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# Functional response of systemic and intrafollicular placental growth factor in cycling mares


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## ORIGINAL ARTICLE



### ABSTRACT

The aim of the study was to assess the physiological reference values for systemic and intrafollicular placental growth factor (PlGF) concentrations in different categories of follicular sizes in cycling mares, according to progesterone ( $P_4$ ) and oestradiol ( $E_2$ ) patterns. Sixty ovaries were taken after slaughter from 30 clinically healthy mares. Regarding their size, the follicles were classified into three different categories, i.e. small (20–30 mm), medium-sized (31–40 mm) and large ( $\geq 41$  mm), and follicular fluid (FF) was sampled from each single follicle. Intrafollicular PlGF concentrations were significantly increased in larger and medium-sized follicles compared to small follicles, and their values were 1.48 and 1.36 times higher than the systemic values, respectively. On the other hand, systemic PlGF concentrations were 1.3 times higher than those in the FF of follicles of small size. Intrafollicular  $P_4$  concentrations were significantly higher in larger follicles than in small ones, and their concentrations were 6.74 and 3.42 times higher than the systemic values, respectively. Intrafollicular  $E_2$  concentrations were significantly higher in large and medium-sized follicles than in follicles of small size, and their concentrations were 21.1, 15.4 and 8.35 times higher than the systemic values, respectively. Intrafollicular and systemic PlGF concentrations were strongly and positively correlated; nevertheless, no correlations between intrafollicular and systemic steroid hormones, PlGF and follicle diameters, PlGF and  $E_2$ , or PlGF and  $P_4$  were observed. This represents the first study to characterise systemic and intrafollicular PlGF concentrations in cycling normal mares, providing evidence that the bioavailability of this factor in follicles of medium and large sizes was higher than in small follicles, independently of steroid hormone concentrations. Further studies are needed to assess the presumable implications of PlGF in follicular angiogenesis in mares, similar to those already observed in women and primates.

### KEYWORDS

follicular fluid, mare, placental growth factor (PlGF), plasma, steroid hormones

## INTRODUCTION

Endocrine, paracrine and vascular control of follicular development and ovulation rate has already been assessed in different farm animal species (Acosta and Miyamoto, 2004; Hunter et al., 2004; McFee et al., 2012), with a specific emphasis on the role of placental growth factor (PlGF). However, it is well known that after the ovulatory gonadotropin surge the follicular PlGF mRNA and protein concentrations increase; likewise, both granulosa and theca cells contribute to the increase of PlGF concentrations in the follicular fluid (FF) just before

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ovulation, as observed in primates (Bender et al., 2018) and in women undergoing fertility treatments (Gutman et al., 2008; Tal et al., 2014). There is evidence to suggest that PIGF is a potent pro-angiogenic factor, contributing to pathological angiogenesis, anovulatory infertility and ovarian hyperstimulation syndrome in women (Fraser, 2006). Indeed, in women with polycystic ovary syndrome (PCOS), the dysregulation of follicular angiogenesis is related to PIGF concentrations of the FF (Tal et al., 2014) and with a 4- to 6-fold FF to serum ratio of PIGF, but there is no correlation with gonadotropin and oestrogen. Moreover, PIGF plays a pivotal role in the angiogenesis process of the granulosa cell layer, as the ovulatory follicle transforms into corpus luteum (CL) in humans (Carmeliet et al., 2001) and in the early morphological luteinisation of the primate follicle (Bender et al., 2018). PIGF also regulates vascular permeability, which is involved in plasma extravasation along luteal development in mice and primates (Carmeliet et al., 2001; Herr et al., 2015). PIGF concentration also increases in the peritoneal fluid of women with endometriosis (Suzumori et al., 2003). The inhibition of PIGF in these patients is related to reduced plasma extravasation (Tal et al., 2014). In mares, other factors such as vascular endothelial growth factor (VEGF) (Watson and Al-Zi'abi, 2002; Al-Zi'abi et al., 2003; Ginther et al., 2004a) and insulin-like growth factor (Ginther et al., 2004b; Müller et al., 2009; Bashir et al., 2016) associated with follicular angiogenesis have also been investigated. What is more, plasma PIGF concentrations have recently been determined in pregnant Spanish Purebred mares (Satué et al., 2018). However, the PIGF concentration of the FF and a related comparison with systemic PIGF concentrations during the ovulatory period in mares have not been documented so far. In view of its clinical importance, we considered it necessary to investigate the physiological role of PIGF to incorporate its modulations into the reproductive pathological model associated with infertility in mares. Considering that the presence of PIGF concentrations in the FF has been unknown to date, and on the basis of its potential role in ovarian function, the aim of this study was to assess the physiological reference values for systemic and intrafollicular PIGF concentrations in different categories of follicular sizes in cycling mares, by taking into account the modifications that steroid hormones (progesterone, P<sub>4</sub>; oestradiol, E<sub>2</sub>) could induce in this factor, and/or vice versa.

