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Pituitary and ovarian responses after an application of a low dose of the luteinizing hormone-releasing hormone (LHRH)-lytic peptide conjugate during the preovulatory period in ewes

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Abstract

The aim of this experiment was to determine the effects of a low dose (0.2 mg/kg body weight i.v.) of the luteinizing hormone-releasing hormone (LHRH)-lytic peptide conjugate (LHRH-Phor21) on the periovulatory ovarian and endocrine events, ensuing luteal function and fertility in an ovine experimental model. We hypothesized that the dense expression of LHRH receptors on the anterior pituitary and ovarian structures would make them highly susceptible to the membrane disrupting ability of LHRH-Phor21. Six sexually mature Rideau Arcott ewes were used to test the effects of the conjugate; seven ewes served as controls. Prior to drug administration, estrus was synchronized with intravaginal medroxyprogesterone acetate (MAP)-releasing sponges that were left in place for 12 days and a single i.m. injection of 750 IU of equine chorionic gonadotropin (eCG) given at sponge removal. LHRH-Phor21 or saline were administered 36 h after MAP sponge withdrawal/eCG injection, around the expected onset of the endogenous discharge of gonadotropins. Treatment resulted in greater ($p<0.05$) peak LH concentrations and an earlier rise ($p<0.05$) in estradiol secretion in LHRH-Phor21-treated than control ewes; however, there were no differences ($p>0.05$) in the mean number of ultrasonographically detected luteal structures and serum progesterone concentrations during the luteal phase post-treatment. There were no differences ($p>0.05$) in the number of ewes that lambed or lamb characteristics between the two groups at lambing 9 months post-treatment. Overall, there was no adverse effect of LHRH-Phor21 on the ovulatory process, luteal function and lamb productivity in the ewes of the present study. With a lack of suppressive effects of LHRH-Phor21 on the pituitary-ovarian axis and fertility, our results support the suitability of the drug as a potential cancer pharmaceutical in reproductive-aged women.

Keywords: Conjugated lytic peptide, luteinizing hormone-releasing hormone, ovulation, luteogenesis, antral follicle development, ewe, transrectal ultrasonography

Introduction

Luteinizing hormone-releasing hormone (LHRH), a decapeptide synthesized by the hypothalamic neurosecretory cells, is a key regulator of the development of reproductive tissues and gonadal function [1-8]. However, the overexpression of LHRH receptors found in reproductive tissue cancers such as breast, prostate, ovarian and endometrial cancer has been associated with the undesirable progression of the disease [9-17]. Although LHRH receptors are densely expressed on the anterior pituitary, earlier studies in rats have shown that after the treatment with maximum tolerated doses of LHRH cytotoxic analogs, the suppressive effect on gonadotropin secretion was transient and reversible [11]. LHRH receptors are also expressed on extrapituitary cells and tissues including lymphocytes, mammary gland, ovary, and prostate [7,8,10,18]. The extrapituitary LHRH receptors may represent an additional target for the lytic action of cell disrupting peptides conjugated with LHRH.

LHRH-lytic peptide conjugates act as molecular cytotoxic transporters carrying lytic peptides to tissues expressing complement receptors [9,15,19]. These synthetic conjugates are linear, positively charged, amphipathic and α-helical molecules. Although not completely understood, the proposed mechanism of action proceeds *via* disrupting the negatively charged cell membranes, creating microscopic pores that allow unregulated ionic flow across the membrane, which ultimately results in cell death [9,20]. The entire process is completed within minutes after the lytic peptide interacts with the membrane [9].

In the present study, we used the lytic peptide Phor-21 (21 amino acids) linked to LHRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2). This is a relatively new conjugate and so no studies have been done to evaluate the anti-cancer effects of this drug. However, previous studies using Phor21 conjugated to a fragment of the beta chain of human chorionic gonadotropin (βCG) showed a significant decrease in tumor burden both *in vitro* and *in vivo* [9,21-23]. Phor21-βCG(ala) was demonstrated

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to destroy human breast cancer xenografts in nude mice at much lower doses (0.2 mg/kg body weight) than other conjugated lytic peptides tested [9]. Another lytic peptide, hecate (23 amino acid), conjugated to LHRH was also shown to be very effective in suppressing the growth of cultured prostate cancer cells and human prostate cancer xenografts implanted in nude mice; in those studies, the growth arrest of tumor cells and marked reduction of tumor burden were observed [9,21-23]. However, LHRH-hecate was found to impair spermatogenesis and damage interstitial cells in the testes of nude mice bearing prostate cancer cell xenografts [24].

