Kisspeptin-10 and gonadotropin inhibiting hormone during pregnancy in dairy cows

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Summary
Recently, two different molecules have been discovered to play an important role in reproduction: kisspeptin (Kp) and gonadotropin inhibiting hormone (GnIH). The aim of this study was to establish the trend of kisspeptin 10 (Kp-10) and GnIH concentrations, during all phases of pregnancy in cattle, in order to understand their possible role in the physiology of pregnancy. To examine the correlation between these hormones and steroid hormones, cortisol and oestradiol 17β (E2) were also analyzed. Eighty pregnant cows were enrolled and the pregnancy was divided into 8 periods of 30 days each (from 30-60 days to 240-270 days). One blood sample was collected from each cow of the groups. Kp-10, GnIH, cortisol and E2 were measured in sera. After an initial plateau, Kp-10 concentrations increased at 90-120 days. It subsequently decreased until 180-210 days, to then further increase until 240-270 days. GnIH concentrations decreased until 90-120 days, then increased until the end of gestation. These trends were opposing until 180-210 days, then concentrations of both increased until the end of gestation. Cortisol concentrations were homogenous at all times, except at the final period, in which they were higher. E2 showed two peaks, at 90-120 days and 240-270 days. The trends in Kp-10 and GnIH concentrations suggest that these two hormones might act to maintain the delicate endocrine equilibrium of pregnancy.

Introduction

Pregnancy is a fragile and complex phase of reproduction, the endocrine status of which has been extensively studied. Progesterone (P4) is the main hormone involved in the maintenance of pregnancy. Blood progesterone levels increase slightly during pregnancy, then decline around 20-30 days prepartum (Noakes *et al.* 2019). Oestrogens (E2) also are important, as levels in the maternal peripheral circulation start to increase from around 90-120 days to 150-180 days, then drop slightly at around 180-210 days. After this time, E2 levels rise again at 240-270 days and decline rapidly to zero, postpartum (Minoia *et al.* 1987, Rizzo *et al.* 2009). An optimal E2/P4 ratio is essential for the promotion of uterine contractility necessary for placental exchanges and function during pregnancy, and for calving at the end of pregnancy (Piccinno *et al.* 2016). The calving event is triggered by foetal stress, which activates the hypothalamic-pituitary-adrenal axis, and increases corticotropin releasing hormone (CRH) release from foetal hypothalamus (Senger 1999). Corticotropin releasing hormone stimulates corticotropin secretion by anterior pituitary corticotropic cells, which in turn leads to a surge in foetal cortisol secretion (Mastorakos and Ilias 2003). The activation of different placental enzymes determines the shift from P4 to E2 production in maternal tissues, with a sharp rise in oestrogens that promote myometrial contractility (Romero *et al.* 1988).

Recently, two different molecules have been discovered to play an important role in reproduction: kisspeptin (Kp) and gonadotropin inhibiting hormone (GnIH) (Wang *et al.* 2018).

Kisspeptin belongs to a family of peptides encoded by the KISS-1 gene, which synthesizes a 145-amino acid peptide which is proteolytically cleaved to a 54-amino acid product and further degraded to one of three shorter peptides of 14, 13 or 10 amino acids in length (kisspeptin-14, kisspeptin-13 and kisspeptin-10). Kisspeptin-10 has greatest affinity for the G protein-coupled receptor 54 (GPR54) and plays an important role in controlling the gonadal axis, since it stimulates luteinizing hormone (LH) release through its effect on GnRH neurons (De Roux *et al.* 2003).

Gonadotropin inhibiting hormone, identified by Tsutsui and colleagues (Tsutsui *et al.* 2000) in the Japanese quail, is a novel hypothalamic dodecapeptide, which acts through the G protein-coupled receptor 147 (GPR147) (Hinuma *et al.* 2000) and belongs to the RFamide-related peptide (RFRP) family. The 173 amino acid precursor is cleaved into two shorter peptides, RFRP-1 and RFRP-3 (Tsutsui *et al.* 2000). RFamide-related peptide-3 inhibits gonadotropin release *in vitro* and *in vivo* (Clarke *et al.* 2008, Kadokawa *et al.* 2009).

Both Kp and GnIH are involved in pregnancy, as demonstrated in different species (Reynolds *et al.* 2009, Sabet Sarvestani *et al.* 2014, Martino *et al.* 2015). In particular, it has been reported that Kp levels increase by 940-fold in the first trimester, and further increase to around 7,000-fold higher in the third trimester, then fall to concentrations comparable with those prior to pregnancy, at 5 days post delivery, implicating a placental source of the peptide (Horikoshi *et al.* 2003).

