AKADÉMIAI KIADÓ

Acta Veterinaria Hungarica

68 (2020) 1, 79-84

DOI:
10.1556/004.2020.00011


ORIGINAL ARTICLE

# Modulation of the renin–angiotensin–aldosterone system by steroid hormones during the oestrous cycle in mares

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Received: May 7, 2019 • Accepted: November 13, 2019
Published online: May 8, 2020

**ABSTRACT**

In women and females of different species of laboratory animals, oestrogens stimulate the renin–angiotensin–aldosterone system (RAAS) by increasing tissue and circulating levels of angiotensinogen and renin during the preovulatory period. Progesterone and cortisol compete with aldosterone for mineralocorticoid receptors, which results in increased $Na^+$ reabsorption during the postovulatory period. The purpose of the current research was to analyse the relationship of oestradiol-17$\beta$, progesterone and cortisol with RAAS in 23 mares during an oestrous cycle. During the preovulatory period, significant positive correlations of oestradiol-17$\beta$ with renin and aldosterone concentrations and negative correlations of progesterone with renin and aldosterone concentrations were found. In contrast, during the postovulatory period, oestradiol-17$\beta$ concentrations were positively correlated with angiotensin concentrations and progesterone was negatively correlated with this component of the RAAS. Cortisol concentrations were not correlated with the hormones of the RAAS, neither before nor after ovulation. This research demonstrates that, as occurs in other species, changes in the RAAS during the periovulatory period in mares may be modulated by variations in the concentrations of steroid hormones.

**KEYWORDS**

oestrus, mare, renin–angiotensin–aldosterone system

## INTRODUCTION

In women and in females of laboratory animals, steroid hormones modify the activity of the renin–angiotensin–aldosterone system (RAAS) during the menstrual/oestrous cycle. In physiologically normal cycles of these species, the concentrations of renin (REN), angiotensin (ANG) and aldosterone (ALD) increase during the luteal phase (Szmuilowicz et al., 2006; O'Donnell et al., 2014). The corpus luteum is the primary source of REN and ALD, and a significant correlation has been described between progesterone ($P_4$), REN and ALD during the dioestrus (García et al., 2008). Some mechanisms dependent on $P_4$, including an increase in plasma flow and in the glomerular filtration rate and the excretion of $Na^+$ and $Cl^-$, cause natriuresis (Chapman et al., 1997). Natriuresis induced by $P_4$ stimulates a compensatory secretion of REN, ANG and ALD (Oelkers, 1996; Szmuilowicz et al., 2006). However, the peak concentrations of oestrogens and $P_4$ during the luteal phase can reach values two or three times higher than those of REN, ANG and ALD. Additionally, the physiological increase of oestrogens during the first half of the cycle does not substantially increase the concentrations of REN or ALD (Szmuilowicz et al., 2006; O'Donnell et al., 2014).

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The expression of angiotensinogen is transcriptionally regulated by oestrogens. The activation of RAAS may be partly related to the synthesis of angiotensinogen, even though this synthesis does not occur in all cycles (Oelkers, 1996; Brosnihan et al., 1999). Combined administration of oestrogens and $P_4$ to ovariectomised mice leads to an increase in REN concentrations without parallel changes in angiotensinogen concentrations. Furthermore, the lack of a significant relationship between REN and angiotensinogen concentrations excludes oestrogens as potential causes of increased REN, despite their participation in ALD secretion (Ojeda et al., 2007).

Although the main source of the circulating active REN is the kidney, the cyclic increase of prorenin during the oestrous cycle seems to come from the ovaries (Sealey et al., 2010). Luteinising hormone (LH) causes a 3 times increase in prorenin concentration, which persists on an elevated level during the first half of the luteal phase. At the end of the dioestrus, the reduction in $P_4$ induces a simultaneous reduction of prorenin (Sealey et al., 1987; García et al., 2008).

