Validation of a luciferase bioassay to detect the progestative activity in gilts whose estrus was induced by an uterotonic herb (Ligusticum chuanxiong)

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\textbf{Abstract}

\textit{Ligusticum chuanxiong} (LC) is an uterotonic herb. Ethanol extract of LC has potent progestative activity. The progestative activity in rat serum was well documented after oral and subcutaneous administration of LC. The objective of this study was to investigate the possibility of LC for estrus synchronization of gilts in place of synthetic progesterone. Eighteen gilts were randomly assigned into three groups, control group fed normal feed, positive control group supplemented with 20 mg of altrenogest (Regumate\textsuperscript{\textregistered}) and treatment group supplemented with 100 g of LC daily for 22 consecutive days in feed. Blood samples were collected from jugular vein of gilts on the 22nd day before altrenogest or LC feeding. Estrus was monitored every day in the morning and the evening by observing the vulva swelling and reddening as well as nose to nose fence line contact between the boar and sow. Estrus was confirmed by testing the standing reflex in the presence of boar. All gilts in LC-fed group and 5 gilts in altrenogest-fed group came in estrus within 2–6 days after withdrawal of feeding, but did not show estrus during the feeding period. Whereas, all gilts in control group came in estrus during the feeding period. Progesterone responsive reporter plasmid was constructed by incorporating the progesterone response elements and TATA box at the multiple cloning site of pGL3 basic vector. Gilts serum progestative activities were measured by using luciferase reporter gene bioassay. Serum analysis showed that progestative activities of altrenogest- and LC-supplemented groups had a similar pattern. It is concluded that LC is a potential feed additive to be used for estrus cycle synchronization in swine industry.

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1. Introduction

The normal estrus cycle in sow is 21 days long (Senger, 2003) and it starts from 5–7 months of age (Brinkley, 1981). Estrus synchronization involves interrupting the natural estrus cycles of female animals so they can be pregnant at approximately the same time. Balanced and feedback mechanism of gonadotropins releasing hormone
(GnRH), follicle stimulating hormone (FSH), luteinizing hormone (LH), estrogen and progesterone are important for ovarian activities such as follicular development, maturation as well as ovulation (Lesoon and Mahesh, 1992). FSH and LH are low at the prepubertal stage in gilts and LH under the stimulation of GnRH for follicle development and ovulation (Driancourt et al., 1995). On the other hand, progesterone inhibits the synthesis and secretion of GnRH which subsequently inhibits the follicular growth and development as well as ovulation (Lesoon and Mahesh, 1992). Protocols have been developed for estrus synchronization of gilts, either by controlling events leading to follicles development, maturation and ovulation or altering luteal phases. Progesterone and its derivatives were not proved fully effective for estrus synchronization of gilts (First et al., 1963). For this reason, non-steroidal progestogen, such as meathallibine (Aimax®, ICI33828, Suisynchron™), allyl-trenbolone (altnrenogest, as Regumate®) as well as other hormones including PG600® (combination of equine chorionic gonadotropin and human chorionic gonadotropin), Prostamate® and Lutalyse® (PGF2α) are commercially used in swine industry (Britt et al., 1989, Estienne et al., 2001; Kaeoket, 2008). Due to the teratogenic effect of meathallibine and Zinc-methalibure, they were banned by US and EU in swine industry. Nowadays, allyl-trenbolone is the only substance with progestative effect used for swine estrus synchronization around the world. Although allyl-trenbolone is highly effective in mature and random cycling gilts, it is not effective for estrus induction in prepubertal gilts. Therefore, it is necessary to find new substances with progestogenic effect.

*Ligusticum chuanxiong* Hort. (family Apiaceae) is known as uterotonic herb and commonly used in traditional Asian folk medicines. The strong progestative activity of its ethanolic extract was well documented. The maximal progestative activity of the LC ethanolic extract was comparable to 95% of 100 nM progesterone with an EC50 of 7 µg/ml (Lim et al., 2006a). Oil fraction of LC extract contains monomeric and dimeric Phthalides and it is prescribed for several diseases including menstrual disorders (Chen, 1992; Zhang et al., 2003). Two phytoprogesterone, 3,8-dihydro-diligustilide and Riligustilide, were isolated from LC extract, and 3,8-dihydro-diligustilide has higher progestative activity. The maximal progestative activity of 3,8-dihydro-diligustilide was equivalent to 180% of 100 nM progesterone with an EC50 of 91 nM (Lim et al., 2006b). Riligustilide was less strong than 3,8-dihydro-diligustilide and its maximal activities was 15% of 100 nM progesterone with an EC50 of 81 µM (Lim et al., 2006a). Phyto-progestagens in LC were efficiently absorbed and the progestogenic activity was detected in rat sera when administrated subcutaneously and orally (Lim et al., 2006b). Phyto-progestagens in LC crude extract act through the same molecular mechanisms of progesterone agonist (Lim et al., 2006a). The progestative activities of LC extracts were fully suppressed by progesterone antagonist, RU486 (Lim et al., 2006b). Therefore, LC could potentially be used as an alternative for progesterone. The objectives of the study were to investigate the possibility of using LC for estrus synchronization of gilts and to develop and validate a bioassay to detect serum progestative activity of LC extract.

