

Gonadotrophin Concentrations in Sheep Plasma after Injections of GnRH with or without a Mixture of Serine and Threonine

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Abstract: This study was designed to test the effect of Serine and Threonine mixture with or without a GnRH dose on plasma LH and FSH concentrations in naturally cycling ewes. Two experiments were conducted. In the first experiment, the ewes were intravenously injected by jugular vein twice at 11.00 am and 2.00 pm as Saline/Saline (S/S, control), Saline/GnRH (S/GnRH), Saline/Amino acids and GnRH (S/AA+GnRH); Amino acids/GnRH (AA/GnRH), Amino acids/Amino acids with GnRH (AA/AA+GnRH). Blood samples were collected at 15, 30 and 60 min intervals from 60 min before, until 360 min following the first injection. In the second experiment, control and treatment groups received five saline or aminoacids mixture at two hours intervals beginning at 10.00 am. Injection of GnRH significantly increased plasma LH concentrations in all groups between 180 and 300 min following the first injections ($P < 0.05$). When amino acid mixture were injected three hours before the GnRH injection or three hours before and then together with GnRH there were further significant increases in plasma LH concentrations in AA/GnRH and in AA/AA+GnRH groups between 180 and 300 min following the first injections. In the second experiment, injection of amino acid mixture at two hour intervals did not cause any significant changes in plasma LH concentration ($P > 0.05$), while plasma FSH concentrations were suppressed ($P < 0.05$). The suppression of FSH concentration might be the result of an increased negative feedback of ovarian derived factors (most likely inhibin) on FSH secretion.

Key Words: Serine, threonine, ewe, GnRH, LH and FSH

Serin ve Threonin Amino Asitlerinin GnRH ile Birlikte veya Ayrı Olarak Enjeksiyonunun Koyunun Kan Plazmasındaki LH ve FSH Konsantrasyonuna Etkisi

Özet: Bu çalışma; Serin ve Threonin amino asitlerinin, GnRH ile birlikte veya ayrı olarak, doğal kızgınlık döngüsü seyrinde olan koyunlara enjekte edilmesi sonucu kan plazmasında meydana gelen LH ve FSH konsantrasyonlarındaki değişimleri ölçmek ve analiz etmek için yapıldı. Birinci deneyde, jugular damardan koyunlara saat 11:00 ve 14:00 da olmak üzere iki kez enjeksiyon yapıldı. İki seferdede tuz çözeltisi enjekte edilen koyunlar (Salina/Salina (S/S)) kontrol olarak tutuldu. Test gurupları: Salina/GnRH (S/GnRH), Salina/Amino asit karışımı ve GnRH (S/AA+GnRH), Amino asit karışımı ve GnRH (AA/GnRH), Amino asit karışımı ve amino asit karışımı ile birlikte GnRH (AA/AA+GnRH). Birinci deneyde, ilk enjeksiyondan 60 dakika önce olmak üzere koyunlardan 15, 30, 60 dakika aralıklarla ilk enjeksiyondan 360 dakika sonrasına kadar kan alındı. İkinci deneyde, saat 10:00 dan başlamak üzere her iki saatte bir 7 koyuna salina enjeksiyonu, diğer 8 tanesine ise amino asit enjeksiyonu yapıldı. İlk deneyde; birinci enjeksiyonu muteakip, 180-300 dakikalar arası kontrol gurubu (S/S) hariç olmak üzere tüm guruplarda plazma LH konsantrasyonu arttı ($P < 0,05$). Amino asitlerin GnRH enjeksiyonundan üç saat önce enjekte edilmesi ve üç saat önce enjekte edilip sonradan tekrar GnRH ile birlikte enjekte edilmesi sonucu AA/GnRH ve AA/AA+GnRH gurubunda 180-300 dakikar arası plazma LH konsantrasyonunda daha büyük bir artış sağlandı ($P < 0,05$). İkinci deneyde, amino asit karışımının iki saat aralıklarla enjeksiyonu LH konsantrasyonunda bir değişikliğe sebep olmaz iken FSH konsantrasyonunu azalttı ($P < 0,05$). Bu azalmanın nedeni, ovaryumlardan salgılanan inhibin ve benzeri faktörlerin hipofiz üzerine negatif geri etkisinin bir sonucu olabilir.

