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The inhibitory effect of physiological pattern of testosterone on luteinizing hormone secretion in sheep

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면양에서 생리적 분비형태의 테스토스테론이 황체형성호르몬의 분비 억제에 미치는 효과

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초록 : 본 연구의 목적은 거세한 숫양에서 테스토스테론의 생리적인 주기적 분비형태가 황체형성호르몬의 분비억제에 미치는 효과를 연구하는 데 있다. 이를 위해, 3개의 서로 다른 실험들을 수행하였다.

실험 1에서는 정상적인 숫양에서 관찰되는 테스토스테론의 분비방식과 동일한 분비형태를 인위적으로 만들기 위해 필요한 테스토스테론의 주입비율과 분비형태를 얻기 위하여 3가지 다른 양(25, 50 그리고 100μg)의 테스토스테론을 정맥에 주입시켜 테스토스테론의 약리동력학을 분석한 결과, 테스토스테론의 평균 제거율상수, 분포용적, 그리고 총 체청소율은 각각 0.18min^{-1}, 0.53l/kg, 그리고 0.09l/min/kg 이었다. 실험 2에서는 테스토스테론의 처리 시간의 경과에 따른 테스토스테론의 황체형성호르몬 평균 농도의 억제 효과를 조사하기 위하여 3가지 다른 분비율(192, 384 그리고 768μg/kg/24h)의 테스토스테론을 주기적으로(4시간 간격) 3일 동안 정맥에 주입시킨 결과, 테스토스테론의 처리 시간이 증가함에 따라 혈액 내 황체형성호르몬의 평균 농도는 서서히 감소하였다. 테스토스테론을 2일 또는 3일간 처리하였을 때는 테스토스테론을 처리하기 전에 비해 황체형성호르몬의 평균 농도는 현저히 감소하였다. 그러나 테스토스테론의 1일간 처리는 황체형성호르몬의 평균 농도를 감소시키지 못하였다. 실험 3에서는 두 가지 다른 분비 형태(지속적 분비 형태와 주기적 분비 형태)의 테스토스테론의 황체형성호르몬의 분비에 미치는 효과를 비교 조사하였다. 지속적 분비 형태를 만들기 위하여 테스토스테론을 3일간 지속적으로(32μg/kg/h) 정맥 주입시켰고, 주기적 분비 형태를 만들기 위하여 테스토스테론을 4시간 간격으로 3일간 주기적으로(128μg/kg/h) 정맥 주입시켰다. 지속적 방법과 주기적 방법 간에 동일한 양(768μg/kg/h)의 테스토스테론이 주입되었다. 혈액은 테스토스테론의 정맥 주입전 4시간 동안과 3일간 정맥 주입 기간 중 마지막 4시간 동안 각각 10분 간격으로 경정맥에서 채취하였고, 황체형성호르몬과 주입된 테스토스테론의 혈액 내 농도는 각각의 방사성면역방법을 이용하여 측정하였다. 황체형성호르몬의 펄스 간격($p<0.034$)과 황체형성호르몬의 평균 분비량은($p<0.045$) 주지적 방법 보다 지속적 방법의 테스토스테론의 주입에 의해 현저히 증가하였다. 황체형성호르몬의 펄스 분비량은 주기적 방법과 지속적 방법 간에 차이가 없었다.

이상의 결과들은 숫양에서 지속적 방법의 테스토스테론이 주기적 방법의 테스토스테론의 보다 황체형성호르몬의 분비를 저하시키는데 보다 더 효과적임을 나타내 보이고 있다.

Key words : testosterone, pharmacokinetics, pulsatile, luteinizing hormone, sheep

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Introduction

It is well established that testosterone(T) inhibits luteinizing hormone(LH) secretion in males. Observations that T replacement decreased gonadotropin-releasing hormone(GnRH) pulse frequency in castrated rams[1,2], taken together with the observation that castration led to an increase in GnRH pulse frequency compared to intact rams[3], provide clear evidence that T reduces LH pulse frequency by reducing GnRH pulse frequency. T also suppresses responsiveness of the pituitary to GnRH in the ram[4-7]. However, in most previous studies, T was administered by either implantation of Silastic tubes filled with T or periodic injection of testosterone propionate. However, all of these treatment procedures produced a relatively constant concentra-tion of T in the circulation. T is secreted in distinct periodic pulses in intact males including sheep[8-11]. Thus constant T replacement dose not reflect the endogenous T secretory pattern and the question arises as to whether constant administration of T produces physiologically normal responses. This question also is raised by the many observations that continuous infusion of peptide hormones causes down regulation or desensitization of the target tissue[12], These observations suggest that a physiologcal pattern of peptide hormone administration is optimal for expression of biological function. In the case of gonadal steroid hormones, however, more effective suppression of LH by a constant pattern of T than a pulsatile pattern of T has been proposed by Hutchison and Goldman[13]. Thus, the aims of the present study were 1) to establish infusion rates and patterns needed to produce normal T secretory profiles found in intact rams, 2) to determine the time-course of the suppressive effect of physiological pattern of T on circulating LH concentrations, and 3) to compare the effectiveness of a pulsatile versus a constant pattern of T to suppress LH secretion in wethers.