Preovulatory increases of angiotensinogen, REN, ANG and ALD have been described in different species, including mares (Satué et al., 2017). In women (Sealey et al., 1994) and in female rats (De Vito et al., 1989), the increase in oestradiol-17$\beta$ ($E_2$) seems to exert a stimulatory effect on angiotensinogen at the hepatic level and the latter, in turn, stimulates REN and ALD concentrations. In contrast, earlier investigations (Sundsfjord and Aakvaag, 1972) rejected the hypothesis of any influence of oestrogen concentrations on the synthesis of REN and ANG during the follicular phase in women. Although these investigations provide contradictory results, both support the hypothesis that the increase in angiotensinogen is the reason for the peak in REN concentrations.

Adrenocorticotrophic hormone (ACTH) is one of the main regulators of ALD secretion during the ovulation period, because of the stimulating effect exerted by oestrogens on the adrenal glands (Szmuilowicz et al., 2006). It has been shown that the infusion of ACTH increases REN concentrations, although this was not accompanied by changes in cortisol (CORT) concentrations during the cycle (Szmuilowicz et al., 2006).

The aim of the current research was to analyse whether the relationships between steroid hormones and RAAS in mares in relation to the phase of the oestrous cycle are similar to those previously described for women and females of laboratory animal species. The hypotheses to check were: firstly, if the increase in $E_2$ synthesis during the preovulatory period would be associated with the activation of the RAAS, and secondly, if the changes in RAAS during the postovulatory period would be correlated with the increase in $P_4$. Understanding the changes of the RAAS under the influence of steroid hormones in healthy breeding mares would improve the knowledge about physiological changes related to hormonal alterations during the oestrous cycle.

## MATERIALS AND METHODS

This research was approved by the Animal Ethics Committee of the CEU-Cardenal Herrera University (Valencia, Spain).

### Mares

The study was carried out on 23 healthy Spanish broodmares aged between 5 and 15 years, belonging to four different farms located in the East of Spain. A total of 30 mares were arbitrarily selected from the farms, provided that they met the following inclusion criteria: (1) absence of reproductive diseases at the clinical examination; (2) absence of inflammatory processes or infections that had required treatment or hospitalisation during the month prior to the start of the study; (3) be updated in vaccination and deworming; (4) be younger than 15 years old, have no conformation defects that affect the perineum and vulva, and lack of previous history of reproductive diseases affecting fertility. The study was performed during the months of February, March and April of 2018.

All animals were subjected to the same conditions of management, feeding and reproductive control. They were fed a diet composed of 4 kg of mixed grains (oats, barley and corn flakes) and 3 kg of alfalfa per day, divided into two intakes. Water was provided *ad libitum* and the animals had access to a mineral block during the study period.

### Reproductive monitoring of the mares

Follicular development was checked by rectal ultrasound examination (Ultrasound: Sonosite 180 Plus) daily until the time of ovulation. After ovulation, daily echographic exams were also performed to confirm the development and maturation of the corpus luteum, up to 5 days after ovulation. Only natural cycles were included.

### Venous blood sampling

Blood samples were taken from the mares at the time when they began to show signs of oestrus. Because the objective of the current study was to establish the relationships between several steroid hormones and RAAS during the periovulatory period, blood samples were taken every day, from day −5 to day +5 of ovulation. To reduce the influence of daily rhythms on the release of hormones, all blood samples were withdrawn between 9:00 and 10:30 AM, always by the same operator and before the mares received their grain ration.

To measure ANG, blood was placed into chilled tubes with 0.125 M EDTA and 0.025 M o-phenanthroline (199 $\mu$l/ml plasma, P-9375; Sigma Chemicals, St. Louis, MO), centrifuged at 1,000 g for 15 min at 4 °C, and the plasma was extracted. The remaining blood was poured into tubes without anticoagulant to obtain serum for the measurement of ALD, $E_2$, $P_4$ and CORT concentrations and into EDTA tubes for the measurement of REN concentrations (5 mL). The samples for ANG measurements were placed in a

CryoPac shipper (−196 °C) cooled in liquid nitrogen and transported to the laboratory, where they were stored at −70 °C until analysis.

### Measurements of hormone concentrations

REN concentrations were measured by an immunoradiometric sandwich technique to detect active REN in plasma. Polystyrene tubes coated with rabbit anti-REN polyclonal antibodies (Biogenesis, Morphosys 7,929–9,930, Cergy Saint-Christophe, France) and a secondary antibody labelled with I-125 (DRG Diagnostics, 10,285, Marburg/Lahn, Germany) were used. This measurement procedure shows a high specificity for REN (95–105% recovery percentage of the sample). The sensitivity of this technique was 1 pg/mL. The intra- and inter-analysis coefficients of variations (CV) were <5 and 15%, respectively.