Studies regarding the involvement of GnIH in pregnancy are scarce. Sabet Sarvestani and colleagues (Sabet Sarvestani *et al.* 2014) have reported persistent GnIH mRNA expression at the dorsomedial hypothalamic nucleus and that glucocorticoids causes an increase in GnIH, during pregnancy in rats.

Based on these data, the aim of this study was to establish the trends in concentrations of these two novel peptides, Kp-10 and GnIH, during all phases of pregnancy in cattle, in order to understand their possible role in the physiology of pregnancy. To examine the correlation between these hormones and steroid hormones, cortisol and oestradiol 17β were also measured.

Materials and methods

All procedures were conducted in accordance with the institutional guidelines on the welfare and use of animals, and with the informed consent of the owner and ethics committee (protocol No. 25/18).

The study was carried out at the Azienda Agricola Posta La Via, located in the countryside of San Giovanni Rotondo (FG). The farm contains approximately 500 cows, of which an average of 180 were lactating. The free-housed animals were fed with total mixed ratio, composed of corn silage, oat hay, medical hay, corn flour, soybean meal, cotton, crushed barley, beet pulp, and vitamin and oligomineral supplements.

Animals

Eighty Italian Friesian pregnant cows, from 4 to 6 year old, free from non-infectious and infectious diseases, were enrolled in this study. All cows were previously bred using artificial insemination approximately 12 h following the onset of standing oestrus. Pregnant cows were identified through farm management software and pregnancy status was confirmed by clinical examination using B-mode ultrasonography (SonoSite MicroMaxx Bothell WA, USA, with a 7.5 MHz linear probe), as summarized by Hughes and Davies (Hughes and Davies 1989). In the experimental group there were no twin pregnancies.

Pregnancy was divided into 8 periods of 30 days each (from 30-60 days to 240-270 days) and the cows were enrolled based on their gestational period.

A single blood samples was collected from 80 cows that were at various stages of gestation, from the coccygeal vein into cold vacutainer glass tubes (BD Vacutainer®, UK). Once in the laboratory, blood was centrifuged at 1,620 g for 10 min at 4 °C. Serum was stored in 1.5-ml Eppendorf tubes at - 80 °C, until further analyses. Serum levels of Kp, GnIH, cortisol and oestradiol 17β (E2) were measured.

Laboratory analysis

Kisspeptin-10 (Kp-10) concentrations were determined using a radioimmunoassay (RIA) kit (Phoenix Pharmaceuticals, Inc. Burlingame, CA, USA; range 10-1,280 pg/ml; specificity 100%; intra assay CV 5.8%, inter assay CV 8.2%), as previously described (Rizzo *et al.* 2018, Rizzo *et al.* 2019). Prior to measurement, the specificity of the human kit for bovine Kp-10 was verified as reported by Mondal and colleagues (Mondal *et al.* 2015), using human samples, bovine samples, mixed human plus bovine samples at varying ratios, and mixed bovine and Kp free plasma samples.

Gonadotropin inhibiting hormone concentrations were determined using a bovine specific ELISA kit, following the manufacturer's instructions (MyBioSource Inc., San Diego, CA, USA; precision intra assay/inter assay CV% < 10; sensitivity 1.0 pg/ml; no significant cross-reactivity with GnIH analogues was observed).

Cortisol concentrations were determined using a competitive bovine specific kit (CSB-E13064B Cusabio; detection range 0.049 ng/ml-200 ng/ml; sensitivity < 0.049 ng/ml; no significant cross-reactivity or interference between bovine cortisol and analogues was observed; intra-assay precision CV% < 8, inter-assay precision CV% < 10).

Estradiol-17β concentrations were determined by an immunoenzymatic method (Estradiol ELISA, Dia. Metra S.r.l., Italy). The cross-reactions of antibodies were as follows: E2 100%, oestrone 2%, oestriol 0.39%, testosterone 0.02%, cortisol < 7 × 10^{-3}%, progesterone < 3 × 10^{-4}% and DHEA-S < 1 × 10^{-4}%. The lowest detectable concentration was 15 pg/ml, at the 95% confidence level.

Statistical analysis

The results are expressed as mean ± standard deviation (SD). Statistical analyses were conducted with SPSS version 19 (IBM, New York, USA). Hormone concentrations in the different phases of the pregnancy were analysed using one-way ANOVA, with Fisher's least significant difference test post hoc. Pearson's test was used for correlations between Kp-10, GnIH, cortisol and E2, in all phases of pregnancy. The values were considered statistically significant at $P < 0.05$.