## MATERIALS AND METHODS

### Animals

A total of 30 clinically healthy mares, aged  $6.6 \pm 1.3$  years, with a body weight paired to  $533 \pm 7.3$  kg, were evaluated. All animals were subjected to the same management conditions and feeding regime, including an additional orchard grass–alfalfa mixed hay and free access to mineral salt and fresh water. The study was performed in the northern hemisphere in the months of April and May 2018 of the

breeding season, to ensure the cyclic activity of the ovaries. The environmental temperature ranged between 27 and 31 °C, with 40–60% of relative humidity. The official veterinarians for each farm and slaughterhouse accepted responsible participation in the present study, and only mares with normal reproductive history regarding their oestrous cycles were included. The veterinary examination of the animals before slaughter consisted of verifying the official documents including farm of origin, sanitary registration number, suitable health status, deworming and vaccination plan, as well as clinical and reproductive history.

All methods and procedures used in this study were in compliance with the guidelines of the Spanish law (RD 37/2014) regulating the protection of animals at the time of slaughter and the EU directive (2010/63/EU) on the protection of animals used for scientific purposes. The Animal Ethics Committee for the Care and Use of Animals of the CEU-Cardenal Herrera University (Spain) concluded that the proposed study did not need ethical approval, as it did not qualify as an animal experiment under Spanish law.

### Collection of blood and ovaries

Before slaughter, blood samples (20 mL) were collected from the jugular vein into heparinised tubes. The samples were centrifuged at 1,200 g for 10 min, plasma aliquots were collected and stored at 4 °C in a portable cooler for successive transportation to the analytical laboratory. After slaughter, the ovaries of all mares were collected within 2 hours, as reported by Hinrichs (2012). All ovaries were placed in containers with 0.9% physiological saline plus penicillin (100 IU/mL) and streptomycin (50 mg/mL), and they were transported to the laboratory in individually labelled plastic bags in thermal containers at 25 °C (Foss et al., 2013).

### Collection of follicular fluid (FF)

Ovaries were washed three times with sterile saline, then all follicles were measured using conventional callipers, and subsequently classified according to diameter as small (20–30 mm), medium-sized (31–40 mm) and large ( $\geq 41$  mm). FF was aspirated using different sterile syringes and needles of 22 G for each follicle. Following collection, the FF samples were centrifuged for 10 min at 1,200 g to eliminate the cumulus–oocyte complexes. Only the supernatant, containing pure FF, was collected and stored in aliquots of 0.5 mL at  $-20$  °C until analysis.

### Determination of placental growth factor (PIGF), progesterone (P<sub>4</sub>) and oestradiol (E<sub>2</sub>)

The intrafollicular and systemic concentrations of PIGF (pg/mL) were determined by means of a sandwich immunoenzymatic technique (PIGF ELISA Demedite DE4529, Demetic Diagnostic GmbH, Kiel, Germany), specifically validated for the equine species (Satué et al., 2018). The technique used two highly specific antibodies with high affinity to PIGF. PIGF in the samples bound to the first

Table 1. Mean values ( $M \pm SD$ ) of systemic and intrafollicular placental growth factor (PIGF), progesterone ( $P_4$ ) and oestradiol ( $E_2$ ) concentrations in follicle of different sizes

