—Research Note—

Study of Enteral *versus* Parenteral Application of the Gonadotropin Releasing Hormone Agonist Gonadorelin[6-D-Phe] (D-Phe⁶-LHRH) on LH Secretion in Goettinger Miniature Pigs

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Abstract. With respect to the assessment of residue situation and as a part of preclinical trials to determine the biological activities of potential gonadotropin releasing hormone (GnRH) residues in porcine organisms the GnRH agonist Gonadorelin[6-D-Phe] (D-Phe⁶-LHRH) was administered either enterally or intramuscularly (i.m.) to female Goettinger miniature pigs in order to evaluate the GnRH-induced luteinizing hormone (LH) surge. Gilts received an (i) enteral application of 10 mg D-Phe⁶-LHRH *via* a probang (*enteral group*, n=7), (ii) i.m. injection of 0.1 mg D-Phe⁶-LHRH (*parenteral group*, n=5), or (iii) saline injection (*control group*, n=4). The GnRH and saline applications were repeated every second day with up to seven repetitions. Blood samples were collected *via* previously fitted jugular catheters immediately before injections, over an 8 h period in 1 h intervals beginning 2 h after injections, and at 24, 26, 28 and 30 h after applications. Enteral application of D-Phe⁶-LHRH induced an LH surge in 23 of 30 treatments. All gilts in the parenteral group exhibited LH release after each D-Phe⁶-LHRH application ($P < 0.05$), whereas no LH surges were observed after saline injection in the control group. A significant ($P < 0.05$) LH rise to mean maximum LH concentrations of 3.25 ± 0.43 and 3.05 ± 0.26 ng/ml occurred in both the enteral and parenteral groups, but there was no difference in the time interval after GnRH (2.6 ± 0.3 vs. 2.3 ± 0.3 h) and the mean duration of LH peak (6.5 ± 0.4 and 6.8 ± 0.3 h) between the treatment groups. In conclusion, (i) enteral application of 10 mg D-Phe⁶-LHRH induced LH release in a physiological range from the pituitary of female minipigs, and (ii) neither an accumulative effect nor a cumulative LH response were found after repeated GnRH application. Furthermore, (iii) in regard to consumer protection and gonadotropin secretion, D-Phe⁶-LHRH residues can be excluded from having long-term effects.

Key words: Enteral gonadotropin releasing hormone (GnRH) application, GnRH agonist, Luteinizing hormone (LH) secretion, Miniature pig

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Several hyperactive analogues of gonadotropin releasing hormone (GnRH) have been synthesized that have been applied to farm animals

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for pro-fertility and anti-fertility intervention [1, 2]. These analogues derive their properties from either increased binding to anterior pituitary receptor sites or through decreased susceptibility to enzymatic degradation [3–6].

GnRH is usually applied parenterally by intravenous, intramuscular, or subcutaneous

injections, but intranasal, intravaginal, and intrarectal applications are also possible [7–10]. Up to now, oral application has been uncommon due to the low bioavailability after degradation of GnRH by buccal and intestine enzymes. Proteases, such as chymotrypsin, elastase and gastricsin, are responsible for GnRH degradation in the intestine [11–14]. Metabolic breakdown of GnRH, however, may lead to biologically active fragments that can be absorbed through the intestine and produce biological effects in receptive tissues [15–17]. Furthermore, the order of degradability of GnRH agonists, e.g., D-Phe⁶-GnRH and D-Trp³-D-Phe⁶-GnRH, is lower compared to native GnRH [18]. GnRH receptors have been detected in gastric smooth muscle and epithelial cells [19–21], and myenteric plexus cells [22], and LHRH-like factors have been found in duodenum [23]. Accordingly, GnRH preparations reaching the gastrointestinal tract may induce LH secretion.

The GnRH agonist D-Phe⁶-LHRH (Berlin-Chemie AG, Berlin, Germany) is widely used in German pig production, and its LH release capability has been tested previously [24–27]. The following experiment was arranged to establish a maximum residue limit and withdrawal period for meat and milk (consumer protection) and as a part of preclinical trials to determine the activities of potential GnRH residues in porcine organisms. D-Phe⁶-LHRH was given either enterally or intramuscularly to Goettinger miniature pigs in order to evaluate the GnRH-induced LH surge. Furthermore, possible GnRH-induced ovulations were checked by laparotomy.

Materials and Methods

Animals and surgical procedures

Altogether 16 female peripubereal Goettinger minipigs (8 months, 23.2 ± 3.3 kg) were used. They were housed in individual pens with visual contact. The animals received a standard feed twice daily and had free access to drinking water.