Anahtar Sözcükler: Serin, threonin, koyun, GnRH, LH ve FSH

Introduction

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) play an important role in controlling reproductive function in females and males (1). The development of reproductive organs and the onset of puberty in sheep are dependent on circulating concentration of gonadotrophins. The secretion of

gonadotrophins must occur at the right time in concert with other physiological changes that occur in the reproductive axis. Therefore, careful regulation of LH and FSH secretion is vital for full reproductive function.

It has been reported that gonadotrophin secretion is accompanied by changes in concentration of metabolic substances such as glucose, insulin and amino acids in the

peripheral circulation (2-4). The effect of specific nutrients (such as branched chain and excitatory amino acids) on gonadotrophin secretion was investigated (5,6). According to Ebling and his colleagues, administration of Excitatory Amino Acids (EAA) to feed restricted lambs increased episodic secretion of LH (7). In another experiment carried out by Recabarren et al. (8) pre-pubertal ewe lambs were injected with 15-30 g L-Arginine. According to their data, short period (6 h) of infusion of L-Arginine was capable of altering LH pulse frequency. The increase in LH pulse frequency during arginine infusion was thought to be a result of an action stimulating hypothalamic gonadotrophin releasing hormone (GnRH) release. Therefore, the effect of amino acids on gonadotrophin secretion was proposed. The same authors did another experiment on prepubertal female sheep to investigate the function of L-Arginine (ARG) or L-Ornithine (ORN) on pulsatile LH secretion (9). According to their data, mean LH concentrations during the 285-min period after infusion were greater in Arginine infused group. Huffman et al. (10) monitored the episodic LH secretion throughout development in eight April-born ewe lambs to determine if a change in LH pulse patterns preceded first ovulation at puberty. They postulated that an increase in the frequency of episodic LH secretion is a key event leading to the onset of ovarian cycle in the lamb.

In this experiment, attention was paid to Serine and Threonine injection since Serine and Threonine amino acids are the major substrates for phosphorylation (1,11,12). Many cellular processes are regulated by phosphorylation of Serine/Threonine into target proteins such as cAMP- Responsive Elements Binding (CREB) proteins and cAMP-Responsive Enhancer element modulator (CREM). These proteins regulate the secretion of second messengers, which play roles in the expression of mRNA for Transforming Growth Factor Beta (TGF β) like peptides. These peptides are capable of modulating the gonadotrophin secretion and the response of the pituitary to GnRH (12).

The identification of Serine and Threonine action mode on ovarian function requires a better understanding of their effect on hypothalamo-pituitary function, which might indirectly affect follicular growth and differentiation. Therefore, the aim of this study is to investigate the effect of a mixture of Serine and Threonine injection with or without GnRH on LH and FSH secretion.

Materials and Methods

Animals and preparation of injections

Sixteen cross bred naturally cycling ewes (Suffolk and Cheviot, 2-6 years old), with an average weight of 63 kg were purchased from a commercial company. According to body weight, animals were divided into 5 groups and penned together in groups of 2, 3 or 4 and fed a diet ad libitum. A mixture of 63.96 g L-Serine (Cat no; 21101-019; Life Technologies, Paisley, Scotland) and 31.98 g L-Threonine (Cat; 103053, ICN, Ohio, USA) were dissolved in 640 ml saline (pH 7.4) to provide 2 g L-Serine and 1 g L-Threonine in each injection. The solution was sterilised by filtration. Human synthetic GnRH was purchased from Sigma chemical company (Cat; L-7134, St Louis, MO, USA).