The effect of lytic peptide conjugates on tumor burden and the restoration of normal physiological function after cessation of treatment paved the way to further research into this modality of the targeted cancer therapy. Investigation of the effects of lytic peptide conjugates on LHRH- mediated processes such as ovulation and the formation of luteal structures in a non-rodent species is still required [25]. Sheep are widely used as a model for human reproductive processes because of their similarities in body size, hormonal profiles during ovulatory cycles and ovarian kinetics [25-27]. Hence, utilizing ewes as an experimental model, the aim of the present study was to evaluate the effect of LHRH-Phor21 on pituitary and ovarian function, and fertility post-treatment. We hypothesized that LHRH-Phor21 would bind to the LHRH receptors on the anterior pituitary and ovarian structures leading to a decreased gonadotropin release and ovarian follicular steroidogenesis. This decrease in reproductive hormone biosynthesis would then result in failure of follicle selection for ovulation or inadequate corpus luteum formation detected *via* ultrasound and hormone measurements.

Materials and Methods

All experimental procedures were in compliance with the Canadian Council on Animal Care guidelines and had been approved by the local Animal Care Committee. The experimental approaches used and drug dosing in the present study were based on the previously validated and published methodology [25]. Clinically healthy, sexually mature Rideau Arcott ewes (n=13) of similar age (mean±SEM: 3.2±0.3 years; range: 3 to 7 years) and weight (mean±SEM: 78.5±1.7 kg; range: 68 to 85 kg) were housed in the Ponsonby Research Station near Fergus, ON, Canada (latitude: 43°35'N, longitude: 80°20'W) during seasonal anestrus (May-June). Housed in sheltered pens away from the rams, they received a diet consisting of alfalfa hay, water and iodized salt licks *ad libitum*. The thirteen ewes were randomly allocated to two groups, six treated and seven control animals.

Intravaginal progestagen-releasing Veramix® sponges (medroxyprogesterone acetate, MAP; 60 mg) were inserted and left in place for twelve days to synchronize estrus and ovulations. At the time of sponge removal, 750 IU of equine chorionic gonadotropin (eCG; Pregnecol™6000, Bioniche Animal Health, Belleville, ON, Canada) were injected i.m. Following this protocol, the ewes are in estrus ~48 h after sponge withdrawal and ovulate ~14 h later. LHRH-Phor21 (0.2 mg/kg body weight i.v.) was administered 36 h after sponge removal, just prior to the excepted pre-ovulatory discharge of gonadotropic hormones. Blood samples were drawn every 2 h, from 24 to 60 h after sponge removal/eCG injection, and then twice daily (from 3 to 7 days after MAP sponge removal/eCG injection) and daily until the end of the 19-day period after estrus synchronization, as previously described [25]. All ewes underwent transrectal ovarian ultrasonography 3 days after drug/saline injection to visualize and count all luteal structures. Ewes were scanned with the high-resolution B-mode ultrasound echo camera (Aloka SSD-3500SD; Aloka Co., Tokyo, Japan) connected to a 7.5-MHz bi-plane linear probe [28,29]. Ovarian images were displayed on the viewing screen of an ultrasound scanner at 1.5x to 2x image magnification, using a linear-array component of the bi-plane probe. Digital images of both ovaries were captured and stored on a compatible DVD recorder (Pioneer® DVD Recorder DVR-510H, Pioneer Electronics of Canada Inc., Markham, ON, Canada) for analysis of ovarian images at a later date.

The serum samples were analyzed for levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol 17-β (E_2) and progesterone (P_4) [25]. The intra-assay coefficients of variation (CVs) for reference sera with mean LH concentrations of 0.44 or 2.33 ng/ml were 10.3% and 4.6% respectively. The intra-assay CVs for reference sera with mean FSH concentrations of 0.52 or 2.90 ng/ml were 4.8% and 3.0% respectively. The intra-assay CVs for reference sera with mean estradiol 17-β (E_2) concentrations of 0.52 or 2.90 ng/ml were 4.8% and 3.0% respectively. The intra-assay CVs for reference sera with mean progesterone (P_4) concentrations of 1.4 or 4.1 ng/ml were 9.4% and 5.5%, respectively.

