0.50 0.01

Fe: iron; Ferr: ferritin; Hepc: hepcidin and E_2: estradiol-17β.

chronic elevation of circulating Hepc; this last condition, consequently, induces a lower availability of plasmatic Fe, which limits the synthesis of hemoglobin and, finally, causes anemia due to inflammation [30,31]. However, mares did not present pathological processes during this experimental period, so the changes in Fe and Ferr levels are probably related to age.

It is well known that E_2 is the most abundant endogenous form of oestrogen in cyclic mares [32], and this study shows that its concentrations progressively decreased with advancing age in Spanish Purebred mares. These results were similar to those reported by Ginther et al. [33], in which during the preovulatory peak, the levels of this hormone were higher in young than in old mares. In addition, 7–9 year old mares showed higher E_2 concentrations on day - 5 than on day +16. This result was surprising since both days correspond to the same period of the estrous cycle. We do not know the origin of these differences since the mares showed normal reproductive cycles and prolonged luteal activity was not identified in any of them. Based on the normal reproductive physiology of the mare, it could be suggested that although hormonal dynamics usually show an individual character, certain common variations may occur linked to age.

In equine reproduction, it is a proven fact that age affects the fertility [34,35]. Indeed, advancing age in the mare is associated with changes in follicular parameters that are closely related to a reduction in the count of antral [35] and preantral [36] follicles. This reduction in E_2 with age simultaneously occurred with the reduction in Fe and an increase in Ferr and Hepc, as confirmed by the existence of negative correlations between E_2 both with Ferr and Hepc, and of its positive correlation with Fe.

Although the exact mechanism by which E_2 participates in Fe regulation remains unknown, some *in vitro* studies in breast (MCF7 and MDA-MB231) [3], ovarian (SKOV3) [37] and liver (HepG2) [38] cancer cell lines of women and other experimental models in rodents suggest that E_2 can positively regulate genes involved in Fe metabolism [1]. In this context, it has been reported that the human E_2 downregulates Hepc synthesis and upregulates Fpn expression, as a means of enhancing intracellular iron efflux [3]. In ovarian cells, the positive regulation of the transcription factor HIF-1α, that regulates a variety of proteins induced by E_2, reduces the expression of the Hepcidin Antimicrobial Peptide gene (HAMP), decreasing the concentration of Hepc [38].

Based on this evidence, and according to on previous study in which it has been shown that serum Ferr is significantly correlated with the concentration of nonheme Fe in the liver and spleen of horses [18], the results of the present study could indicate that the reduction of E_2 in older compared to young adult mares decreases the inhibitory effect on Hepc, increasing probably the Fe store (Ferr) and at the same time, decreasing the mobilization of Fe to plasma. We do not know the mechanisms by which E_2 could regulate Hepc synthesis in the mare, so future research is necessary to clarify these complex physiological mechanisms. However, other investigations have reported an increase in HAMP gene expression and Hepc synthesis in response to E_2 treatment [39,40].

The relationship between E_2 and Fe homeostasis seems to be specific to the cell type. Indeed, in uterine cells, E_2 plays an

important role in reducing Hepc expression and in supporting Fe turnover during E_2-induced cell growth and development. In contrast, in immune cells, mid-cycle increases in E_2 may enhance the proinflammatory response in macrophages and dendrites, increasing Hepc and Fe sequestration as part of the anti-inflammatory response [1,40].

The results observed in the mare seem surprising, since in young mares in which E_2 levels are high and Hepc seems to be suppressed, Fe and Ferr concentrations are lower than in older mares. However, as age advances and E_2 decreases, Hepc secretion increases, although Fe reserves increase, while free Fe concentration decreases, as confirmed by the existence of negative correlations between Hepc both with Ferr and Fe. This last decrease could explain the greater tendency to anemia in elderly animals; indeed, low-grade anemia is common finding in older horses. A combination of less exercise, lower regenerative capacity of bone marrow, the decrease in metabolic rate and the presence of chronic diseases may result in a lower requirement for oxygen in senior horses, as previously suggested in this species [41].

We do not know the mechanisms by which estrogens reduce the synthesis of Hepc, and favor the greater mobilization of Fe from reserves or if the absorption of this mineral can increase during the estrous cycle in young adult mares compared to older ones; however, the results obtained clearly demonstrate a close relationship between estrogens and the metabolism of Fe.

5. Conclusion

In cyclic Spanish Purebred mares there is a clear and direct relationship between estradiol and the systemic metabolism of Fe, so an “estrogen-iron axis” could be suggested. In young adult mares, estrogen dominance in the pre- and ovulatory periods allows for a more efficient iron status, mediated by hepcidin inhibition. The reduction of the inhibitory effect of hepcidin, due to the decrease in estrogen in older mares, tends to increase hepcidin and ferritin levels, although iron levels decrease. Future research is required to clarify these metabolic and hormonal interrelationships in equine species.

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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