Materials and Methods

Experiment 1

Animal : Two Hampshire ovariectomized-ewes were used in Experiment 1. They were fed grass or alfalfa hay and grain, and given free access to water and sheep mineral blocks.

Experimental protocol : Doses of 25, 50 or 100μg of T in 5ml of ethanol-saline solution(final ethanol concentration was less than 10% of final volume) were used for intravenous injection. On day 1, 25μg and 50μg of T were injected intravenously via one jugular vein to each animal. On day 2, 50μg and 100μg of T were injected intravenously to each aninal.

Collection of samples : Single blood samples were collected from the other jugular rein both prior to and 1, 3, 5, 10, 15, 30, 45, 60, 90, 120, 150 and 180 mins after T injection. Blood samples were maintained in ice bath and centrifuged within 30 min after samples were collected, and then the plasma was removed and stored at -20℃ until assay.

Hormone assay : Testosterone was measured in duplicate sample using a previously described radioimmunoassay[14], validated direct, double antibody procedure[15]. The T antibody AS-0116 was purchased from Immuno Corp, Montreal, Canada. Assay sensitivity was approximately 20pg. Intra- and interassay coefficients of variation were 4.4% and 12.7%, respectively.

Analysis of the data : Within the aid of a computer program, the volume of distribution, half-life, total body clearance, and infusion rate of T were estimated.

Experiment 2

Animal : Six adult Hampshire wethers(ranged from 70 to 82kg BW) were used in Experiment 2. Animals had been castrated at least 5 months before the first infusion. They were fed grass or alfalfa hay and grain, and given free access to water and sheep mineral blocks.

Experimental protocol : Doses of 192, 384 or 768μg/kg/24h of T in propylene glycol-heparinized saline solution were used for intravenous infusion. Each dose of T was given into 2 different animals. For pulsatile infusion, the T solutions were infused as six, 1h duration, pulses each 24h. Thus T was infused

only for every first 1h at the flow rate of 15ml/h at 4h intervals for 3d at doses of 192, 384, or 768μg/kg/24 h.

Infusion protocol : On the day before the infusion, Tygon catheters(Norton Co, Akron, OH, USA; 1.02mm id×1.78mm od) were inserted into each jugular vein of the animals under local anesthesia (Lidocaine Hydrochloride; Anthony Products, Arcadia, CA, USA). One was used to administer T and the other to collect peripheral blood. The animals then were placed in metabolism cages with food and water, and allowed to acclimate for 16 to 18h prior to the infusion. Crystalline T was dissolved in 100% propylene glycol(Phoenix Pharmaceutiacl, Inc, St Joseph, MO, USA) for stock T solution. T-infusates consisted of propylene glycol: sterile heparinized saline solution(50:50). The final concentration of heparin in each infusate was 60IU/ml. T was delivered by a pulsatile infusion with the aid of peristaltic pumps(Gilson Medical Electronic, Inc, Middleton, WI, USA) using PVC Manifold Tubing(Rainin Instrument Co, Woburn, MA, USA; 2.28mm id). Each pump was controlled by an electric timer(ChronTrol; Lindburg Enterprises, San Diego, CA, USA) for a pulsatile infusion.

Collection of samples : Blood samples were taken at 10-min intervals for 4h both prior to infusion(day 0), and during the last 4h of the infusion of day 1, day 2 and day 3. Blood samples were centrifuged within 30 to 40 min after samples were collected, and then the plasma was removed and stored at -20℃ until assay.

Hormone assay : LH was measured in duplicate plasma samples using a previously described radioimmunoassay[16]. The sensitivity was 0.1ng NIH LHS20. Intra- and interassay coefficients of variation were 3.2% and 4.8%, respectively.

