Non-disturbing of Decidual Response by Steroid Hormonal Complexes of Pig Testis

JaHyun Yoo[1], JeeHyun Byun[1], So Ra Jeon[1], Dong-Mok Lee[2], Taehoon Chun[3], Ki Ho Lee[4], Inho Choi[2] and Yong-Pil Cheon[1,†]

[1]School of Biosciences and Chemistry, Center for NanoBio Applied Technology, Institute of Basic Sciences, Sungshin Women's University, Seoul 136-742, Korea

[2]School of Biotechnology, Yeungnam University, Gyeongsan 712-749, Korea

[3]School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea

[4]Dept. of Biochemistry and Molecular Biology, Eulji University School of Medicine, Daejeon 301-832, Korea

ABSTRACT : Sex steroid hormones are key molecules to prepare the decidual response and their levels are important in this process. Imbalances of the levels of steroid hormones are cause of implantation failure and other diseases including physical weakness. Androgen replacement therapy or selective androgen receptor modulator are used to overcome various diseases but long-term use may cause of side effects. In previous report, it is suggested that the steroid hormonal complexes derived from pig enhance the proliferation of satellite cell. Therefore, to evaluate the possible usage of steroid hormonal complex derived from pig testis (tS-C), the effects of tS-C on uterine response were studied using the model of artificial decidua. tS-C did not disturb the rhythmical estrus cycle. Artificial-induced decidual response was normally induced in tS-C administered mice. The histological characters of the decidua of tS-C administered mice were not different from the vehicle. The expression patterns of molecular markers of decidua were not different between vehicle and tS-C group. Collectively these results suggested that tS-C does not disturb the uterine responsibility to the embryo. In addition, our results suggested that tS-C can be applied to overcome the various problems such as loss of muscle mass and anemia.

Key words : Testis derived steroid hormonal complex, Artificial decidualization, Uterine responsibility.

INTRODUCTION

We have previously demonstrated the possible roles of steroid hormonal complex which derived from pig testis (tS-C); tS-C stimulates the expression of androgen receptor, myoD, desmin and myogenin in myogenic satellite cells (Lee et al., 2010). Based on that result, it was suggested that tS-C can be applied to human disease such as muscle loss. However, it could have adverse effects because the main components of tS-C are androgen and estrogen including nandrolone.

Although nandrolone is considered as a useful medicine for gain in strength and muscle mass, and anemia, it has adverse effects in various organs including liver, cardiovascular system, male and female reproductive system, and central nervous system (Ishak & Zimmerman, 1988; Lukas 1993; Rockhold, 1993). Therefore, its prescription is very restricted especially in women (Mustafa & Hashimi, 2006).

Estrogen is indispensible in the establishment of pregnancy. They are involved in the regulation of uterine sensitivity to blastocyst, control of the progressive phases of embryo-uterine interaction, and induction of endometrial differentiation (Psychoyos, 1986; Carson et al., 2000; Cheon et al., 2002). Embryo implantation is initiated when the embryo adhere to the luminal epithelium. Attachment of blastocyst is critical to decidualization induction and is dependent on proper interaction between embryo and maternal

† Corresponding author: Yong Pil Cheon, School of Biosciences and Biochemistry, College of Natural Sciences, Sungshin Women's University, 249-1, Dongseon-dong, 3-ga, Seongbuk-gu, Seoul 136-742, Korea. Tel: +82-2-920-7639, Fax: +82-2-920-2093, E-mail: ypcheon@sungshin.ac.kr

endometrium. Decidualization is rapid remodeling of the uterine stroma cells. It is the process by which the uterine stroma cells proliferate and differentiate into morphologically and functionally distinct decidual cells. Differentiation of cell into decidua depends on ovarian steroid hormones especially estrogen and progesterone. The development of mutant mouse models lacking estrogen or progesterone receptors support the role of steroids in those events (Ozturk & Demir, 2010). Collectively, this process is under the control of a sequential interaction between steroid hormones (Cheon et al., 2002; Bazer et al., 2010; Ozturk & Demir, 2010).