Experiment 1

Animals were injected intravenously, twice (at 11:00 and at 14:00) with 20 ml combinations of saline (S), L-Serine and L-Threonine mixture (AA) and 150 ng GnRH in 2 ml of saline. Blood samples (4 ml) were taken at 15 min intervals. The experiment was repeated twice to give the following treatments (I) S/S (n=8), (II) S/GnRH (n=6), (III) S/AA+GnRH (n=5), (IV) AA/AA+GnRH (n=6), (V) AA/GnRH (n=7). Ewes were bled beginning one hour before the first injection and continued for seven hours at 15 min intervals. Blood (4 ml) was collected through a jugular catheter and placed in heparin containing tubes on ice at 4 °C. Then the tubes were immediately carried to the laboratory and centrifuged at the same temperature at 2900 RPM for 20 min. After centrifuging, supernatant containing sheep plasma was removed by a transfer pipette and transferred in test tubes and stored at -70 °C until subsequent analyses.

Experiment 2

The ewes were divided into two groups and injected with either saline (Control, n=7) or a mixture of Serine and Threonine (Treatment, n=8), using the same dose as used in Experiment 1, every two hours beginning at 10:00 am for a total of 5 injections. Blood samples (4 ml) were taken via a jugular venous catheter during the 9 hours at 20 min intervals starting at 10:00 am.

LH and FSH assay

Label (Na¹²⁵I) was obtained from Mr Michael Avella, Royal Veterinary College, UK. Ovine LH and FSH, for iodination, were obtained from NIDDK (Cat; AFP7071B

and Cat; AFP 4117A, Beltsville, USA). These proteins were iodinated by using the Chloramine-T method (13). Ovine LH for use in the reference preparation was obtained from NIDDK-NIH, oLH-26 (Cat; APF192279, Beltsville, USA). Standards were prepared in concentrations between 0.2 ng/ml and 100 ng/ml. Ovine FSH (APB-4117A) was obtained from USA-BARC-Reproduction Lab-Beltsville, USA. Standards were prepared in assay buffer in concentrations of 0.05-50.00 ng/ml.

Plasma LH concentrations were measured by RIA. The antibody for LH was developed in a rabbit (R 29). Rabbit IgG (Cat; S0022-220) and Normal Rabbit Serum (Cat; S030-220) was obtained from Scottish Antibody Production Unit, Low Hospital Carlisle, Lanarkshire, Scotland. 100 µl of unknown plasma was placed in assay tubes in duplicates with 200 µl of assay buffer and 100 ml of the first antibody (R29) and incubated for 2 days at 4 °C, 100 µl label was then added, giving 12000-15000 CPM. The tubes containing buffer, antibodies and the label were incubated at the same temperature for another 2 days. The second antibody and normal rabbit serum were then added and incubated one more day. Then 1 ml 0.01 M phosphate buffered saline (PBS) was added and assay tubes were immediately centrifuged at 2900 rpm for 1 h, decanted and counted in a gamma counter.

For FSH concentrations, anti-ovine FSH developed in the rabbit (Cat; APF-C5288113, NIDDK-NIH, Beltsville, USA) was used in a dilution of 1/12000. Anti-rabbit IgG (second antibody) and normal rabbit serum were the same as used for LH assay. Buffers, incubation time and other assay procedures applied in FSH assay were also identical to the LH assay.

Intra- and inter-assay coefficients of variation were calculated for each quality control at maximum, median and minimum concentration of LH. The sensitivity of LH assay was 0.22 ± 0.01 ng/ml. Intra-assay coefficients of variation for high, medium and low were 10.5, 11.1 and 11.4. Inter-assay coefficients of variation for high, medium and low were 20.2, 10.5 and 16.2, respectively.

The sensitivity of FSH assay was 0.055 ± 0.005 ng/ml. Inter-assay coefficients of variation for high, medium and low were 7.4, 7.6 and 12.0. Intra-assay coefficients of variations were 4.8, 16.9 and 17.9, respectively.

Statistical analysis

Data were analysed by using analysis of variance (ANOVA). Total variation, variation between and within groups were calculated. Groups were compared at the 0.05 probability level. Last Significant Difference (LSD) method was used to identify which groups are significantly different, at any time of sampling.