A comparison of the single time-point observations obtained from LHRH-Phor21–treated ewes and control animals was done using Student *t*-test (SigmaStat®2.0 for Windows®; SPSS Inc., Chicago, IL, USA). Two-way ANOVA was used to compare serum concentrations of gonadotropic and steroid hormones over time (from 36 to 60 h after sponge removal/eCG injection) and between treatment and control groups. Statistical significance was regarded as $p<0.05$. Fisher's protected least significant difference (LSD) was used after ANOVA to test the differences between individual means. In addition, the time from treatment to peak concentration, the maximum concentration and the area under the curve (AUC) for serial concentrations were calculated for serial LH, FSH and estradiol-17β concentrations in individual ewes. AUC was calculated using NCSS Statistical Software (Kaysville, UT, USA; http://www.ncss.com). Data are expressed as mean±SEM.

Results

Three days following the injections in the treatment and control groups, luteal structures were detected with ultrasonography

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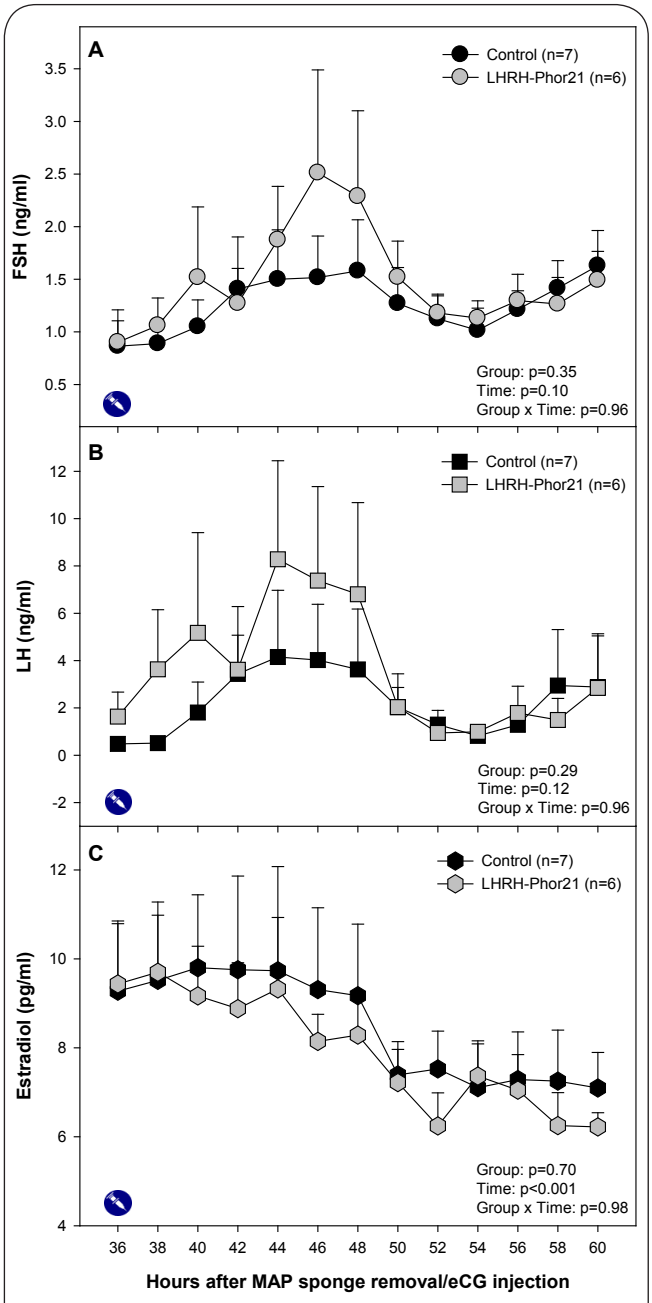

Figure 1. Serum FSH (A), LH (B) and estradiol-17β (C) concentrations determined by frequent sampling (conducted every 2 h) during the 24 to 60 h period after MAP sponge removal/eCG injection. Six ewes were treated with LHRH-Phor21 at a dose of 0.2 mg/kg body weight i.v. Statistical comparisons were only performed on hormone concentrations obtained during the period after drug/saline injection.

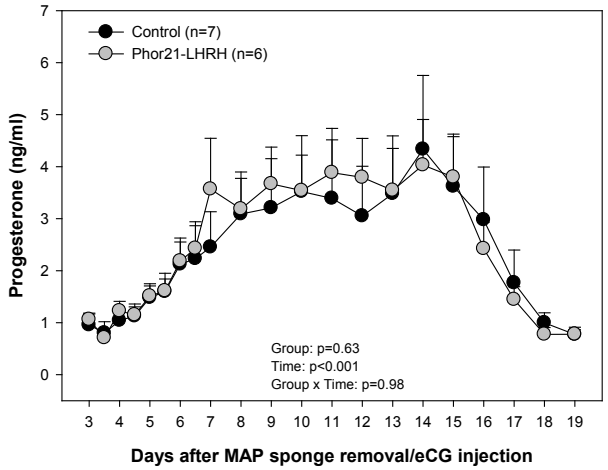

Figure 2. Systemic progesterone concentration from days 3 to 19 after MAP sponge withdrawal/eCG injection in ewes that received LHRH-Phor21 (0.2 mg/kg body weight i.v.) or saline only 36 h after sponge removal.