2. Material and methods

2.1. DNA vectors, chemicals and reagents

The pGL3 basic and pSV-β-galactosidase control vector, luciferase enzyme assay system, β-galactosidase enzyme assay system and restriction enzymes were purchased from Promega (Madison, WI, USA). Progesterone, estradiol-17β, testosterone, dexamethasone and mifepristone (RU486) were bought from Sigma Aldrich (St. Louis, MO, USA). Lipofectamine 2000, Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serums (FBS) were obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Design of animal experiment

Eighteen crossbred (Landrace – Duroc × Yorkshire) gilts with an average age of 6–8 months and average body-weight of 120 kg were used. When observing the first estrus in pig farm, gilts were randomly divided into three groups with 6 animals each, control group fed only normal commercial feed, positive control group supplemented with 20 mg altnrenogest once daily in the morning and treated group supplemented with 100 g LC powder twice daily in the morning and the evening, in the feed for 22 consecutive days. All gilts were fed a commercial feed at 2 kg/gilt in individual trough twice a day at 8 AM and 5 PM. Water was available ad libitum. Estrus detection was performed twice every day, starting on the first day of feeding until 7 days after withdrawal of altnrenogest and LC. Estrus was recognized by three healthy 10–12 months aged boars which were housed other end of the breeding barn from the gilts. Gilts were exposed to the boar two times daily, in the morning and the evening for 20–30 min each time, boars were allowed to move through the alley of each gilt pen, nose to nose fence line contact between the boar and each sow was observed, and then back pressure act by hand was practiced to confirm the immobility of gilts. In addition, red and swollen vulvas were also monitored every day in the morning and the evening. Before feeding on the last day of experiment, 6 ml of blood sample was collected from jugular vein of each gilt, allowed for clotting on ice and then centrifuged at 1500g for 30 min at 4 °C and kept at −20 °C.

2.3. Preparation of LC extracts

Rhizome chuanxiong (*L. chuanxiong*) roots, purchased from Sun Ten Pharmaceutical Co. Ltd. (Taichung, Taiwan), were grinded and soaked with 100% ethanol for 72 h at RT. The extracts were filtrated and kept inside a hood for rapid evaporation of ethanol. Dried extracts were diluted at the 100 mg/ml stock concentration and kept at −80 °C.
2.4. Construction of reporter plasmid pGL3-2PRE-TATA

The reporter plasmid pGL3-2PRE-TATA was constructed in two steps. In the first step, two 104-mer oligomers, antisense to each other, were synthesized by incorporating a minimal promoter, two progesterone response elements upstream of the promoter, and HindIII restriction site at both ends. These oligomers were annealed by gradually reducing the temperature from 99 °C to 25 °C to synthesize a double-stranded DNA. In the second step, 86-bp DNA fragment was inserted into the HindIII restriction site on the pGL3 basic vector to construct the recombinant plasmid, pGL3-2PRE-TATA (Fig. 1).

2.5. Cell transfection and luciferase assay

Human breast adenocarcinoma cell line, T47D, was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 1% sodium pyruvate, 3.5 g/L sodium bicarbonate, and 10% FBS. For assay, cells were maintained in a phenol red-free DMEM supplemented with 1% sodium pyruvate and 10% dextran coated charcoal FBS or 10% gilt serum. All cells were cultured at 37 °C and 5% CO2 in a humidified incubator. Two days before transfection, T47D cells were plated at 2.5 × 10^5 cells per well into six well plates with DMEM containing 2% dextran coated charcoal FBS and incubated for 48 h. Cells were co-transfected with 2 μg of pGL3-2PRE-TATA and 1 μg of pSV-β-galactosidase control vector using lipofectamine 2000, according to manufacturer’s protocol. The transfected cells were treated with different concentrations of progesterone, anti-progesterone, others steroids, LC extract, 100% ethanol (final concentration 0.1%, as control) or 10% gilt serum. After 24 h, cells were lysed and cell lysates were used to determine luciferase and β-galactosidase activities by using luciferase and β-galactosidase assay kits according to the manufacturer’s instructions. Proteins content was determined by Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). Luciferase activity was normalized by β-galactosidase activity and proteins content.

