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Effect of 19-Norandrostenedione on the Spermatogenesis in Rat Testis

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19-Norandrostenedione이 흰쥐 정소내 정자형성에 미치는 영향

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INTRODUCTION

Testosterone(T) in relatively low-dose inhibits secretion of gonadotropins and thus supresses spermatogenesis. However, high dose of testosterone stimulates spermatogenesis, even though pituitary gonadotropin secretion is depressed(Clermont & Harvey, 1967 ; Reddy & Rao 1972 ; Berndtson et al., 1974 ; Steinberger & Smith 1977 ; Rea et al., 1986 a, b ; Matsumoto, 1988, 1990). These results suggest that low dose of testosterone adequate to maintain the functions of accessory sex glands and libido, is insufficient to maintain spermatogenesis.

The anabolic steroid 19 - nortestosterone (NT), widely used in horse and human, has the potential for the fertility control since high dose of NT supress spermatogenesis. NT, replacing T action, has not shown serious toxic side effects in horse and in over 20 years of clinical uses(Nieschlag et al., 1981 a, b; Schuermeyer et al., 1983, 1984; Belkien et al., 1985), such as in the treatment of conditions caused by defects in androgen synthesis or metabolism, disorders of sexual development, and male antifertility agents(Nieschlag et al., 1981 a, b). On the other hand, 19-norandrostenedione (NORA) is a major steroid in equine and porcine follicular fluids (Khalil & Walton, 1985; Khalil et al., 1989), and inhibits the oocyte maturation in pig(Daniel et al., 1986). NORA is syntuesized by aromatase from andvostenedion(A) in porcine granylosa cell and by microsomal aromatase in equine testis(Gaillard & Silberzahn, 1987; Silberzahn et al., 1988). However the synthesis, metabolism and mechanism of NORN are not clear yet.

A number of systemic routes of administration are currently available for delivering agents to the testes for the study of the effects of androgens on spermatogenesis. Many of these routes such as intraperitoneal (i.p.), intravenous(i.v), intramuscular(i.m.), oral etc are in common usage and have been validated. However intratesticular(i.t.) injection is a seldomly used techinique, that requires further validation and the acceptibility may be questioned eventhough Russell et al(1987) had thoroughly validated to study the direct effects of the agents of unknown toxicity on the testis.

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The i.t injection offers the potential benefit over other route administration because i.t. injected androgens are not systemically metabolized(Weinbauer et al., 1985; Russel et al., 1987; Yoon and Yang, 1988).

Therefore, the present study was designed to study the direct effect of 19-nortestosterone on the spermatogenesis comparing with that of 19-nortestosterone and testosterone enanthate and the efficacy of intratesticular injection of androgens.

MATERIALS AND METHODS

ANIMALS: Adult male Sprague-Dawley rats weighing 450-490g were under conditions of controlled temperature and 14h light: 10h darkness cycle and given free access to rat chow and water.

CHEMICALS : 19 - nortestosterone(NT). 19-norandrostenedione (NORA), testosterone enanthate(TE), were purchased from Sigma, (Inben, Korea). 1, 2, 6, 7-^{3}H-testosterone(sp. act. 85Ci/mmol) and 1β, βb-^{3}H androst-4-ene-3, 17-dione(sp. act 50Ci/mmol), were purc-hased from New Nuclear England.

TREATMENT PROTOCOL : Groups of ten rats were treated thrice per week for 12 weeks with either vehicle(50% v/v propylene glycol : water) or 50μg/day(representing a dose of approximately 150μg/kg body wt per day) of the potent GnRH antagonist, (N-AC-D-Nal(2)1, D-pC1-Phe2, D-Trp3, D-hArg(Et2)6, D-Ala10)-GnRH (Syntex Research, Palo Alto, CA). In addition, groups of vehicle -and antagonist-treated rats received 300μg of testosterone, NT, or NORA by i.t injections. After 4 weeks of continuous treatment with GnRH, when serum GTH levels were at below the limit of detection, androgens were supplemented by i.t. injection using a 26 or 30 gauge needle and precision volume(Hamilton) syringe. At the end of the treatment period, the rats were killed by decapitation under light ether anaesthesia and testes, epididymis, accessory organs such as seminal vesicles, prostate and copulating gland were removed and weighed. One testis and one epididymis from each animal were processed for histological examination. The remaining tissues were frozen and stored at -70℃ for intratesticular steroid hormones. Trunk blood was collected for the determination of serum testosterone, luteinizing hormone(LH) and follicle stimulationg hormone(FSH) by radioimmunoassays(RIA).