The tS-C includes various steroid metabolites such as nandrolone, 4-androsten-17β-ol-3-one and 17-β-estradiol. It has been considered as a good candidate for getting muscle mass (Lee et al., 2010). However, anabolic steroids could interfere with negative feedback control for endogenous steroid hormone synthesis. On the other hand, it is also known that the functional properties of a sex steroid hormone are dependent on the physiological properties which are constructed by other steroid hormones (Cheon et al., 2009). Therefore, it is useful to evaluate the potential toxicity of tS-C using mouse model. To know where there is toxicity in female reproduction, uterine responsibility to the embryo was examined using an artificial induced decidualization model.

MATERIALS & METHODS

1. Experimental Animals and Steroid Hormonal Complex

All experimental animals studied followed to the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health. Animals were maintained under standard conditions at the animal facility in Sungshin Women's University with diurnal rhythm kept under the 14L:10D schedule with light-on at 06:00 and clean room system. Animals were fed a standard rodent diet and water *ad libitum* from weaning at 21 days of age. The testis-derived steroid-complexe (tS-C) was obtained kindly from Dr. Choi in Yeungnam University (Korea).

2. Vaginal Smear

In order to examine the effects of tS-C on female reproductive cycle, estrus cycle was analyzed by a vaginal smear method. To choose female mice with regular estrus cycles, vaginal smear was performed 8 days before tS-C administration. CD1 female mice (6 wks) were subjected to 0.1 ㎖ injection of tS-C for 21 days (s.c.). Vaginal smear was daily performed until the day that mice were sacrificed. Analysis was undertaken to define by the presence of epithelial cells and leukocytes.

3. Ovariectomy and tS-C Administration

Female mice were subjected to bilateral ovariectomy for the remove the ovarian steroid hormones estrogen and progesterone. Two weeks following ovariectomy, mice were injected subcutaneously with 0.1% tS-C or vehicle (cotton seed oil) for two weeks. Components of tS-C are presented the Table 1.

4. Artificial Decidualization and Tissue Collection

Artificial decidualization was induced as described previously (Cheon et al., 2004). Briefly, ovariectomized female mice were continuously injected (s.c) with 0.1 ㎖ tS-C until those were sacrificed. After 7 days of ovariectomy, 100 ng 17β-estradiol was administered for 3 days. Following two days the mice kept free from hormonal administration. After then 6.7 ng 17β-estradiol and 1 ㎎ progesterone were administered every day until mice

Table 1. Amount of the steroid hormone extracts from porcine testis

Components	
Nandrolone	2.604 (㎍/g)
Testosterone	10.416 (㎍/g)
Androstenedione	325.08 (ng/g)
Estradiol	547.18 (ng/g)
Estrone	320.38 (ng/g)

were sacrificed. At third day, artificial decidualization was induced with mechanical trauma: insertion of a blunt needle into the uterine horn just proximal to the cervix and longitudinally scratching the entire length of the uterine horn along the antimesometrial side. The other horn was left unstimulated. Induced female mice were sacrificed 96 hr later. For histological analysis, half of the simulated and unstimulated horns were fixed with 4% paraformaldehyde and further processed for histological analysis. To isolate total RNA, the remaining uterine parts were quickly frozen with liquid nitrogen and kept at −80℃ freezer until used.

5. Histological Analysis

Fixation of uterine sample was done for overnight with 4% paraformaldehyde and the samples were transferred to 70% ethanol. To make paraffin block, water was removed and replaced with paraffin. Dehydrated tissues were put into paraffin for overnight. Using microtome the blocks were sectioned (4 ㎛ thickness) and put on slides.

6. Immunohistochemistry

Paraffin-embedded sections were deparaffinized in xylene, rehydrated through a series of ethanol washes, and rinsed in water. The antigen retrieval was performed by immersing the slides in 0.1 M citrate buffer solution, pH 6.0, and subjected to microwave heating for 20 min. The sections were allowed to cool, washed in PBS for 5 min. Endogenous peroxidase activity was blocked by incubating sections in 0.3% hydrogen peroxide for 10 min at room temperature. The slides were washed in PBS-Tween 20 (PBST), and then incubated in a blocking solution containing 10% normal goat serum for 1hr before incubation in primary antibody overnight at 4℃. Immunostaining was performed using VECTASTAIN ABC Reagent for rabbit primary antibody. Sections were stained with hematoxylin, eosin, mounted, and examined under bright field. Brown deposits indicate the sites of immunostaining.