Analysis of the data : Mean LH concentrations were compared by multifactor analysis of variance using a split-plot design with repeated measures, in which doses of T were assigned to the whole plot and the period of infusion to the subplots within each whole plot. Significant differences in treatment means between period of infusion, or between period of infusion within same dose of T were determined using least significant difference test[17].

Experiment 3

Animal : Five adult Hampshire wethers were used in Experiment 3. Animals had been castrated at least 5 months before the first infusion. They were fed grass or alfalfa hay and grain, and given free access to water and sheep mineral blocks.

Experimental protocol : Five wethers were randomly assigned to each of 2 patterns of treatments in a cross-over design. The 2 treatments were 1) constant-T(C-T); pulsatile-T(P-T). For constant infusion, T(32μg/kg/h) was continuously infused at the flow rate of 5ml/h for 3d. For pulsatile infusion, T was infused as six, 1h duration, pulses each 24h for 3d. To achieve pulses, T(128μg/kg/h) was infused only for the first 1h of each 4h period at the flow rate of 20ml/h. Under these infusion protocol, same amounts of T(768μg/kg/24h) were delivered for C-T and P-T.

Infusion protocol : Infusion protocol used in Experiment 3 was same as that in Experiment 2, with the exception that T was delivered by either a constant infusion or a pulsatile infusion with the aid of peristaltic pumps on which PVC Manifold Tubing(0.76mm i.d. for a constant infusion and 2.28mm i.d. for a pulsatile infusion) was installed. One pump was used for a constant infusion and the other controlled by an electric timer for a pulsatile infusion.

Collection of samples : Blood samples were taken at 10-min intervals for 4h both prior to infusion(day 0), and during the last 4h of the infusion(day 3). Blood samples were centrifuged within 30 to 40 min after samples were collected, and then plasma was removed and stored at -20℃ until assay.

Hormone assay : LH and T were measured in duplicate plasma samples using previously described radioimmunoassay(see Experiment 1 and 2 in Materials and Methods).

Analysis of the data : LH pulses were identified and described with the aid of the Pulsar algorithm[18] using G values of 1.5, 1.4, 1.3, 1.2 and 1.1 for G(1) through G(5), respectively. Differences of means between the treatment groups either during pre-treatment, during treatment, of between pre-treament and

during treatment were compared using Student's t-test[17]. Differences of $p<0.05$ were considered significant.

Results

Experiment 1 : The disappearance of exogenous T from the plasma of ovariectomized ewes with time after T intravenous injection is shown in Fig 1. The biexponential curve indicates that T is distributed into 2 compartments: central compartment and tissue compartment. After intravenous injection, T is more rapidly diffused into the central compartment. Once equilibrium is established, T is eliminated from the central compartment.

The pharmacokinetic data of T are listed in Table 1. The volume of distribution of T(V_c: the apparent volume in the body in which T is dissolved) is calculated by the following equation: $V_c=D_O/C_{PO}$

where D_O =injected T dosage

C_{PO}=plasma T concentration at the time of injection

Half-life of T averaged approximately 10min(data not shown). Total body clearance(Cl_T: the sum total of all the clearance pathways in the body including renal and hepatic clearance) of T is calculated from the following equation: $Cl_T=K_{el}\times V_c$, where K_{el} is elimination rate constant. The infusion rate(R) is calculated from the following equation: $R=(C_P-0.5)\times Cl_T$, where C_P is the target concentration of T(ng/ml).

From the study to validate the T-infusion performance(unpublished data), a significant($r=0.95$, $p<0.01$) linear relationship between T dose and plasma T concentrations was observed(Y=-0.42+0.31 X= where, T dose and Y=circulating plasma T concentrations). Using the equation as shown above, it is estimated that intravenous infusion of T at rate of 32μg/kg/h produces mean circulating concentration of 10ng/ml of T. This mean concentration of T is similar to that observed in the ram during the breeding season[8,10,19].

Experiment 2 : Mean LH concentrations before and during 3d of 3 different doses of T infusion are

Table 1. Elimination rate constant(K_{el}), volume of distribution(V_c), and total body clearance (Cl_T) of testosterone(T) estimated from the disappearance curves of exogenous T after intravenous injection of T in ovariectomized ewes

T-dose[1]	K_{el} (min^{-1})	V_c (l/kg BW)	Cl_T (l/min/kg BW)
0.36	0.23	0.44	0.10
0.69	0.18	0.55	0.10
0.71	0.20	0.53	0.10
1.37	0.11	0.60	0.07
Mean±SEM	0.18±0.02	0.53±0.03	0.09±0.01

[1]Dose is expressed as μg/kg BW in 5ml of ethanol-saline solution.