HISTOCHEMICAL OBSERVATION FOR GERM CELLS: For histological preparation, we adapted a modified method of Bansal and Davis(1986): The testes were dissected out and fixed in Bouin-Hollande fixative for 48 hours. The tissues were embedded in paraplast wax after rou-tine dehydration Ribbons of 4 μm thick sections were stained by the periodic acid-Schiff's reaction. and counter-stained in Harris's haematoxylin and mounted in DPX. Cell nuclei lying in transverse sections of tubules at stage Ⅶ of the tubules were counted (Leblond & Clermont, 1952). Nuclei of Sertoli cells and type A spermatogonia were scored only when nucleoli were visible. Sections of tubules not showing a lumen or having axial ratios greater than 2 were not used (Davies et al., 1974). The error to underestimate the number of the germ cells because of not counting the samll particles, was reduced by counting the sections of nuclei or nucleoli whose apparent diameter was judged to be larger than the true radius. A correction for the size of the diameter was then made by a modification of Aberecrombie's method(Davies, 1973).

HORMONE ASSAYS: Serum FSH and LH were determined by double antibody RIA using rat hormones (LH-I-5, LH-RP-1, FSH-I-6, FSH-RP-1) and antisera (anti-LH-S-9 and anti-FSH-S-11), kindly

supplied from National Homone and Pituitary Programme through Dr. MA Rea, Max-Planck Instute, Muenster, FRG. The minimum detectable dose of LH was 5ng/ml, and intra-assay variation (WAV) was 7.5 %. The inter-assay variation (BAV) was 15.3%. The minimum detectable level of FSH was 70ng/ml of serum. WAV and BAV for FSH assays were 6.3% and 11.6% respectively. Pituitary contents of LH or FSH were determined in 50μl each aliquots of the pituitary homogenates after dilution of 1:1,000 and 1:100 respectively, using a modified method of Rea et al (1986 a). All serum and puitary samples were assayed in a single RIA batch for gonadotropin estimation.

The steroids 19NT, T, and DHT were fractionated as previously described (Bals-Pratsch et al., 1986; Yoon & Kim, 1987; Yoon et al., 1987). To determine NT only, we used the celite chromatography using the fractions (48-55 fractions), eluted by pure isooctane. Testosterone, progesterone(P) and estradiol(E) concentration were determined by RIAs (Yoon et al., 1981, 1987).

STATISTICALL ANALYSIS: Each experiments was repeated four times. Untransformed data are represented as means $\pm$SD for quadriplicate analyses. Statisticall analysis of the data was by one-way analysis of variance and Duncan's multiple range test.

RESULTS

1) Effects of NORA on Male Reproductive Organs

Testis weight was reduced significantly after 4 weeks of antagonist treatment and also significantly after 12 weeks of TE treatment. NORA, NT and T supplementations with GnRH antagonist for 12 weeks significantly prevented the loss of testis weight (Table 1). One way analyses of variance on data from all treatment groups indicated significant effects on testis ($p < 0.001$). Treatments for 12 weeks with 300μg doses of NORA, NT and TE increased in the weights of the accessory glands of the rat treated with GnRH antagonist. However, they did not increase the weight of completely prevented the reductions of epididymal weight induced by GnRH antagonist.

2) Effects of NORA on Serum Gonadotropins

Table 1. Changes of weights of body weight, testis, accessory glands, and epididymis after 12 weeks treatment with vehicles or GnRH with or without 19-norandrostenedione, 19-nortestoserone and testosterone enanthate.

Steroids	Control	NORA	NT	TE
Body weight(g)	472±16	371±17	435±19	386±23
Testis weight				
Vehicle	1.7±0.1	2.0±0.6	1.5±0.2	1.2±0.1
GnRH	0.3±0.05	1.3±0.1	1.5±0.1	1.8±0.3
Accessory Weight(g)				
Vehicle	1.8±0.1	2.2±0.1	2.5±0.2	3.8±0.4
GnRH	0.3±0.06	0.6±0.1	3.1±0.7	3.5±0.9
Epididymis				
Vehicle	0.7±0.05	0.8±0.07	0.7±0.25	0.9±0.07
GnRH	0.1±0.06	0.5±0.31	0.6±0.23	0.6±0.04

The weights of accessory glands are included the weight of seminal vesicles, prostate, coagulating glands. Values are single organs and are the means +/−S.D. of between 10 to 20 animals. *$p<0.05$ compared with the vehicle-treated group without androgen (ANOVA followed by Mann-Whitney U test). NORA; 19-norandrostenedione. NT; 19-nortestosterone. TE; testosterone enanthate.