Results

Serum Kp-10 and GnIH, and Cortisol and E2 concentrations are shown in Figure 1 and Figure 2, respectively. The correlations between different hormones are shown in Table I.

Mean serum Kp-10 concentrations, at all timepoints, were higher than those described in the postpartum period (Rizzo *et al.* 2019). After an initial plateau, Kp-10 levels increased at 90-120 days, decreased until 180-210 days ($P < 0.05$), and increased again until 240-270 days ($P < 0.05$).

Regarding GnIH, there are no reported values in the literature for either humans or animals. In our study, concentrations decreased until 90-120 days, then increased throughout the remaining gestation period.

Cortisol concentrations were within the expected range for the species (Patel *et al.* 1996) and showed an homogeneous trend in all periods, except during the final period (240-270 days), in which concentrations were significantly higher ($P < 0.05$).

Concerning oestradiol 17β levels, an initial peak was noted at 90-120 days, and a second at 240-270 days.

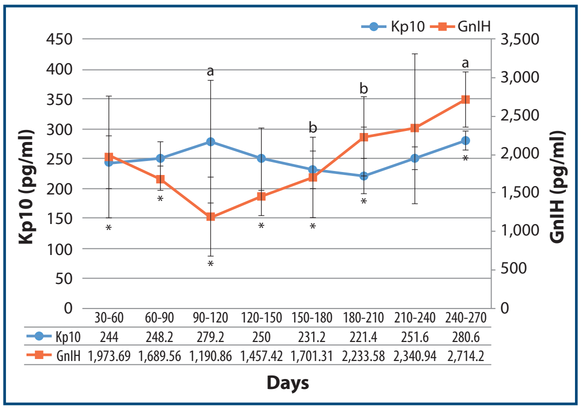

Figure 1. *Concentrations (mean ± S.D.) of Kisspeptin 10 and GnIH during different phases (8 periods of 30 days each, from 30-60 days to 240-270 days) of dairy cow pregnancy.* For Kp-10: a, b: $P < 0.05$. For GnIH: *: $P < 0.05$.

Discussion

The present study investigated blood levels of Kp-10 and GnIH, throughout bovine pregnancy, and examined their correlation with cortisol and E2. The complex interplay between the endocrine system and pregnancy has been widely studied, even though data on these novel hormones are scarce

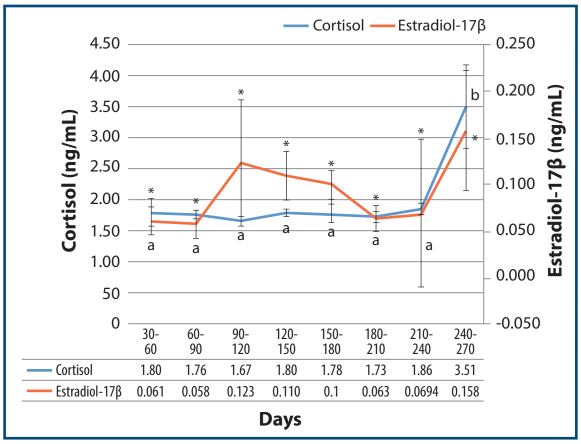

Figure 2. *Concentrations (mean ± S.D.) of cortisol and estradiol 17β during different phases (8 periods of 30 days each, from 30-60 days to 240-270 days) of dairy cow pregnancy.* For cortisol: a, b: $P < 0.05$. For estradiol 17β: *: $P < 0.05$.

Table I. *Pearson' correlation among Kisspeptin 10 (KP), GnIH, cortisol and estradiol 17β (E2) concentrations detected during different phases (8 periods of 30 days each, from 30-60 days to 240-270 days) of dairy cow pregnancy.*

Days	Hormones	Pearson' correlation	Significance
30-60	E2 - GnIH	r = - 0.893	P < 0.01
60-90	E2 - KP	r = + 0.893	P < 0.01
90-120	KP - GnIH	r = - 0.778	P < 0.01
	E2 - KP	r = + 0.739	P < 0.01
120-150	Cortisol - GnIH	r = + 0.682	P < 0.05
150-180	KP - GnIH	r = - 0.833	P < 0.01
	Cortisol - GnIH	r = + 0.814	P < 0.01
240-270	E2 - KP	r = + 0.717	P < 0.05

were similar to those reported by other authors (Piccinno *et al.* 2016).