7. Total RNA Extraction and cDNA Synthesis

Total RNAs of uteri were extracted using TRIzol Reagent according to the manual of manufacture (Invitrogen). First strand cDNA was synthesized using First-strand synthesis system (Invitrogen). We used the following mixture for first-strand cDNA synthesis; reaction regent 1.0 ㎕ oligo (dT) primer (0.5 ㎍/㎕), 0.5 ㎕ random primers (0.1 ㎍/㎕), 1.0 ㎕ dNTP mix, 7 ㎕ total RNA (5 ㎍/㎕), 2.0 ㎕ standard buffer (10×), 4.0 ㎕ $MgCl_2$ (25 mM), 2.0 ㎕ DTT (0.1 M), 1.0 ㎕ RNase Inhibitor, 1.0 ㎕ SuperScript™ II ^{RT} and 0.5 ㎕ DEPC treated water. The mixtures were incubated at 65℃ for 5 minutes and then at room temperature for 10 minutes for the primers to anneal to the RNA. The tubes were incubated at 42℃ for 1 hr and at 70℃ for 15 minutes to terminate cDNA synthesis.

8. Screening the mRNA Expression of Decidual Markers

Transcripts of target genes were detected by RT-PCR using appropriate primers (Table 2). For quantitative PCR (qPCR), all PCR reactions were performed in a 20 ㎕ reaction volume using the SYBR Green RCR Master Mix and each specific primer. Dissociation curves were run on all reactions to ensure amplification of a single product with the appropriate melting temperature.

9. Statistical Analysis

The *t*-test was performed to evaluate statistical significance between controls and experiment groups. Results were presented as MEAN±SED. A p-value less than 0.05 was considered to be significantly different.

Table 2. Sequences of specific molecular marker for decidua

Alkaline phosphatase	S	TTA AGG GCC AGC TAC ASS ACA A
	AS	TTC ACC CGA GTG GTA GTC ACA A
CTLA-2β	S	ACA GAA GAC TCA TGT GGG AGG AGA
	AS	TTC TCA GCT TTC TGT GGG CA
Connexin 43	S	TCC TTT GAC TTC AGC CTC CAA G
	AS	TGG GGA AGG ACT TGT CAT AGC A
Doc-1	S	TGG CTG AAA CGG AAC GGA AT
	AS	GGG GAC AAA CAA TAG CTT GCT G

RESULTS

1. tS-C Did Not Disturb the Estrus Cycle

We have examined whether tS-C could affect patterns of estrus cycle in female mice. Most mice in control group showed regularly cycling estrus cycle. Comparably, most mice treated with tS-C also had regular cycles with a few unusual patterns such as no epithelial cell dominant phase (Fig. 1).

2. Uterine Responsibility was Saved in tS-C Administration

Uterine preparation for embryo implantation is controlled totally by the sex steroid hormones. tS-C is a candidate in modulator of uterine physiology because it is a mixture of steroid hormones. The tS-C pretreated uteri could respond to the artificial decidualization signals. The patterns were similar with the control. The phenotypes of uteri were same between the control and the tS-C (Fig. 2A).

The histological characters in uterine section of tS-C treated mice were same with that of the control; The large cells were observed in deciduoma. To conform the decidualizing of stroma cell, immunohistochemistry was performed with anti-mouse prolactin antibody. Prolactin specific signals were localized the decidua cells (Fig. 2B-b).