ANG concentrations were measured with a competitive ELISA protocol based on the biotin-streptavidin-peroxidase system. ELISA assay plates coated with a primary antibody (polyclonal antibody anti-ANG-II, RAB 002-12, Phoenix Pharmaceuticals, Inc., Belmount, CA, USA), secondary antibody (Phoenix Pharmaceuticals, Inc., Belmount, CA, USA), biotinylated ANG-II (Phoenix Pharmaceuticals, Inc., Belmount, CA, USA) and streptavidin-peroxidase (EK-SA-HRP, Phoenix Pharmaceuticals, Inc., Belmount, CA, USA) were used. This laboratory procedure shows high specificity for ANG-II (percentage recovery = 98.5%). The sensitivity of the technique was 100 pg/mL, while the intra- and inter-analysis CVs were <5% and 14%, respectively.

ALD concentrations were measured with a competitive ELISA using polyclonal antibody AD97 and the combination ALD 3CMO-HRP (Endocrinology Laboratory, Complutense University of Madrid, Spain). This test showed high specificity for ALD (percentage recovery = 97.6%). The sensitivity of this technique was 15 pg/mL. Intra- and inter-analysis CVs were 4.7–6.4% and 8.5–9.6%, respectively.

$P_4$ levels were analysed by solid-phase I-125 radioimmunoassay (RIA) (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, USA). The CV inter- and intra-assay for P4 were as follows: 16.1% and 4.3% at 3.5 nmol/L; 7.3% and 8.5% at 22.5 nmol/L; 23.3% and 6.4% at 54.8 nmol/L. The minimal assay sensitivity of P4 was 0.1 ng/mL. The concentrations of E2 (ng/mL) in plasma were determined by a competitive enzyme-linked immunosorbent assay (E2 Sensitive, Demeditec ELISA DE4399) validated specifically for the equine species. The limit of detection was 1.4 ng/mL. The percentage of recovery in plasma was 98.72%. The intra- and inter-analysis CVs at low and high concentrations were 7.87 and 5.52% and 8.78 and 6.78%, respectively.

Total cortisol (CORT) concentrations were measured by competitive immunoassay using C97 polyclonal antibodies (Endocrinology Laboratory, Complutense University Madrid, Madrid, Spain). This laboratory procedure shows high specificity for cortisol. The sensitivity of the technique was 3 pg/100 $\mu$L. The intra-assay CVs were between 3.7% and 6.63% at low concentrations and between 3.92% and 9.93% at high concentrations.

### Statistical analysis

Statistical analysis was carried out using SPSS 12.01 for Windows (SPSS Inc.). Normality of the data and equality of variances were assessed using Shapiro-Wilk's and Levene's tests, respectively. Data were not normally distributed. Correlations between variables were analysed with a Spearman rank correlation test. The probability level was fixed at $P < 0.05$.

## RESULTS

Correlations between RAAS and steroid hormones during the pre- and postovulatory period are presented in Table 1. During the preovulatory period, significant correlations were found between $E_2$ and $P_4$ with REN and ALD (positive with $E_2$ and negative with $P_4$). During the postovulatory period, $E_2$ and $P_4$ showed moderately significant correlation with ANG, and $P_4$ was also correlated with ALD. Cortisol showed non-significant correlation coefficients with the components of the RAAS.