Weekly serum GTH values were determined during 12 weeks of GnRH antagoinst -suppressed treatment group are shown in Table 2. After 4 weeks of treatment, serum FSH concentration were at or below the detection limits. However NORA and TE supplementation were sufficients to maintain serum T concentrations at levels about 2 to 4 times higher and prevented the loss of serum FSH. At the end of treatment period, serum FSH values with NORA supplemented group were not significantly different with the control group. Furthermore, pituitary FSH content which was reduced to about 10% of the control value in GnRH antagonist treated group was not significantly different from the control values in any of the supplemented groups(data not shown).

On the other hands, both serum concentration and pituitary content of LH were not substantly reduced after antagonist treatment. A further reduction ($p<0.05$) in pituitary and seum LH content after NORA

Table 2. Serum gonadotropins and steroid hormones and intratesticular testosterone concentration in vehicle and gonadotropin-releasing hormone antagoist-treated rats.

Hormones	Control	Treatment	
		NORA	TE
Serum LH(ng/ml)			
Vehicle	16.2± 6.1	ND	ND
GnRH	ND	ND	ND
Serum FSH(ng/l)			
Vehicle	266.2±34.5	245.7±13.4	153.7±12.9
GnRH	<70.5±11.5	121.7±14.6	151.4±16.9
Serum T(nmol/l)			
Vehicle	12.7± 4.5	26.4± 7.8	49.1± 4.6
GnRH	<1.7± 1.4	16.7± 9.8	36.3± 5.8

Valuse are means +/− S.D. of ten between twenty animals. *$p<0.05$ compared with the vehicle-treated group (ANOVA followed by Mann-Whitney U test. ND; not detectable. NORA; 19-norandrostendione. TE; testosterone enanthate.

Table 3. Numbers of A1 spermatogonia, preleptotene spermatocytes, pachytene spermatocytes and step 7 spermatids after 12 weeks of continuous treatment of rats with vehicle or GnRH antagonist either with NORA and TE supplementation.

Germ cells	Control	Treatment	
		NORA	TE
A1 spermatogoina			
Vehicle	0.54±0.01	0.51±0.05	0.49±0.01
GnRH	0.75±0.01	0.44±0.10	0.47±0.11
Preleptotene spermatocytes.			
Vehicle	17.5±2.7	17.9±2.6	16.7±3.5
GnRH	15.6±3.2	16.4±3.9	17.4±4.1
Pachytene spermatocytes			
Vehicle	9.3±1.1	8.5±3.3	11.5±2.7
GnRH	4.7±1.3	7.5±2.1	9.6±3.8
Step 7 spermatid			
Vehicle	29.4±1.8	17.9±3.5	16.5±3.1
GnRH	8.3±1.2	16.5±1.7	17.9±1.7

Cell counts were corrected according to the Davis (1973) formular. Values are means +/− S.D. of ten between twenty animals. *$p<0.05$ compared with the vehicle-treated group without testosterone (ANOVA) followed by Mann-Whitney U test. NORA; 19-norandrostenedione. TE; testosterone enanthate.

or TE supplementation could not be observed. NORA and TE supplements could not elevated the serum levels of LH in rat treated with GnRH antagonist.

3) Effects of NORA on Germ Cells

The corrected cell counts in 10 transverse sections of seminiferous tubles, after applying diameter corrections, are given in Table 3. The over all effects of NORA and TE were significantly on step 7 spermatids ($p < 0.05$) as given by one-way analysis of variance on data from all the treatment groups. There were reductions in the number of step 7 spermatid. The depression of intratubular germinal cell number was greater after 12 weeks of treatment than after 8 weeks. NORA and T supplementations prevented partially the reductions of step 7 spermatids and pachytene spermatocytes in rat treated with continuous GnRH.

DISCUSSION

19-norandrostenedione has been identified as a major steroid in porcine ovarian follicular fluid (Khalil & Walton 1985; Khalil et al., 1989), but never been identified in plasma of untreated subjects. In stallion testis, NORA could be detected as an intermediate of NT aromatization (Silberzahn et al., 1988). These results suggest that testicular NORA is an intermediate in an accessory pathway from T to estradiol (see " Introduction" for selected citations).