Results

Experiment 1

In all groups, plasma LH concentrations increased significantly between 180 and 300 min compared with the control group. The plasma peak levels of LH in S/S, S/GnRH, S/AA+GnRH, AA/GnRH and AA/AA+GnRH groups were 0.54 ± 0.07 , 1.34 ± 0.35 , 1.40 ± 0.44 , 3.57 ± 0.99 and 4.16 ± 0.73 ng/ml, respectively.

Plasma LH concentrations were significantly higher in AA/AA+GnRH group than the plasma LH concentrations of all other groups, except AA/GnRH group, between 180 and 210 min. The differences in plasma concentrations of LH between the AA/GnRH and AA/AA+GnRH groups are not significant ($P > 0.05$) between 180 and 210 min (Figure 1).

After the injection of GnRH, in S/GnRH and AA/GnRH groups, between 180 and 270 min, FSH secretion increased ($P < 0.05$). The differences in FSH secretion between the S/S and AA/AA+GnRH groups were not significant ($P > 0.05$). Plasma FSH concentration was significantly suppressed in S/AA+ GnRH group between 180 and 240 min following the second injection ($P < 0.05$) (Figure 2).

Experiment 2

The injections of amino acid mixture at two hour intervals did not cause any significant changes in LH secretion (Figure 3).

Plasma FSH concentrations of the sheep that received amino acids were lower than those of the saline injected control group. Secretions of FSH were significantly suppressed ($P < 0.05$) by the injection of amino acid mixture (Figure 4).

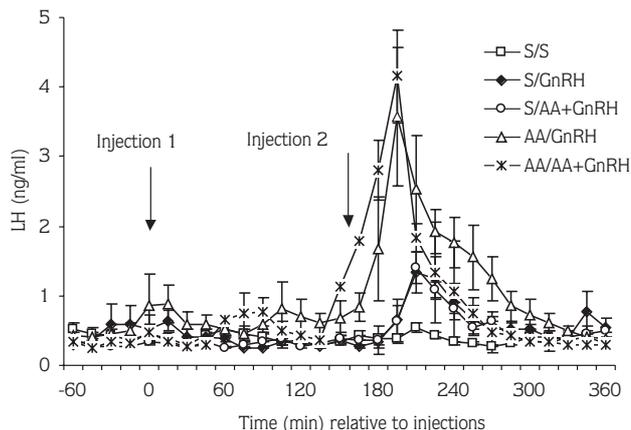


Figure 1. Plasma LH concentrations (Mean ± SEM) in control and treatment groups before and after injections during seven hours. (S): saline; (AA): Amino acid mixture; (GnRH): Gonadotrophin releasing hormone. Letters before and after (/) indicate treatment at the first and second injections respectively. Following the second injections, plasma LH concentration increased significantly over the S/S group ($P < 0.05$). Differences in LH concentration after the injection of (AA), between 0 and 150 min, are not significant ($P > 0.05$). Increases in LH concentration following the second injections are significant compared with S/S group ($P < 0.05$). Differences in LH concentration after the second injections, between AA/GnRH and AA/ AA+GnRH groups, are not significant. Also the differences in LH concentration between S/GnRH and S/AA+GnRH groups following the second injections are not significant ($P > 0.05$). Other differences are significant ($P < 0.05$).