	N	Follicle diameter (mm)	PIGF (pg/mL)	$P_4$ (ng/mL)	$E_2$ (pg/mL)
System	30		392.1 $\pm$ 41.5 (293.6–442.7)	2.98 $\pm$ 0.37 (2.20–3.50)	82.0 $\pm$ 19.5 (58.7–113.6)
Small	20	20–30	302.4 $\pm$ 240.6 <sup>b</sup> (114–832)	10.3 $\pm$ 1.69 <sup>b</sup> (8.0–14.0)	685.3 $\pm$ 177.7 <sup>b</sup> (465.2–989.4)
Medium	20	>31–40	587.7 $\pm$ 307.8 <sup>a,b</sup> (217–1,157)	9.70 $\pm$ 5.49 <sup>b</sup> (6.5–23.0)	1,266.7 $\pm$ 148.1 <sup>a,b</sup> (1,123.0–1,634.7)
Large	20	>41	528.2 $\pm$ 299.4 <sup>a,b</sup> (154.0–1,076)	20.1 $\pm$ 1.81 <sup>a,b</sup> (18.5–23.0)	1737.2 $\pm$ 114.9 <sup>a,b</sup> (1,510.2–1876.4)

Different superscripts indicate significant differences: a = vs. small follicles ( $P < 0.01$ ); b = vs. systemic values ( $P < 0.001$ )

antibody immobilised in the microplate wells. Subsequently, the second biotinylated anti-PIGF antibody and the streptavidin–peroxidase enzyme conjugate, binding the PIGF, bound to the first antibody in the wells. Finally, the substrate chromogen TMB (Neogen, USA) was added, developing a colour that will be of an intensity proportional to the PIGF concentration in the sample. The values of the standard curve were 25, 50, 125, 500 and 1,000 pg/mL. The detection limit of the technique was 1,062 pg/mL. The percentages of recovery in FF and plasma were paired to 99 and 105.1%, respectively. The intra- and inter-assay coefficients of variation (CV) were 5.23–5.87% and 6.75–6.81% in plasma and 5.5–6.5%, and 7.3–7.9% in FF, respectively. The technique was linear in all the dilutions used in both plasma and FF (1:25, 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160).

The concentrations of  $P_4$  (ng/mL) in FF were determined using a single antibody RIA kit (Coat-a-Count<sup>®</sup> TKPG-1; DPC, Los Angeles, CA). The values of the standard curve were 0.1, 0.5, 2.0, 10, 20 and 40 ng/mL. The limit of detection was 40 ng/mL. The intra- and inter-assay CV were 6.12 and 4.11%, respectively.

The concentrations of  $E_2$  (ng/mL) in FF were determined by a competitive enzyme-linked immunosorbent assay (Estradiol sensitive ELISA Demeditec DE4399) validated specifically for FF in the equine species. The values of the standard curve were 3, 10, 50 and 200 pg/mL. The limit of detection was 1.399 pg/mL. The percentage of recovery was 99.5%. The intra- and inter-assay CVs at low and high concentrations were 7.87 and 5.52% and 8.78 and 6.78%, respectively.

### Statistical analyses

Statistical investigations were performed using Statistica 12.0 (StatSoft Inc., Tulsa, OK, USA). Means ( $\pm$  SD) for PIGF,  $P_4$  and  $E_2$  in FF of small, medium-sized and large follicles were calculated. Normality was verified in all data groups using the Kolmogorov–Smirnov test. To determine the magnitude of variation in systemic and FF PIGF concentrations in follicles of different diameter, the data were subjected to one-way analysis of variance (ANOVA). *Post-hoc* comparisons were performed using Tukey's test. The relationships between FF and systemic PIGF concentrations, FF and systemic steroid hormones, PIGF and follicle diameters, and

PIGF and steroid hormones were examined by linear regression analysis, and expressed by Pearson's correlation coefficient. Differences were considered to be statistically significant when  $P < 0.05$ .