*Table 1.* Spearman rank correlation coefficients during the pre- and postovulatory period in 23 mares

| | REN | ANG | ALD | $E_2$ | $P_4$ |
|---|---|---|---|---|---|
| Preovulatory period | | | | | |
| ANG | 0.397 | | | | |
| ALD | 0.784[a] | 0.450 | | | |
| $E_2$ | 0.859[a] | 0.360 | 0.834 [a] | | |
| $P_4$ | −0.903[a] | −0.371 | −0.863[a] | −0.938[a] | |
| CORT | 0.281 | 0.468 | 0.365 | 0.218 | −0.257 |
| Postovulatory period | | | | | |
| ANG | −0.545 | | | | |
| ALD | 0.595 | −0.704[a] | | | |
| $E_2$ | −0.430 | 0.711[a] | −0.584 | | |
| $P_4$ | 0.480 | −0.852[a] | 0.663[a] | −0.850[a] | |
| CORT | 0.513 | −0.512 | 0.585 | −0.314 | 0.381 |

REN, renin; ANG, angiotensin; ALD, aldosterone; $E_2$, oestradiol-17$\beta$; $P_4$, progesterone; CORT, cortisol.
[a] Statistically significant at $P < 0.05$.

## DISCUSSION

The present study evaluated the relationships between steroid hormones and the components of the RAAS during the oestrous cycle, in an attempt to reveal endocrine aspects scarcely investigated in the mare. We hypothesised that $E_2$ concentrations would be the main determinant of the RAAS before ovulation, whereas P4 would determine the functionality of the axis after ovulation. These hypotheses have been confirmed but our results also suggest that $P_4$ modulates the RAAS, with a negative effect on it, both before and after ovulation. In addition, it has been demonstrated that before ovulation, steroid hormones were more related to REN and ALD, whereas after ovulation, the relationships were marked with ANG.

Studies carried out in women have demonstrated that oestrogens activate tissue or plasmatic mechanisms directly related to an overexpression of angiotensinogen and REN, with an inhibition of the angiotensin-converting enzyme and ANG-II. Under these circumstances, angiotensinogen, prorenin and REN increase simultaneously until the time of ovulation, with positive correlations between them, while ANG-II decreases (Owonikoko et al., 2004; Komukai et al., 2010). Similar results have been previously reported in mares by Satué et al. (2017). The results of these authors are in agreement with those found in the present study, since we found significant positive correlations of $E_2$ with REN and ALD in the preovulatory period.

The mechanism by which oestrogens participate in the synthesis of angiotensinogen is based on promoting the region of the gene that encodes angiotensinogen, making itself sensitive to oestrogens. The administration of oestrogens favours the synthesis and hepatic release of these proteins. This experimental evidence has been documented in hormone replacement therapies in menopausal women and in oral contraceptive treatments (Oelkers, 1996; Giribela et al., 2015).

Despite the positive significant correlations between $E_2$ and REN and ALD, in the present research significant correlations between $E_2$ and ANG were not found in the preovulatory period. This apparent dissociation between ANG with REN and ALD in the preovulatory period has been previously reported in women (Oelkers, 1996), bitches (Owonikoko et al., 2004) and laboratory animals (Macova et al., 2008). It has been attributed to a reduction in the mRNA expression of the angiotensin-converting enzyme, reducing tissue sensitivity to ANG-II (Prime et al., 2007) and reducing the expression of AT1 receptors in the tissues. At the central level, oestrogens suppress the expression of AT1 receptors in the pituitary gland, reducing the synthesis of ACTH and the production of ALD.

Among the factors related to the dissociation between REN and ALD, the most relevant are the negative feedback effects of ANG-II in the synthesis of REN (Xu et al., 2008) and the modifications in the synthesis pattern of ANG-derived peptides at the tissue level, as is the case with ANG(1–7). This deviation of peptide synthesis by inhibiting the activation of angiotensin-converting enzyme halts the activation of the RAAS (Brosnihan et al., 1999).

The close relationship between $E_2$, REN and ALD during the preovulatory period in the mare seems to suggest that the activation of the RAAS is in part related to these steroids. The correlation between ANG and ALD was mild ($r$ = 0.450), lower than expected, suggesting that the contribution of ANG to the synthesis of ALD was quantitatively lower compared to the effect of $E_2$. These results contrast with those found by us in pregnant mares (Satué and Domingo, 2011).