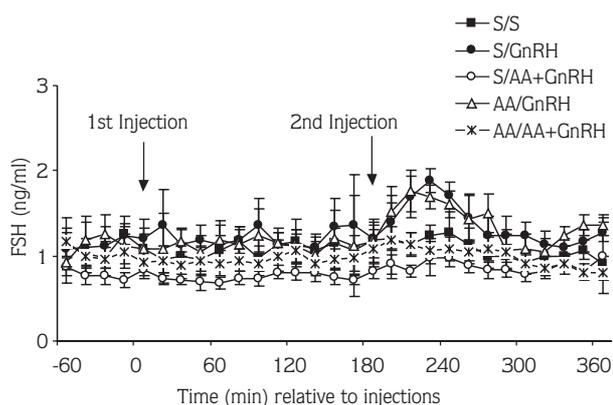


Figure 2. Plasma FSH concentrations (Mean ± SEM) in control and treatment groups. (S): Saline; (AA): Amino acid mixture; (GnRH): Gonadotrophin Releasing Hormone; Letters before and after (/) indicate treatment at the first and second injections respectively. FSH secretion was suppressed ($P < 0.05$) after the second injection in Saline/AA+GnRH group as compared with Saline/saline group between 180 and 225 min. In Saline/GnRH and AA/GnRH groups, FSH concentration between 195 and 240 min was significantly increased after by the injection of GnRH ($P < 0.05$). Other differences are not statistically significant.

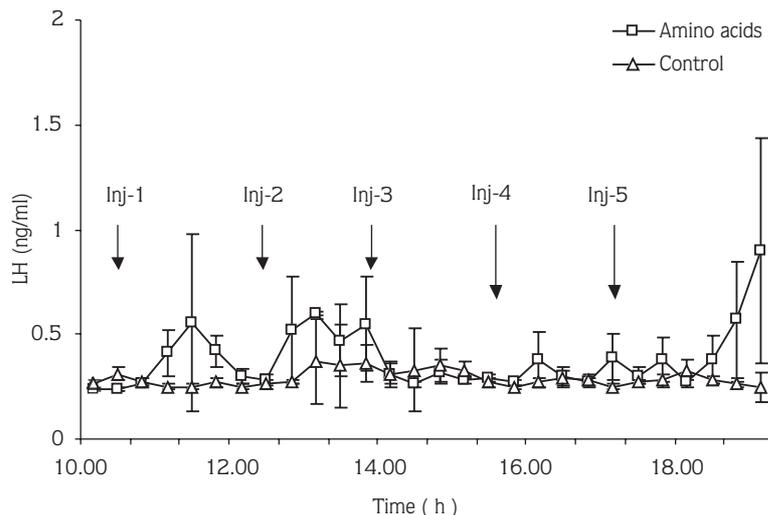


Figure 3. Plasma LH concentration in saline and amino acid mixture injected groups. Injection of amino acids at two hour intervals did not cause any significant changes in plasma LH concentrations during 9 hours ($P > 0.05$).

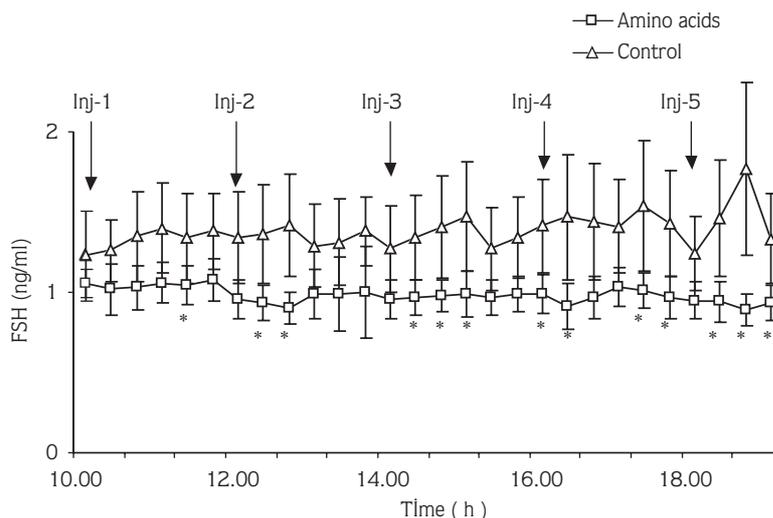


Figure 4. Plasma FSH concentrations (Mean \pm SEM) in control and treatment groups. The samples significantly different in FSH concentration are indicated by stars (*). Amino acid injection significantly reduced plasma FSH concentration ($P < 0.05$).