Three days before start of the experiment, intravenous jugular catheters were surgically implanted into the Vena cephalica antebrachii [28] of the animals under ketamine/xylazine anaesthesia (45 mg/kg BW Ursotamin and 4 mg/kg BW Xylazin; Serumwerk Bernburg, Bernburg, Germany). The catheters were flushed and filled

with 3 ml of 3% Na-citrate.

All procedures involving animal handling and treatment were approved by the Committee for Animal Use and Care of the Scientific Senate Department of Berlin, Germany.

Experimental design and sampling procedures

The gilts were randomly allotted to the following treatment groups: (i) the enteral group, which received enteral application of 10 mg D-Phe⁶-LHRH (Berlin-Chemie; n=7); (ii) the parenteral group, which received intramuscular injection of 0.1 mg D-Phe⁶-LHRH (n=5); and (iii) the control group (n=4). In the enteral group, 10 mg D-Phe⁶-LHRH (0.4 mg/kg BW) diluted in 10 ml of saline was applied via a probang (2.1 mm in diameter; Braun-Dexon, Spangenberg, Germany). Therefore, gilts were held by one person while another inserted the probing, which was connected to a 10 ml-syringe, through the throat into the esophagus. The gilts in the parenteral group received 0.1 mg D-Phe⁶-LHRH (0.004 mg/kg BW) diluted in 10 ml saline i.m. into the neck. The control gilts were injected intramuscularly with 10 ml of saline. GnRH and saline applications were repeated every second day with up to seven repetitions (1st to 7th application, respectively). Each application was conducted at 0800 h.

Blood samples were collected immediately before GnRH or saline application, over an 8 h period in one hour intervals, and at 24, 26, 28 and 30 h thereafter. Prior to blood collection, the citrate within the catheter and the first 3 ml of blood were discarded. At each sampling, 5 ml of blood was collected into ice-cooled plastic syringes containing EDTA-potassium as an anticoagulant (KABE, Nümbrecht-Elsenroth, Germany). The samples were centrifuged at $1,800 \times g$ for 15 min at 4 °C, and the plasma was portioned into aliquots and stored until analysis at –20 °C.

The gilts were injected with a lethal dose of T61 (embutramide, mebezonium iodide and tetracaine hydrochloride; Intervet, Unterschleissheim, Germany). Thereafter, the abdominal cavity was opened and the ovarian features were recorded [29].

LH analysis

The plasma concentrations of porcine LH were determined in duplicate in 100 µl plasma samples using the homologous double-antibody

Table 1. Numbers of repeated GnRH or saline applications and respective blood collections in the enteral, parenteral and control groups

Enteral group		Parenteral group		Control group	
Gilt	Number of applications	Gilt	Number of applications	Gilt	Number of applications
E1	7	P1	6	C1	7
E2	6	P2	6	C2	4
E3	5	P3	3	C3	6
E4	1	P4	3	C4	6
E5	4	P5	6		
E6	3				
E7	4				
Total	30		24		23

radioimmunoassay described by Kanitz *et al.* [30]. The intra- and inter-assay coefficients of variation were 7.9 and 8.0%, respectively, and the assay sensitivity was 0.2 ng/ml.

Statistical analysis

The secretion characteristics of LH [maximum plasma LH concentration of the induced LH surge (LH_{MAX}), duration of the induced LH surge, LH area over baseline (LH_{AOB}), and interval from GnRH agonist administration to LH_{MAX}] were calculated by PULSAR [31]. The following G-values were used: 3.8 for G1, 3.2 for G2, 2.7 for G3, 2.4 for G4 and 1.8 for G5. The respective secretion parameters were calculated using a 10% criterion of variation and the appropriate assay parameters (sensitivity and frequency of sampling).

The LH data was evaluated by repeated measures analysis of variance (ANOVA) using the MIXED procedure of the SAS/STAT software in SAS System for Windows, release 8.02 (SAS Institute, 1999). The model for the response variable plasma LH concentrations repeatedly measured at time points (0, 2, 3, 4, 5, 6, 7, 8, 24, 26, 28 and 30 h) after the 1st, 2nd, 3rd, 4th, 5th and 6th applications of D-Phe⁶-LHRH or saline contained the fixed effect of the treatment groups (enteral, parenteral and control); the repeated factors, application and time; and the corresponding interactions. Repeated measures on the animals were taken into account by the repeated statement of the MIXED procedure and the subject=animal option in order to specify the unstructured residual covariance matrix in the model, as estimated by the MIVQUE0 method (minimum variance quadratic unbiased estimation of the covariance parameters).

In addition, LS Means and their standard errors (SE) were calculated and tested for each effect in the model.

Repeated measures ANOVA was conducted for plasma LH concentrations using the GLM procedure of the SAS/STAT software and two models. One model used the fixed effect of the treatment groups and repeated factor application by each time point, and one model used the fixed effect of the treatment groups and the repeated factor time by each application.