Testosterone enanthate supressed gonadotropin secretion and then for Treplacement therapy to maintain the accessory sex organs, libido and potency. We demonstrated that concurrent NORA supplementation prevented the drastic reductions in the serum concentrations of FSH but not of LH (Rea et al., 1986a, b), suggesting that NORA may actually stimulate FSH synthesis at the expense of LH reduction. These results suggest that the androgenic effect on gonadotropin secretion of NORA is not more sufficient than that of testosterone derivatives. The mechanism by which testosterone and NORA support the FSH synthesis and secretion in the absence of GnRH-mediated hypothalamopituitary regulation remain to be determined. Since aromatase activity has not been convencingly demonstrated in pituitary tissue, NORA seems to be responsible for the synthesis or secretion of pituitary FSH.

The testicular histology after GnRH antagonist treatment has been known to be resembled that of surgically hypophysectomized rats (Rea et al., 1986a). Present results also confirmend that seminiferous tubule diameter and luminal volume are visibly reduced and spermatogenesis did not proceed beyond the level of step 7 spermatid by T supplementation. The numbers of pachytene spermatocytes were reduced by GnRH treatment but NORA and T recovered to the normal levels. It is generally known that the process of spermatogenesis can proceed to step 7 spermatids without hormonal support. However the efficiency of spermatid production is reduced in the absence of gonadotropins. Thus we can assumed that NORA and NT does not seem to act directly to the spermatogenesis but act through the elevation of gonadotropin especially FSH secretion. The present result also raises one possibility that NORA may capable of supporting of FSH synthesis or secretion in rat pituitary cells as does T (Rea et al., 1986a).

The current investigations showed that serum T concentration were increased 3.5 times in mice 24 hour after the last injection of TE (Bansal & Davis, 1986). The present result shows the similar increase of T concentration at 2 to 4 times. The serum concentration of LH became undetectable and FSH was partly suppressed when thrice 300

μg dose i.t injection was adminstered to rats. This degree of gonadotropins suppression seems to be due to the elevated serum concentration T.

The present study adopted the intratesticular injection method to exposure androgens directly to germ cells. This techniques offers several potential benefits to various agents to study the direct effects of unknown toxic agents, because i.t. injected agents are not systemically metabolized. Alternatively i.i injection shows the very specific and well documented effects of agents to observe the consequences of disruption of specific pathways/organells, because systemic injections of very toxic substances or cellular inhibitors such as colchicine often cause mortality before testicular effects are noted (Russell et al., 1981). On the other hands, radiolabelled compounds or bioactive substances, whose cost are very high and their availavility are limited (Weinbauer et al., 1985; Russell et al., 1987; Yoon and Yang, 1988). Therefore, we suggest that the direct effects of androgens such as NORA or synthetic androgens on spermatogenesis should be treated by this method.

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= 국문초록 =

19-nortestosterone(NORA)와 19-norandrostenedione(NT)는 정소내 aromatization과정중 중간대사물로 검출된다. 본 연구는 이들을 장기간 투여하여 정소 및 부속기관의 무게, 혈청내 Testosterone(T)의 농도, 정자형성에 미치는 영향을 조사하였다. NORA, NT 및 TE를 300/50㎕농도로 주당 3회씩 12주간 정소에 직접 주사(intratesticular injection, i.t.)하였다. 또한 GnRH antagonist (RS68439)를 처리하여 혈청내 생식소자극호르몬 (GTH)을 감소시킨후 위 호르몬들을 동일방법으로 처리하여 이들의 보상작용을 조사하였다. NORA는 정소무게를 감소시키지 않았으나 GnRH antagonist를 처리하여 감소된 정소무게를 현저하게 보상하였다($P<0.05$). NORA, NT, TE는 모두 부속기관의 무게를 증가시켰으며, RS68439가 감소시킨 부속기관의 무게를 현저히 증가시켰다. 그러나 이들은 부정소(epididymis)에는 영향을 주지않았으며 RS68439처리군에서는 보상작용을 나타내었다. 이들의 처리로 혈청내 LH농도는 완전히 감소하였으나 FSH의 농도에는 영향을 나타내지 않았다. 그리고 혈청내 T의 농도를 증가시켰다. NORA는 정자형성과정중 7단계의 spermatid의 수를 현저히 감소시켰다. 위 결과로 보아 NORA는 GnRH antagonist로 FSH의 분비가 억제된 쥐의 FSH분비를 촉진하며, T의 농도를 증가시켜 정자형성과정을 억제하는 것으로 추론된다.

Key Words: 19-norandrostenedione, 19norsterosids, Spermatogenesis, LIA, Rat, Testis.