Discussion

The main aim of this study is to investigate the effect of injecting sheep with Serine and Threonine amino acid mixture with or without a GnRH on gonadotrophin secretion. Gonadotrophins are important in the control of follicle selection and ovulation rate in the ewe (14). Many components of blood including amino acids have been tested as potential factors affecting the secretion of gonadotrophins. Therefore, sheep were infused with leucine, isoleucine, valine, tryptophan, tyrosine or thyrosine plus phenylalanine, but infusions of these amino acids did not cause any significant changes in plasma concentrations of LH and FSH (15,16). In another experiment, prepubertal sheep were infused intravenously with 30 g L-Arginine for six hours and it was reported that infusion increased LH pulse frequency. The reason for increased LH secretion was attributed to the increase in hypothalamic GnRH release due to the arginine infusion (9). Some amino acids act as neurotransmitters in the central nervous system and stimulate secretion of GnRH. In an experiment, aspartic acid analogue, N-Methyl-D,L-aspartic acid (NMA) injected intravenously to cycling ewes and injection caused an immediate release of LH. Similar results were also obtained in rodents and primates (17). In this experiment, Serine and Threonine amino acids different from the amino acids used in other studies were injected. Serine and Threonine amino acids are phosphorylated

within the intracellular proteins regulating the secretion of second messengers playing a role in the expression of mRNA for Transforming Growth Factor Beta (TGF β) like peptides. These peptides are capable of modulating the gonadotrophin secretion and the response of the pituitary to GnRH (12). In this study, no immediate effect of Serine and Threonine mixture injection on LH secretion was observed. However, the most significant increases in LH concentrations were observed in AA plus GnRH treated (AA/GnRH and AA/AA+GnRH) groups. This significant increase could be attributed to AA injections in advance, which might affect GnRH secreting neurones in hypothalamus and cause an increase in hypothalamic GnRH release. On the other hand, the LH increase in GnRH treated groups might be a direct result of GnRH injection. The effect of short term GnRH administration is well known to increase pituitary secretion of LH (18). To the best of our knowledge, because of the absence of similar data, it is hard to make a plausible conclusion for the findings.

No immediate effect of Serine and Threonine mixture injection on FSH secretion in this study was observed either. Besides, FSH concentrations were lower in AA+GnRH injected groups than only GnRH injected groups at the second injection. The increases in FSH concentrations in the latter groups could be a direct result of GnRH treatment (19). An antagonistic effect of AA injection when they are administered with GnRH could be

speculated. This aspect of AA effect on FSH secretion requires further studies.

The results obtained in the second experiment support the findings in the first experiment that no immediate effect of single or repeated serine and threonine mixture injection on LH secretion exists (Figure 3). On the other hand, plasma FSH secretion was suppressed when amino acids were injected every two hours (Figure 4). It is possible that Serine and Threonine amino acids affect the expression of TGF- β like factors within the ovary since these amino acids are capable of being phosphorylated by protein kinases within the cells. Unfortunately, no work has been done on sheep to measure the effect of Serine and Threonine injection on ovarian TGF β 1 synthesis. According to the data from an experiment in the rat, injection of L-Arginine resulted in an increase in mRNA expression for TGF β 1 (20). The same analogy may apply

in sheep as well. TGF β 1 is structurally related to inhibin which is well characterized on the basis of its capacity to specifically inhibit the secretion of FSH while it increases pulsatile secretion of LH in sheep (21,22). Inhibin diminishes the stimulatory effect of TGF β 1 on FSH-release (23). It has been reported that TGF β stimulates basal and FSH stimulated inhibin production (24,25).

Whether the increase in LH and suppression in FSH secretion are just a direct result of amino acid injection is not presently understood. It seems that there are some complex interactions between the hypothalamic, hypophysial, and ovarian derived factors and Serine/Threonine amino acids. To understand whether Serine and Threonine injection affect FSH secretion via inhibin production it is required to determine changes in mRNA expression for inhibin after injection.

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