2.6. Data analysis

Progestogenic or anti-progestogenic activities were determined based on the luciferase activities from different treatments normalized by that from vehicle group. All bioassay data represent the results from at least three independent observations each of the vehicle control, positive control, progesterone (P), anti-P control (P plus RU486), background control (vehicle plus RU486), and all other treatment groups. For agonists, which stimulate luciferase expression, treatments were compared to the vehicle (media plus ethanol) control group or to the relative response Charcoal stripped FBS control. For P antagonist, RU486, which blocks P4-induced luciferase expression, was compared to the P positive control group. Relative luciferase activities were converted to fold induction above the vehicle control value. Data were analyzed by ANOVA (main effects being replicated [nuisance factor] and treatment) and Fisher’s least-significant differences (LSD) method was used to compare among the groups. A significant level of 0.05 was adopted. Fisher’s Exact Test was used to evaluate the effect of treatments on the estrus synchronization rate.

3. Results and discussion

3.1. Validation of sensitivity and ligand specificity of reporter plasmid

Constructed plasmid, pGL3-2PRE-TATA, was responsive to progesterone in a dose-dependent manner (Fig. 2). The minimal and maximal luciferase activities were found when cells were exposed to 1 nM and 1 μM of progesterone, respectively (Fig. 2). Progesterone levels are less than 1 nM before puberty and in follicular phase in animals.
Therefore, this constructed plasmid is sensitive enough to be used to detect the animal reproductive status concerning progesterone activity. Other steroid hormones, such as estrogen, testosterone and dexamethasone failed to induce (p > 0.05) luciferase activity (Fig. 3a), although all steroid hormone receptors belong to the same superfamily (Lydon et al., 1995). Progesterone antagonist RU486 suppressed the progestative activity by blocking the binding of progesterone to its receptor (Hazra and Pore, 2001). In Fig. 3b, it is shown that 100 nM RU486 completely suppressed 100 nM progesterone-induced luciferase activity. It is indicated that the constructed plasmid was specifically responsive to progesterone, its agonist and its antagonist. In this study, T47D, human breast cancer cell line, was used because it naturally expressed both progesterone receptors A and B. Progesterone, its agonist or its antagonist binds to nuclear progesterone receptors (Lydon et al., 1995) and form receptor–ligand complex, which binds to progesterone response elements on pGL3-2PRE-TATA plasmid to induce luciferase expression. Constructed plasmid promoter was 86 bp consisting of two progesterone response elements and no others cis-acting element. That is why the constructed plasmid was specifically sensitive to progesterone, its agonist and its antagonist and not sensitive to other steroid hormones.

For practical implication of the constructed plasmid, the ethanolic extracts of L. chuanxiong were used. The oil fraction of LC contains dimeric phytoprogesterone, such as 3,8-dihydro-diligustilide and Riligustilide (Lim et al., 2006a). In our study, we found that ethanol extract of LC induced luciferase activity in a dose dependent manner but biphasic in nature (Fig. 4). Luciferase activity gradually increased dose dependently from 20 to 40 µg/ml, however, above 40 µg/ml of LC extract luciferase activity gradually decreased. It indicated that over doses of LC extracts may be cytotoxic. Highest induced luciferase activity was observed with 40 µg/ml of extracted (Fig. 4) and it was comparable to 100 nM of progesterone induced luciferase activity. Our results were supported by Lim et al. (2006b) with the finding that the progestative activity of 50 µg LC extract was comparable to 80% of 100 nM progesterone induced. The difference in progestative activities of LC between Lim et al. (2006a) and this study may be due to the differences of cell lines, constructed plasmid, LC source and extraction methods.