The frequency data of the animals in the treatment groups with an induced LH surge (enteral and parenteral GnRH application) was analyzed using the GENMOD procedure of the SAS/STAT software.

Results

The numbers of repeated GnRH applications and blood collections per animal were different during the experiment due to the intactness of the jugular vein catheters of the minipigs (Table 1). An LH surge was induced in 23 out of 30 treatments after enteral application of 10 mg D-Phe⁶-LHRH, whereas all gilts of the parenteral groups exhibited LH release after each application of 0.1 mg D-Phe⁶-LHRH ($P < 0.05$).

No differences were obtained regarding the initial LH concentrations before each GnRH or saline application. A significant ($P < 0.05$) LH rise to maximum concentrations occurred 2.6 ± 0.3 h (enteral group) and 2.3 ± 0.3 h (parenteral group) after GnRH. However, there were no differences in this time interval and the mean duration of the

Table 2. Mean ($\pm$ SE) maximum LH concentrations (ng/ml) in the Goettinger minipig gilts after repeated enteral (10 mg) or parenteral (0.1 mg) application of D-Phe⁶-LHRH

Group	1st	2nd	3rd	Application 4th	5th	6th	7th
Enteral (Range)	3.74 $\pm$ 0.99 (1.12 to 6.40)	5.29 $\pm$ 0.82 (2.36 to 7.95)	1.46 $\pm$ 0.00 (1.46 to 1.46)	3.23 $\pm$ 1.75 (1.07 to 6.70)	2.33 $\pm$ 0.98 (1.22 to 4.27)	1.81 $\pm$ 0.37 (1.11 to 2.39)	2.00 $\pm$ 0.50 (1.50 to 2.50)
Parenteral (Range)	4.08 $\pm$ 0.57 (2.34 to 5.56)	3.37 $\pm$ 0.84 (1.34 to 6.41)	3.12 $\pm$ 0.45 (1.42 to 4.05)	1.97 $\pm$ 0.22 (1.75 to 2.19)	1.77 $\pm$ 0.16 (1.61 to 1.92)	2.55 $\pm$ 0.18 (2.26 to 2.88)	2.56 $\pm$ 0.19 (2.37 to 2.74)

induced LH peak (6.5 ± 0.4 and 6.8 ± 0.3 h) between the treatment groups. The results for the mean maximum LH concentrations of the subsequent applications are presented in Table 2. The maximum concentrations of LH fluctuated greatly, with values ranging from 1.02–7.95 ng/ml and 1.34–6.41 ng/ml in the enteral and parenteral GnRH-treated gilts, respectively. However, the mean LH_{AOB} did not differ between treatment groups (10.3 ± 2.0 vs. 9.3 ± 1.1 ng/ml). Furthermore, both repeated GnRH application (application $\times$ time interaction) and method of treatment (treatment $\times$ application interaction) had no influence. Representative individual LH release patterns for saline- and enteral and parenteral GnRH-treated minipigs in seven consecutive treatments are provided in Fig. 1.

No LH surge was observed in the control group around the time of saline injection. However, endogenous LH peaks were observed in one gilt on day 1 of treatment and in another one on day 4, with maximum peak values of 6.46 and 4.63 ng/ml. The duration of this endogenous LH release was more than 24 h. Additionally, a total of 12 LH pulses (1 to 3 pulses/animal) were observed in the control minipigs during the experiment with magnitudes of pulse release between 1.04 and 2.04 ng/ml LH.

The overall mean LH secretion after GnRH or saline application for each treatment group is shown in Fig. 2.

Evaluation of ovaries by laparotomy revealed that 80% of the gilts were pubertal and that they possessed ovarian features associated with the estrous cycle (Table 3). However, not all induced LH surges were followed by ovulation. A clear relationship was identified in six out of 11 GnRH-treated gilts in relation to the induced LH surge, i.e., after the first GnRH application in two gilts from the enteral group and one gilt from the

parenteral group. The other three gilts ovulated after the 3rd, 5th or 6th application. In two control gilts, ovarian development was in accordance with the detected endogenous LH surge.

Discussion

The rapid deactivation of GnRH and its analogues by enzymatic degradation, especially by endopeptidases, and the necessary high dose of D-Phe⁶-LHRH usually exclude oral applications [12, 14]. Nevertheless, biotransformation of peptides in the intestine may produce shorter active polypeptides [16]. A previous study showed that enteral application of LHRH to rats had half of the intravenous LHRH response [17]. Furthermore, intranasal application of buserelin to lactating women presented measurable amounts of the GnRH agonist into the breast milk which was then digested by infants [32]. Based on these results, the extent to which consumption of products from farm animals treated with GnRH stimulates an LH response in humans should be examined. Although the chance of this type of LH response occurring after oral applications is limited, in view of consumer protection, GnRH and/or its residues should be excluded from stimulating LH release in humans. Therefore, the dynamics of LH release after GnRH application were examined in the present study using minipigs.