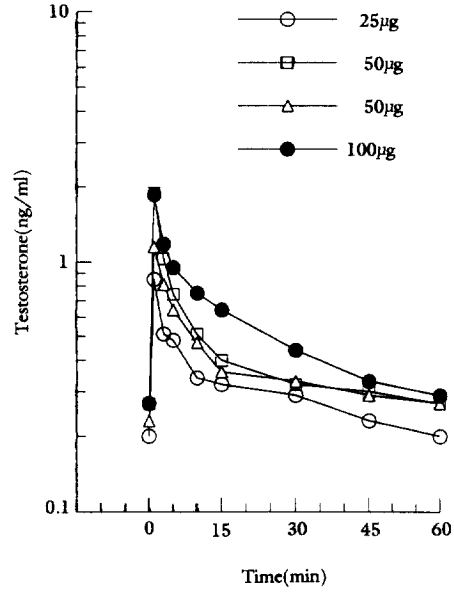

Fig 1. Plasma testosterone(T) profiles in ovariectomized ewes after intravenous injection of 3 different doses(25, 50 or 100 μg) of T.

Table 2. Mean LH concentraions in wethers before(day 0) and during treatment with 3 different doses of pulsatile testosterone(T) infusion for 3d[1]

T-dose[2]	Mean LH concentrations(ng/ml)[3]			
	Day 0	Day 1	Day 2	Day 3
192	$12.54\pm0.65^{A,B}$	14.35 ± 0.84^{B}	9.36 ± 1.29^{A}	9.56 ± 0.26^{A}
384	25.38 ± 5.89^{A}	22.67 ± 5.20^{A}	16.56 ± 3.40^{B}	14.60 ± 2.70^{B}
768	12.35 ± 2.29^{A}	17.76 ± 4.17^{B}	11.94 ± 2.49^{A}	9.17 ± 0.24^{A}
Mean±SEM	16.76 ± 3.18^{A}	18.26 ± 2.31^{A}	12.62 ± 1.71^{B}	11.11 ± 1.31^{B}

[1]For pulsatile T infusion, T was infused as six, 1h duration, pulses each 24h. Thus T was infused only for every first 1h at the flow rate or 15ml/h at 4h intervals.

[2]Dose is expressed as μg/kg/24h.

[3]Values are mean±SEM of 2 observations.

[A,B]Values in the same row with different superscripts are significantly different($p<0.01$).

shown in Table 2. Since a significant main effect(the effect of different doses of T on mean LH concentrations) was not observed, the data were pooled to analyze the effect of different durations of T infusion on mean LH concentrations.

Mean LH concentrations before and during the treatment of T are shown in lower panel of Table 2. As duration of T infusion increased, mean LH concentrations gradually reduced. Mean LH concentrations were significantly lower at day 2 or day 3 than at day 0. However, mean LH concentrations did not differ between day 0 and day 1 or between day 2 and day 3.

Experiment 3 : It was observed that during a pulsatile T infusion, circulating T concentrations were rapidly increased within 20-30min after start of infusion, whereas the concentrations were relatively stable during a constant T infusion. Mean concentrations of T

Table 3. LH pulse parameters in wethers before(day 0) and after treatment for 3 d(day 3) with either constant testosterone(C-T) or pulsatile testosterone(P-T) infusions[1,2]

Treatment	Mean LH concentration(ng/ml)		
	Day 0	Day 3	(Day 0-Day 3)
C-T	13.48±1.51	11.00±1.41	2.48±0.43
P-T	11.92±1.33	7.68±0.92	4.23±0.58
	C-T at Day 0 vs C-T at Day 3:		p=0.005
	P-T at Day 0 vs P-T at Day 3:		p=0.002
	C-T(Day 0-Day 3) vs P-T(Day 0-Day 3):		p=0.045

Treatment	LH Amplitude(ng/ml)		
	Day 0	Day 3	(Day 0-Day 3)
C-T	3.40±0.92	3.45±0.86	-0.05±0.94
P-T	3.00±0.75	5.30±1.41	-2.30±1.32

Treatment	LH Inter-pulse Interval(min)		
	Day 0	Day 3	(Day 0-Day 3)
C-T	31.29±0.77	45.12± 3.25	-13.83±3.96
P-T	32.96±1.76	81.50±10.05	-48.54±9.62

C-T at Day 0	vs	C-T at Day 3:	p=0.025
P-T at Day 0	vs	P-T at Day 3:	p=0.007
C-T(Day 0-Day 3)	vs	P-T(Day 0-Day 3):	p=0.034

[1]For C-T infusion, T(32μg/kg/h) was continuously infused at the flow rate of 5ml/h for 3 d. For P-T infusion, T (128μg/kg/h) was infused only for the first 1h of each 4h period at the flow rate of 20ml/h. Thus same amounts of T(768μg/kg/24h) were given.