3.2. Occurrence of estrus and serum progestative activities

All gilts in LC-fed group showed estrus within 4 days after LC withdrawal, whereas 5 gilts in altrenogest-fed group came in estrus within 6 days (Table 1). Although there was no difference in percentages of gilts showing estrus until 6 days after the end of treatment supply, a higher cumulative percentage (P < 0.05) of LC-fed gilts showed estrus until 4 and 5 days compared to altrenogest-fed group (Table 1) Similar results were found in previous studies (Bates et al., 1991; Kaoket, 2008), there gilts showed estrus within 5–7 days after the last day of altrenogest treatment. Estrus was observed in all gilts of control group at least once during the experimental period, and even two gilts in control group showed estrus twice during that period. Gilts of altrenogest-fed and treatment groups did not show estrus during 22 days of feeding period. It is indicated that phyto-progestogens,
such as 3,8-dihydro-diligustilide and riligustilide, present in LC suppressed hypothalamic GnRH synthesis as gonadal progesterone (Bashour and Wray, 2012; Skinner et al., 1998), consequently suppressed the pituitary gonadotropin secretion (Lesoon and Mahesh, 1992), follicular growth (Adams et al., 1992) and ovulation (Driancourt et al., 1995). Progestin altrenogest does not prevent luteolysis, but can block the onset of estrus after luteolysis (Horsley et al., 2005; Kirkwood, 1999). In this study, phyto-progesterone was found to suppress the ovarian activity. Within the estrus cycle, serum progesterone level remains higher than 1 nM from Day 3 or 4 to Day 14 or 15 (Stabenfeldt et al., 1969). Similar results were found in control gilts in this study. Serum progestative activities of control gilts were equivalent to or higher than 10 nM of progesterone activity (Fig. 5a). Since the control gilts had been in estrus, they had active corpora lutea at different stages explaining their higher progesterone activity. Exogenous progesterone blocks the onset of estrus through the same negative feedback mechanism of gonadal progesterone, which inhibits the release of GnRH from hypothalamus. Gilts of altrenogest- and LC-fed groups did not come in estrus because their serum progestins level was high (Fig. 5b and c). Fig. 5b and c showed that serum progestative activity of altrenogest- and LC-fed groups were equivalent to or lower than 10 nM of progesterone but more than 1 nM induced progestative activity. Stevenson and Davis (1992) demonstrated that feeding altrenogest for 14 consecutive days is enough for corpus luteum to regress and for gilt estrus synchronization. In this study, the gilts were fed for 22 days to investigate whether phytoprogesterone suppress the ovarian activity and to give sufficient time for corpus luteum to regress. The high percentage of gilts (83%) showing estrus within 4–6 days after altrenogest withdraw corroborates the results of previous studies (Horsley et al., 2005; Koutsotheodoros et al., 1998; Martinat-Botte et al., 1990) in which 93–95% of gilts showed estrus after altrenogest feeding for 14–18 days. Gilts in LC-fed group came in estrus earlier than those in altrenogest-fed group, and this may be due to the bioactive compound in LC being rapidly degraded in animal body (Lim et al., 2006b). This is a potential advantage for practical use of LC in estrus synchronization of gilt. L. chuanxiong was supplied twice daily at the amount of 50 g each at 12-h intervals to ensure the availability of progesterone in blood serum. Before gilt estrus synchronization study, the in vivo validation of progestative activity of LC extract in castrated pigs was performed. Serum progestative activities were significantly higher in castrated pigs after feeding LC extract than that in the control (data not shown). Lim et al. (2006a) also observed that serum progestative activity was significantly higher in rat after oral administration of LC extract.

Animal, plant (Iino et al., 2007) and synthetics are the three sources of progesterone. Other steroid hormones, chemicals and environmental wastage have progestative or anti-progestative activity (Chatterjee et al., 2008). Therefore, to understand the steroidogenical characteristics of plants crude extracts or in animal serum, a suitable method to detect additive effects of known and unknown compounds is needed. In this study, a luciferase reporter gene bioassay was developed to detect the kinetic action of progesterone

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**Table 1**

<table>
<thead>
<tr>
<th>Group (No. of gilts)</th>
<th>D-1</th>
<th>D-2</th>
<th>D-3</th>
<th>D-4</th>
<th>D-5</th>
<th>D-6</th>
<th>D-7</th>
<th>Total gilts in estrus within 7 days (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Altrenogest-fed (6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>5 (83)</td>
</tr>
<tr>
<td>LC-fed (6)</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6 (100)</td>
</tr>
</tbody>
</table>

Control group gilts showed estrus during 22 days of feeding whereas gilts of altrenogest-fed and LC-fed group did not show estrus before the withdraw of these substances. Cumulative percentages of gilts showing estrus until D3 and D4 were significantly different between Altrenogest- and LC-fed gilts (P < 0.05).

**Fig. 5.** Serum progestative activities of gilts. T47D breast cancer cells were cotransfected with reporter plasmids. Transfected cells were treated with 10% sera of (a) control (b) altrenogest-fed and (c) LC-fed gilts. The 10 nM progesterone (P) was used as positive control and charcoal stripped FBS used negative control. C1–C6, R1–R6 and LC1–LC6 are gilt from control, altrenogest-fed and LC-fed group, respectively. Progestative activity is presented as the mean fold induction ± SEM of folds compared to negative control.
and LC extracts, and then the serum progestative activity was evaluated after feeding altrenogest and LC. Therefore, the total progestative activities in serum were detected.

4. Conclusion

LC is a potential feed additive to be used for estrus synchronization in swine industry. Further study will be needed to understand the effect of LC on follicle development and maturation, follicular atresia, number of ova shed, fertilization and implantation of embryo in synchronized gilts.

Conflict of interest statement

None of the authors have any conflicts of interest to declare.

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