We did not expect LH release from the pituitary after enteral application of D-Phe⁶-LHRH with a similar release pattern compared to that of intramuscular application. Furthermore, half of the enteral GnRH-treated minipigs ovulated in response to one of the applications. We assume that the extremely high dose of the enteral applied D-Phe⁶-LHRH and possibly altered degradation of the decapeptide are responsible for this result. In

Fig. 1. Individual LH release patterns of three female minipigs after oral (●, gilt E1) and parenteral GnRH application (○, gilt P1), and after saline treatment (▲, gilt C3) in seven consecutive treatments (1st to 7th application).

the present experiment, the minipigs received a hundredfold dose of D-Phe⁶-LHRH that is usually given to sows for synchronization of ovulation [27, 33], and in terms of the minipigs' bodyweights, this dose was about five hundredfold higher. Since only a small dose of GnRH is necessary to stimulate LH secretion, part of the enteral applied D-Phe⁶-LHRH that avoided enzymatic degradation may have reached the blood circulation and stimulated LH release from the pituitary. In the literature, only limited data is available on oral GnRH

Fig. 2. Mean ($\pm$ SE) LH secretion of female minipigs ($n=16$) after saline and enteral or parenteral GnRH application (a, b: $P<0.05$).

application. In adult men, ingestion of 0.6 mg buserelin dissolved in cow milk had no biological effect on both serum and urinary levels of LH [32]. On the other hand, 2–4 month old male infants receiving a single oral dose of 35 μ g of the GnRH agonist buserelin mixed with breast milk showed an increase in urinary LH concentrations within 4–6 h after treatment [34]. These findings suggest that a sufficient quantity of buserelin escapes gastrointestinal inactivation probably due to immaturity of enzyme function.

However, with regard to the short half-life of GnRH and its agonists, even if the half-lives of GnRH agonists are relatively longer due to higher receptor affinity and resistance to inactivation [4, 6], given the extremely low dosage per animal necessary to achieve an appropriate clinical effect, it is doubtful that GnRH residues in farm animal products stimulate gonadotropin release in humans.

It is interesting to note that after both enteral and parenteral D-Phe⁶-LHRH application, irrespective of the endocrine situation, no desensitization of the pituitary occurred. LH secretion was stimulated in 47 of 54 applications without significant alterations in LH release (treatment $\times$ application interaction, $P>0.05$). This is different from the results of repeated GnRH application in barrows [35]. The parameters of the induced LH surge, i.e. increase, maximum concentrations, and duration, in the enteral and parenteral D-Phe⁶-LHRH-treated

Table 3. Ovarian features at laparotomy (17 days after first treatment) of gilts in the different treatment groups

Group	Gilt No.	Ovarian features	Stage of the estrous cycle	Relation to GnRH application
Enteral	E1	Follicles 4–5 mm, c.a. ¹⁾	Early follicular phase	–
	E2	Corpora lutea	Luteal phase	1 st application
	E3	Corpora lutea, c.a.	Luteal phase	1 st application
	E5	Follicles 4–7 mm, c.a.	Early follicular phase	–
	E6	Ovulated and preovulatory follicles, c.a.	Commencement of ovulation	6 th application
	E7	Follicles 2–4 mm	Peripubertal	–
Parenteral	P1	Corpora lutea, c.a.	Early luteal phase	5 th application
	P2	Follicles 3–5 mm, corpora lutea	Late luteal/early follicular phase	1 st application
	P3	Ovulated and preovulatory follicles, c.a.	Commencement of ovulation	3 rd application
	P4	Follicles 2–4 mm	Peripubertal	–
	P5	Follicles 2–4 mm	Peripubertal	–
Control	C1	Follicles 4–5 mm, c.a.	Follicular phase	Endogenous LH peak
	C2	Follicles 1–4 mm, c.a.	Early follicular phase	–
	C3	Corpora lutea, c.a.	Luteal phase	Endogenous LH peak
	C4	Follicles 2–3 mm, c.a.	Early follicular phase	–

minipigs were comparable with those of Landrace gilts after synchronization of ovulation [26].

In conclusion, (i) enteral application of 10 mg D-Phe⁶-LHRH induced LH release in a physiological range from the pituitary of the female minipigs, and (ii) neither accumulative effect nor a

cumulative LH response were found after repeated GnRH application. Furthermore, (iii) in regard to consumer protection and gonadotropin secretion, D-Phe⁶-LHRH residues can be excluded from having long-term effects.

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