### RESULTS

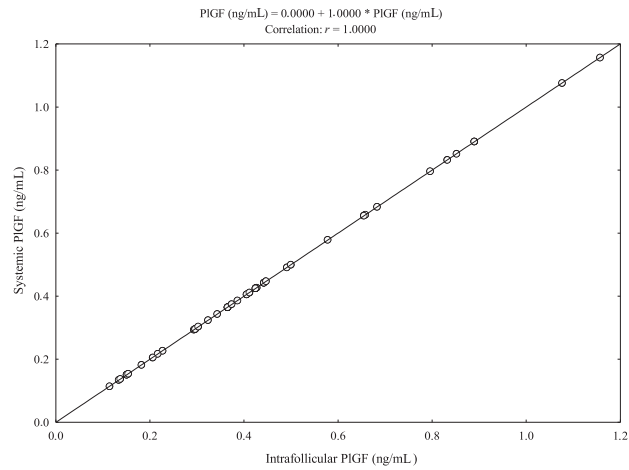
Table 1 shows the mean  $\pm$  SD and range concentrations of PIGF,  $P_4$  and  $E_2$  according to follicle sizes and the specific phase of reproductive cycle. PIGF concentrations were higher in large and medium-sized follicles compared to follicles of small size ( $P < 0.01$ ). Intrafollicular PIGF concentrations in medium-sized and large follicles were 1.48 and 1.36 times higher than the systemic PIGF values ( $P < 0.001$ ). Systemic PIGF concentrations were 1.3 times higher than PIGF concentrations in the FF of small follicles ( $P < 0.001$ ). Intrafollicular  $P_4$  concentrations were significantly higher in large follicles than in small ones, and the concentrations were 6.74, 3.25 and 3.42 times higher in large, medium-sized and small follicles than the systemic values. Intrafollicular  $E_2$  concentrations were significantly higher in large and medium-sized follicles than in small ones, and their concentrations were 21.1, 15.4 and 8.35 times higher, respectively, than the systemic values.

Intrafollicular and systemic PIGF concentrations were strongly and positively correlated ( $r = 1$ ;  $P < 0.001$ ; Fig. 1) in all follicle size categories, while no correlations were observed between intrafollicular and systemic steroid hormone concentrations, PIGF and follicle diameter ( $r = 0.18$ ), PIGF and  $E_2$  ( $r = 0.25$ ), and PIGF and  $P_4$  ( $r = 0.13$ ).

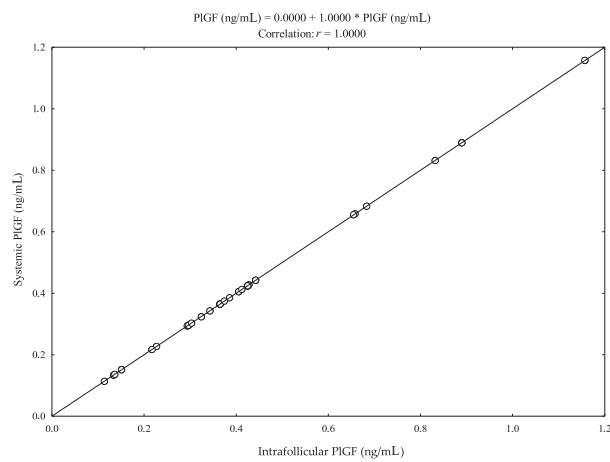
### DISCUSSION

This is the first study on intrafollicular and systemic PIGF concentration ranges in healthy cycling mares. The higher PIGF concentrations found in the FF of medium-sized and large follicles compared to those in small follicles and the systemic concentrations are consistent with similar findings reported in humans (Hou et al., 2014), although both intrafollicular and systemic PIGF concentrations were found to be higher in humans than in mares. In addition, FF PIGF