Blood levels of Kisspeptin-10 found in the present study were higher than those found postpartum (Rizzo *et al.* 2019), probably due to the importance of Kp in placentation and maintenance of pregnancy. In fact, Martino and colleagues (Martino *et al.* 2015) demonstrated that the Kiss-1R/KPs system is involved in the regulation of proliferation of bovine placental cotyledon cell lines isolated during the first trimester of pregnancy. Thus, the high Kp-10 levels found in the present study are likely to be partially derived from placental production also, as demonstrated in human females (Reynolds *et al.* 2009).

Kisspeptin-10 peaked at 90-120 days and at 240-270 days. These peaks coincided with the sharp increase in oestrogen noted to occur during bovine pregnancy. At 90-120 days of pregnancy, the high levels E2 function to adjust the histotrophic environment necessary for conceptus growth and development (Piccinno *et al.* 2016), whereas the peak in E2 at 240-270 days is necessary for proper uterine contractility approaching calving (Senger 1999). With this in mind, the increase in Kp-10 could act to directly stimulate GnRH nuclei, and/or the hypophisis, to produce gonadotropins that, in turn, increase E2 production. This hypothesis is also supported by the positive correlation found between E2 and Kp-10, at 90-120 days and 240-270 days, in the present study.

Regarding GnIH in pregnancy, no data for either humans or animals are currently available. The values reported in the present study represent the first in bovine blood. In contrast to Kp-10, the GnIH trend decreased until 90-120 days, then significantly increased until 240-270 days of gestation. The nadir of the curve occurred at 90-120 days, at the point where Kp-10 peaked. This can be explained by the inhibitory effect on gonadotropin secretion and synthesis exerted by GnIH on the pituitary (Wang *et al.* 2018). In fact, it has been shown that GnIH inhibits GnRH-elicited gonadotropin release, and decreases LH pulse amplitude in female sheep (Iwasa *et al.* 2017). Moreover, it was demonstrated that treatment with progesterone, or treatment with oestradiol plus progesterone, significantly decreased activation of GnIH cells in the Syrian hamster (Benton *et al.* 2018). Similarly, elevated oestrogen lowers GnIH mRNA levels in male and female mice (Poling *et al.* 2012). In the present study, GnIH was low at 90-120 days, when Kp-10 peaked and E2 increased. As confirmation, a negative correlation between Kp-10 and GnIH was found at 90-120 days.

It is known that there is a close correlation between GnIH and stress. In fact, many studies have shown that psychological and immunological stress induces an increased detection of GnIH-immunoreactive cells and GnIH gene expression in the hypothalamus (Kirby *et al.* 2009, Iwasa *et al.* 2017). Gingerich and colleagues (Gingerich *et al.* 2009) reported that corticosterone administration increased GnIH mRNA expression levels in a cell line derived from rat hypothalamus. Moreover, a study in female sheep showed that chronic and acute stress activated GnIH cells in the preoptic region of the brain, due to the effect of cortisol (Clarke *et al.* 2016). These results strongly indicate that GnIH cells play an important regulatory role in stress response, in the mammalian hypothalamic-pituitary-gonadal axis. The results of the present study support this indication, as GnIH peaked just at the end of gestation, when glucocorticoid levels increase to trigger calving. Cortisol, in fact, is a key hormone activating a series of adaptive physiological responses to induce parturition. Moreover, a positive correlation between GnIH and cortisol, at 120-150 and 150-180 days, was found in the present study.

Conclusions

In conclusion, this is a preliminary study on blood Kp-10 and GnIH concentrations throughout bovine pregnancy. Opposite trends in the levels of these novel neuropeptides until 180-210 days of gestation, and a simultaneous increase in both parameters until the end of pregnancy, were observed in the present study. These results suggest these two hormones might act in the fragile process of pregnancy. It is known that during the first gestational period, Kp-10 stimulates the increase of steroid hormones, particularly E2, whereas GnIH levels must be low to allow this. This cooperation is also demonstrated by the negative correlations found between these two hormones in the present study. At the end of pregnancy, the inverse trend observed might be related to the effect of foetal stress, which is likely to have stimulated GnIH release, even in the presence of high Kp-10, acting to increase E2 concentrations for calving to occur. The levels of all hormones found in this study might also have a placental origin and this could explain the observed concentrations that were a little bit higher than those reported by other authors.

Besides being the first to report bovine blood Kp-10 and GnIH concentrations throughout pregnancy, this study may provide key pointers for the wider comprehension of both physiological and pathological aspects of pregnancy in the dairy cow. Moreover, it confirms and emphasizes the close correlation between GnIH and stress, such as that which occurs at calving.

Further studies are necessary to confirm the expression of these hormones in placenta during all phases of physiological pregnancy and compare these values with pathological conditions.

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