[2]Values are mean±SEM of 5 observations.

produced by P-T did not differ from the concentrations of T produced by C-T.

LH pulse parameters at day 0 and day 3 in 2 different treatment groups are shown in Table 3. Neither mean, pulse amplitude, nor inter-pulse intervals of LH differed between the treatment groups at day 0. Treatment with either pulsatile or constant patterns of T for 3d significantly suppressed mean LH concentrations. LH amplitude in either P-T or C-T groups did not differ between day 0 day 3. Treatment with either pulsatile or constant patterns of T for 3 d significantly increased LH inter-pulse interval. Effects of treatment on LH secretion were evaluated by analyzing changes of LH pulse parameters between day 0 and day 3. Changes in LH pulse amplitude did not differ between P-T and C-T groups. However, changes in mean LH(p=0.045) and LH inter-pulse interval(p=0.034) were greater in C-T group than P-T group.

Discussion

The results show that mean LH concentrations were more reduced by C-T than by P-T and LH inter-pulse interval was more increased by C-T than by P-T, demonstrating that a constant pattern of T is more effective than a physiological pulsatile pattern of T in suppressing LH secretion in the ram. These observations are in agreement with the data form Hutchison and Goldman[13] who found that LH concentrations were much lower in castrated rats receiving continous T infusion in one experiment than those in animals receiving single bolus injection of same amount of T in another experiment.

The results from Experiment 3 that both T patterns reduced mean LH and increased LH inter-pulse interval but did not alter LH pulse amplitude confirm the results from the previous studies[1,2].

It is clear for peptide hormones that normal physiological function is more effectively produced by exposure to a physiological pattern than a continuous

pattern of GnRH[20-22]. But very little is understood about the effects of patterns of steroid hormones in negative feedback system.

The mechanism underlying more effective feedback inhibition by C-T than by P-T in suppressing LH secretion is not understood, but it may be explained by the difference in relative number of androgen receptors induced by two different patterns of T. T increases androgen receptor number at hypothalamus or pituitary in males in a dose-dependent manner[23]. Suppression of LH secretion by T is, at least in part, mediated by androgen receptor in males[24,25]. Thus, these observations propose that less effective inhibition of LH secretion by the P-T infusion in the present study may result from the short duration of T pulse which may not be long enough to stimulate appeareance of androgen receptors. However, it remains to be understood to what extent androgen receptor number and/or affinity are regulated by different patterns of T.

Summary

The objective of the present study was to investigate the inhibitory effect of physiologically pulsatile pattern of testosterone(T) on luteinizing hormone(LH) in wethers. To do this, 3 separate experiments were conducted. Infusion rates and patterns needed to produce normal T secretory profiles found in intact rams were established in Experiment 1, the time-course of the suppressive effect of T on circulating LH concentrations was determined in Experiment 2, and the effectiveness of a pulsatile versus a constant pattern of T to suppress LH secretion in wethers was compared in Experiment 3. In Experiment 1, three different doses(25, 50 or 100μg) of T were injected intravenously to animals to do pharmacokinetic analysis of T. Elimination rate constant, volume of distribution, and total body clearance of T averaged 0.18min^{-1}, 0.53l/kg BW, and 0.09l/min/kg BW, respectively. In Experiment 2, three different doses(192,384, or 768μg/kg/24h) of T were infused at 4h intervals for 3 days into animals to evaluate the timecourse of the inhibitory effect of T on mean LH concentration. As duration of T infusion increased, mean LH concentrations gradually reduced. Mean LH concentrations were significantly lower at day 2 or day 3 than at day 0. However, mean LH concentrations did not differ between day 0 and day 1 or between day 2 and day 3. In Experiment 3, animals were subjected to two different intravenous infusion regimens for 3 days: constant T(768μg/kg/24h) and pulsatile(one pulse every 4h) T(768μg/kg24h). Blood samples were collected at 10-min intervals for 4h both prior to infusion and during the last 4h of the infusion. Mean LH was more suppressed($p=0.045$) by constant T than by pulsatile T. LH pulse amplitude was not affected by constant T or pulsatile T. LH interpulse interval was increased more($p=0.034$) by constant T than pulsatile T.

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