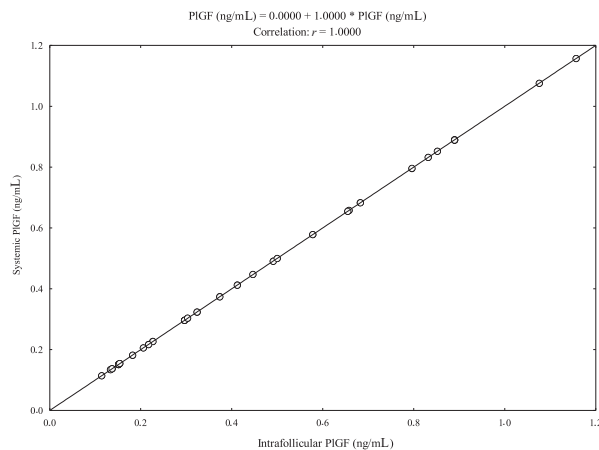




a)



b)



c)

Fig. 1. Scatter plot graph of correlations between systemic and intrafollicular placental growth factor (PIGF) concentrations in small (a) ( $r = 1.0$ ;  $P < 0.001$ ), medium-sized (b) ( $r = 1.0$ ;  $P < 0.001$ ) and large (c) ( $r = 1.0$ ;  $P < 0.001$ ) follicles

concentrations were 4–6 times higher than systemic PIGF concentrations in women (Tal et al., 2014) as well as in women undergoing *in vitro* fertilisation (Gutman et al.,

2008). A possible explanation for differences in the ability to determine PIGF in the blood samples between the various studies was the different type of biological medium used

(serum vs. plasma). FF represents a product of diffusion of molecules secreted by granulosa and theca cells, and of blood constituents crossing the blood–follicle barrier. Although the source of PlGF is unknown in the mare, in other species, such as humans and primates, there is consistent evidence that ovarian granulosa and endothelial cells represent the main source of PlGF (Tal et al., 2014; Bender et al., 2018). Moreover, in women a positive correlation between intra-follicular PlGF concentrations and follicle size was shown (Hou et al., 2014), while in mares no such correlation has been observed. It appears that PlGF can be regulated independently within each follicle size category. Therefore, the highest PlGF concentrations found in medium-sized and large follicles seem to indicate a higher need for angiogenic factors for maturation; indeed, local PlGF concentrations were found to be involved in ovarian follicle angiogenesis and may contribute to oocyte development, maturation and dominant follicle selection (Hou et al., 2014). Until now, the only study on PlGF in the equine species has been conducted in pregnant Spanish Purebred mares (Satué et al., 2018); in that study, the systemic concentrations of PlGF were higher than those measured in the mares of the present study. Although we cannot specify the origin of these differences, we suggest that the gestational state could have conditioned this increase. Namely, PlGF is an essential factor for cardiovascular adaptations along the pregnancy; in addition, embryo implantation and placentation require the development of an appropriate vascular network to ensure the proper exchange of nutrients between mother and fetus (Gourvas et al., 2012; Vrachnis et al., 2013). Moreover, the dominant follicle, the larger follicles and the follicles secreting the highest  $E_2$  concentrations were those that had a complete vascularisation and a higher concentration of VEGF in their compartments (Grazul-Bilska et al., 2007). Bashir et al. (2016) showed that the VEGF concentrations in the FF of 40-mm, impending-ovulation follicles were lower than those observed in follicles of 25–35 mm in diameter. Moreover, VEGF shared a primary structure, such as a limited amino acid sequence homology, with the A and B chains of PlGF; this similarity was also functionally evident in their ability to promote endothelial cell proliferation in the same form (Araújo et al., 2013). Taking into account the changes induced by VEGF in the ovary of mares and in the FF during the development of the dominant follicle, a similar evolution of PlGF in the FF could be expected. In women undergoing fertility treatment, hCG administration triggered an increase of PlGF mRNA and protein in granulosa cells, according to previous reports (Gutman et al., 2008; Tal et al., 2014). The delay between the administration of hCG and the increase of PlGF mRNA in granulosa cells suggested that a factor involved in the ovulatory gonadotropin surge could regulate the production of PlGF (De Falco, 2012). In primates, Bender et al. (2018) reported that when PlGF was neutralised, VEGF remained available to stimulate angiogenesis. The temporal patterns of PlGF and VEGF accumulation in the follicle could be due to their different roles in ovulatory angiogenesis, as observed in humans (Trau et al., 2016). Bender et al. (2018) showed that