Table 1. Pituitary and ovarian endocrine responses in LHRH-Phor 21-treated (0.2 mg/kg body weight i.v.) and control ewes.

Hormone/ Group/ ***Variable***	***Time to peak (h)****	***Peak concentration*****	***AUC for serial hormone concentrations*****
LH			
Control (saline) (n=7)	44.8±5.1	9.24±3.40a	58.6±35.3
LHRH-Phor21 (n=6)	42.0±4.7	18.08±3.60b	126.5±51.1
FSH			
Control (saline)	44.6±4.7	2.73±0.48	5.6±1.2
LHRH-Phor21	42.3±4.5	3.97±0.71	6.5±1.1
Estradiol-17β			
Control (saline)	47.4±2.8a	12.3±2.1	95.1±7.0
LHRH-Phor21	40.0±1.7b	11.7±1.4	103.6±15.7

* Time from MAP sponge removal/PMSG injection to maximum hormone concentration

** LH and FSH (ng/ml) and estradiol-17β (pg/ml)

*** AUC-Area under the Curve (from 36 to 60 h after MAP sponge removal/eCG injection)

ab Within columns, means for each hormone denoted by different letters are different (p<0.05).

in all but one treated ewe. The total number of luteal structures per ewe (2.1±0.3) did not differ (p>0.05) between the two subsets of animals. The time elapsed from MAP sponge removal/eCG injection to the peak of estradiol-17β secretion was less in treated compared to control ewes (40.0±1.7 and 47.4±2.8 h, respectively; **Table 1**). The peak LH concentration was greater in LHRH-Phor21-treated than control ewes (18.08±3.60 and 9.24±3.40 ng/ml, respectively; **Table 1**). No other characteristics of the periovulatory secretion of LH, FSH and estradiol-17β differed between the two groups of ewes (p>0.05; **Table 1** and **Figure 1**). There were no differences (p>0.05) in the mean daily progesterone concentrations between the two groups during the luteal phase post-treatment (**Figure 2**). Lastly, there were no differences (p>0.05) in the ovulation rate, the percentage of

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Table 2. Fertility results at lambing 9 months following the treatment of ewes with LHRH-Phor21 (0.2 mg/kg body weight i.v.) or saline (controls). F-female lamb.

Group	Ewes lambing	No. of live births/ewe	Birth weight (kg)	Sex ratio (% male)	Related Information
Control (saline) (n=7)	4/7	2.0±0.4	4.2±0.2	33.3±11.8	-
LHRH-Phor21 (n=6)	4/6	2.5±0.6	3.8±0.2	20.8±12.5	1F stillborn + retained placenta (6 ml Procillin + 2.5 ml Anafen i.m.)

ewes that lambed or lamb characteristics between the treated and control ewes at lambing 9 months post-treatment (**Table 2**).

Discussion

The purpose of this study was to determine the effect of a lytic peptide conjugate LHRH-Phor21 on the anterior pituitary function and dynamic ovarian processes (ovulation, luteogenesis and ensuing luteal function) in an ovine experimental model. We hypothesized that LHRH-Phor21 would target receptors on the anterior pituitary and ovary, transiently decreasing the LH release and affecting the LH-mediated ovarian processes, including ovulation and ovarian steroidogenesis. However, our results did not indicate a truncated LH release or a significant negative effect on terminal antral follicle development and luteogenesis. Alternatively, a moderate stimulatory effect of LHRH-Phor21 on the phasic release of LH and follicular estradiol was observed.