A positive moderate correlation between ALD and $P_4$ ($r$ = 0.663) was found after ovulation in the mares, which is a result opposite to that found during the preovulatory period, when a negative correlation existed between these two hormones. The binding of ALD to the mineralocorticoid receptor situated at cell level in the distal tubules allows resorption of $Na^+$ and fluid retention. This receptor has the same affinity for $P_4$ and ALD, so that when it joins $P_4$, which is a competitive ALD inhibitor, it antagonises its effects, inducing a transient natriuretic effect (Oelkers, 1996). This initial natriuretic state induced by $P_4$ triggers the compensatory activation of the RAAS and an increase in ALD (O'Donnell et al., 2014). These relationships are based on results derived from experimental studies in rodents in which the addition of $P_4$ to isolated cells from the glomerulus zone *in vitro* favours the synthesis of ALD (Szmuilowicz et al., 2006). Likewise, treatment with exogenous $P_4$ during extended periods of time also significantly increases ALD in women (Stachenfeld and Taylor, 2005), without causing modifications to plasma REN activity and ANG concentrations. However, previously we found a lack of significant correlations between $P_4$ and ALD ($r$ = 0.002) in pregnant mares (Satué et al., 2011), suggesting that the physiological retention of $Na^+$ cannot be explained based on antagonism, so this must be due to different physiological mechanisms of $P_4$ actuation depending on each phase of the reproductive cycle.

Nevertheless, the simultaneous administration of $P_4$ and oestrogens increases plasma REN activity, ANG and ALD concentrations and decreases $Na^+$ excretion in women (Stachenfeld and Taylor, 2005), suggesting that both $P_4$ and oestrogens could be responsible for the activation of the RAAS after ovulation. This evidence in women appears to contrast with that found in the mares of our study, with lower significant correlations between $E_2$ and ALD. These results might suggest that, during the postovulatory period, the activation of the RAAS appears to be more linked with $P_4$, with a lower contribution from oestrogens. Nevertheless, in women Seely et al. (2004) described that $P_4$ was the only hormone to induce ALD via RAAS activation, as oestrogen inhibits this response; these results seem to be more in accordance with those found in our mares. In mares it is unknown whether additional mechanisms exist which would contribute to the increase of ALD during the oestrous cycle. Future studies are needed to further define ALD modifications mediated by $P_4$ and other factors during the complete luteal period.

In the current research, correlations of CORT with RAAS components, as well as with $E_2$ and $P_4$, did not reach the level of statistical significance. In contrast, Asa et al. (1983) documented a negative correlation between CORT and $E_2$ and follicular diameter, and a positive correlation between CORT and $P_4$. This negative relationship between CORT, the degree of follicular growth and $E_2$ concentrations suggested a possible inhibitory influence of CORT on follicular growth. This relation might have indicated that the synthesis of endogenous $E_2$ is not a stimulating factor in the adrenal liberation of this glucocorticoid. In the present study, correlations showed that the influence of $E_2$ secreted by the preovulatory follicle exerted a very limited effect on the adrenal synthesis of CORT. Consequently, other factors should be considered in order to explain increased CORT concentrations during this period. Our results are not in agreement with the ovulatory peak of CORT found in women (Wolfram et al., 2011). This last study gave evidence of a simultaneous CORT peak alongside $E_2$, occurring earlier in rats than in women. Ovariectomy leads to a sudden reduction in CORT, which later can be compensated by the exogenous administration of $E_2$. Oestrogens take part in the synthesis of CORT by means of a direct stimulating effect on the recipient protein for this glucocorticoid (Qureshi et al., 2007). In heifers, this increment was related to an increase in the activity of the enzyme 11-hydroxysteroid dehydrogenase (11-HSD), which acts in the conversion of cortisone to CORT. However, there is a lack of consistency between the results reported by different authors, probably due to the wide variety of methods used, study protocols, sample size, influence of diurnal rhythms, animal management, nutrition, etc.

In conclusion, during the preovulatory period, the positive correlations between $E_2$, REN and ALD might suggest that the activation of the RAAS is partly associated with the release of oestrogens. During the postovulatory period, changes in ANG concentrations appear to be related both to the reduction of $E_2$ and to the increase in $P_4$, and ALD concentrations were associated with $P_4$. The results of the present study confirmed the modulatory effect exerted by steroid hormones on the activation of the RAAS in different moments of the oestrous cycle in the mare.

## ACKNOWLEDGEMENTS

The authors sincerely thank the owners of the mares for their collaboration in carrying out this study.

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