PlGF and VEGF were not accumulated in granulosa cells; rather, they were rapidly released after their synthesis. The concentrations of VEGF in the FF peaked early in the ovulatory interval, whereas the concentrations of PlGF peaked just before ovulation. This temporal pattern is in line with the concept that endothelial cell migration occurs before capillary stalk formation (Jakobsson et al., 2010). These results indicate that local VEGF and PlGF gene expressions are likely to mediate ovarian follicular angiogenesis, contributing to the development and maturation of oocytes and dominant follicle selection. In humans, Nejabati et al. (2017) have recently shown that the PlGF/sFlt-1 ratio was significantly higher in high-responder patients to assisted reproduction programs than in poor responders. In the bovine species, gene expression profiling by a combination of microarray and quantitative real-time PCR (QPCR) analysis showed that two genes (PlGF and TSP2) that regulate the angiogenesis process were differentially expressed in larger ( $10.7 \pm 0.7$  mm) compared to small follicles ( $7.8 \pm 0.2$  mm) (Hayashi et al., 2010). These findings suggest that PlGF may contribute to angiogenesis in the theca of healthy follicles via paracrine/autocrine activity. In the absence of regulated angiogenesis, disorders of ovulation and luteinisation can occur in both women and primates, including the anovulatory infertility and the ovarian hyperstimulation syndrome (Fraser, 2006). Although in mares there is no evidence to date, in women with PCOS 4 to 6 times elevated concentrations of PlGF were measured in the FF (Artini et al., 2006, 2009; Tal et al., 2014). From the clinical point of view, follicular growth and CL development and their endocrine functions are closely correlated with the development of new capillaries, and thus their dysfunction could be related to a deficient vascularisation and infertility in mares, as reported previously (Ferreira-Dias et al., 2006). Likewise, it would be interesting to elucidate the changes that PlGF could promote in the FF of mares with ovulation disorders, such as anovulatory follicles, ovarian tumours or persistence of the CL.

The intrafollicular  $P_4$  concentrations found in this study were similar to those obtained by Tsukada et al. (2008) and Satué et al. (2019), and lower than those reported by Watson and Hinrichs (1988), who obtained significantly lower  $P_4$  concentrations in follicles of 32–34 mm than in follicles >35 mm. In addition,  $P_4$  concentrations in the FF found in this study were 20 times higher than those reported previously (Ginther et al., 2007). These differences found in  $P_4$  values between studies could be explained by the different analytical methods used.

The intrafollicular  $E_2$  concentrations measured in this study were consistent with those obtained by Bashir et al. (2016) and Satué et al. (2019) in similar follicle categories; nevertheless, they were higher than those reported by Watson et al. (2003) and Bridges et al. (2002), and lower than those measured by Gastal et al. (1999). These apparent differences in intrafollicular  $E_2$  concentrations among studies could be due to inter-individual differences between mares (e.g. different nutrition), as well as to the different types of assays used and the cross-reactivity of each assay



with other oestrogens. However, the assay used in this study had low cross-reactivity with other oestrogens, as documented previously (Satué et al., 2019).

No correlations among PIGF, E<sub>2</sub> and P<sub>4</sub> in the FF were observed in the mares of this study, which is consistent with previous findings in women (Tal et al., 2014). It is unknown which ovarian compartment may be responsible for PIGF production, but since there is a strong evidence that ovarian granulosa and endothelial cells are the main sources of the production of VEGF during hyperstimulation (Soares et al., 2008), it is reasonable to hypothesise that PIGF could be produced by similar mechanisms (Tal et al., 2014).

The aim of this pilot study was to characterise both systemic and intrafollicular PIGF concentrations in cycling normal mares. PIGF is locally synthesised, and its bioavailability is higher in medium-sized and large follicles than in follicles of small size, despite the fact that this factor acts independently of P<sub>4</sub> and E<sub>2</sub> dynamics. Thus, a more comprehensive view involving other mechanisms or factors should be considered according to PIGF changes during follicular angiogenesis also in mares, as has been done in humans and primates.

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