Conjugated lytic peptides were developed to target and suppress the proliferation of malignant tumor cells while leaving somatic cells unharmed. Their selectivity is not confined to the ligand-receptor interactions; the lytic peptides also exhibit a preference for binding to cell membranes with abundant negatively-charged phospholipids [**9**,**20**]. In their native and immune role, lytic peptides preferentially lyse bacterial cell membranes while sparing normal/healthy mammalian cells [**12**], this affinity is a result of negatively charged acidic phospholipids contained within bacterial cell membranes. Compelling evidence regarding the selectivity of conjugated lytic peptides for negatively charged membranes with elevated content of phosphatidylserine groups in cancerous tissues was provided by a study comparing the lysing potential of conjugated lytic peptides on CHO (Chinese Hamster Ovary) cells transfected with luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptors and hCG receptor-positive MDA-MB-435S breast cancer cells; the conjugated lytic peptides were less effective at destroying the neutral CHO cells [**12**]. Those earlier results and our present observations may be interpreted to suggest that the cell surface charge is a determining factor for triggering lytic actions of the conjugated lytic peptides anchored to target cells by the hormone carriers.

Significant in the interpretation of our results may also be the consideration of the multiple types of GnRH molecules and, to some extent, of GnRH receptors. Although it is undisputed that the principal target and source for GnRH is the anterior pituitary and hypothalamus respectively, there is now significant documentation of extra-hypothalamic origin of LHRH as well as extra-pituitary LHRH receptors [**26**]. Furthermore, a second form of GnRH, GnRH-II, was first identified in the chicken brain and is structurally conserved in vertebrate species ranging from fish to humans [**18**,**20**,**30**,**31**]. Hence in mammals two forms of GnRH coexist: GnRH I that regulates gonadotropin secretion and GnRH II, a neuromodulator that mainly stimulates sexual behavior [**32**]. GnRHs can also be detected in normal reproductive tissues and the reproductive tissue tumors in which their paracrine and/or autocrine roles are postulated [**32**]. A unique feature of the GnRH receptor is the lack of a carboxy-terminal cytoplasmic tail, which is believed to be involved in desensitization and internalization of other G protein-coupled receptors [**6**]. In contrast to the type I receptors, the type II GnRH receptors expressed in reproductive tissues and in the central nervous system are rapidly internalized and also propagate a distinctly separate signaling pathway [**6**]. Interestingly, in man and some other mammalian species (*e.g.*, chimp, sheep and bovine), the type II GnRH receptor has been evolutionary silenced [**32**]. This phylogenetic variation makes the sheep a suitable species for studying the effects of GnRH as a model for human beings. Nevertheless, GnRH I and GnRH II still appear to have distinctive roles in signalling differentially through the type I receptor and may produce dissimilar downstream effects. The increase in peak concentrations of LH and FSH during certain key intervals in the sheep of the present study may be attributable to GnRH-Phor21 targeting the non-internalized GnRH type I receptors in the posterior pituitary (GnRH I-like effects) but the specific actions of the conjugate exerted at the ovarian level and in non-gonadal segments of the reproductive tract remain to be elucidated.

The present results clearly indicate that the hormonal portion of the conjugated lytic peptide has the ability to bind to its receptor (type I GnRH receptor) and to stimulate typical hormone-dependent responses in healthy (non-cancerous) cells *in vivo*. It would now be imperative to examine if similar effects can be seen during the treatment of reproductive tissue cancers as stimulation of the endocrine function of gonads may be undesirable in patients undergoing cancer therapy. The systemic hormonal therapy involving administration of GnRH antagonists/superagonists, anti-estrogens and/or aromatase inhibitors is typically used to treat ovarian stromal and epithelial tumors [**33**,**34**]. However, a moderate increase in steroid and FSH production leading to increased synthesis and affinity of LH/hCG receptors may be beneficial in targeted

cancer therapy using the lytic peptides conjugated with the βCG fragment [25]. Future studies should also address the effects of the LHRH-Phor21 peptide in males. In the course of prostate cancer, for example, hormone therapy may include the application of LHRH agonists, anti-androgens and the drugs that block adrenal steroidogenesis (ketoconazole and aminogluthimide) [35]. Estrogens were used in the past to reduce testicular androgen production but because of the risk of serious side effects they are seldom used nowadays in the treatment of prostate cancer. Both androgens and estrogens are synthesized by testicular cells [36] and so the effects of the LHRH-lytic peptide conjugates on the pituitary function and gonadal steroidogenesis in males would be of interest.

Conclusion

Contrary to our expectations, there was no disrupting effect of a low dose of LHRH-Phor21 on the pituitary function and ovarian activity during the follicular phase of the ewes' estrous cycle. Although our findings are generally supportive of the safety of LHRH-Phor21 as a cancer therapeutic in women of a reproductive age, the potential for individual variation, dissimilar cellular effects that GnRH mediates in different target cells/organs as well as a slight increase in gonadal steroidogenesis post-treatment warrant further studies.

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Competing interests

The authors declare that they have no competing interests.

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