3. Decidua Specific Gene Expression Profile of tS-C was Similar with that of Vehicle

Further evaluation of the uterine responsibility was performed with the molecular marker with real-time PCR

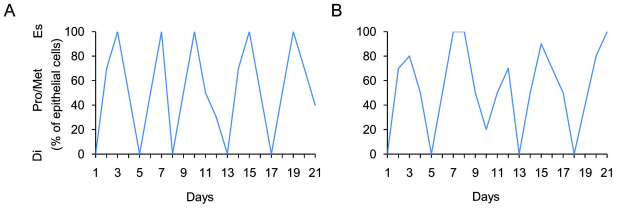

Fig. 1. Patterns of estrus cycle in tS-C treated mice. A, the pattern of estrus cycle in vehicle; B, the pattern of estrus cycle in tS-C. Vehicle and tS-C were administered during 21 days. Estrus cycle was analyzed daily by vaginal smear.

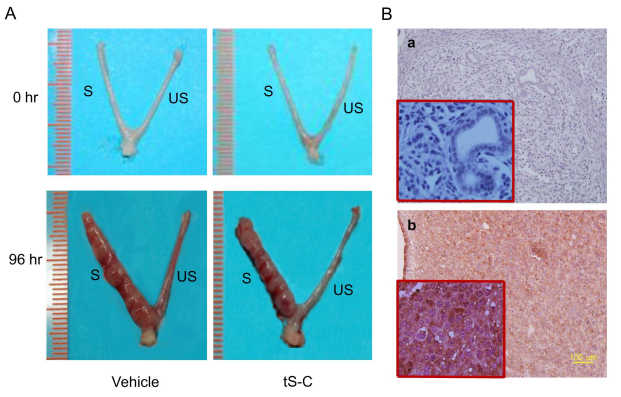

Fig. 2. tS-C kept the uterine responsibility to the decidual stimulation. A, ovariectomized mice were administered with tS-C for 1 wk and induced artificial decidualization according to the standard method. B, Photomicrograph of unstimulated (a) and stimulated (b) uterine section after 96 hr of decidual induction in tS-C administered mice (magnification, 100×). Insert shows the magnified photomicrograph of immunohistochemistry (400×). Prolactin was detected with anti-mouse prolactin Ig. Brown color showed the prolactin specific signal.

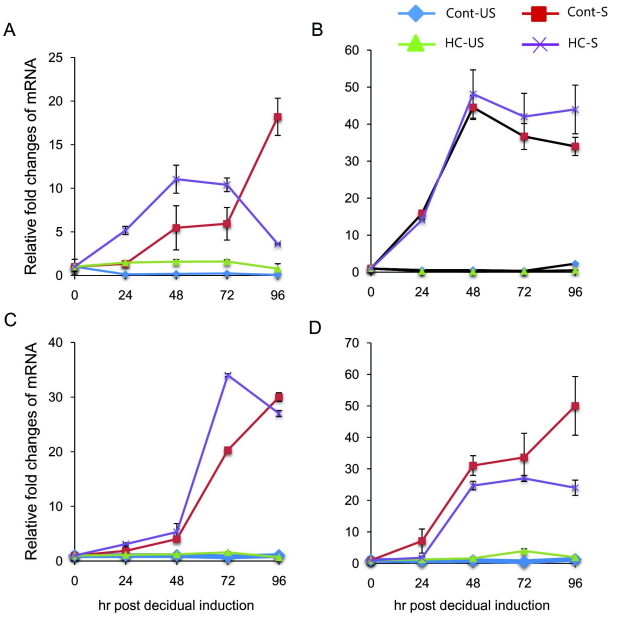

Fig. 3. Expression levels of some genes which are expressed during decidualization. The expression profiles of these genes were analyzed with real-time PCR method in decidualized uteri of vehicle and tS-C administered mice. The uteri were samples at 24 hr, 48 hr, 72 hr, and 96 hr post tS-C administration. A, alkaline phosphatase; B, Connexin 43; C, doc-1; D, CTLA-2beta.

methodology. Alkaline phosphatase, connexin 43, doc1, and CTLA2-beta specific mRNAs were detected both in vehicle and tS-C and the levels were also similar until 72 hr. After 72 hr of decidual induction the levels of alkaline phosphatase, doc-1, and CTLA-2beta specific mRNAs were decreased in tS-H but not in vehicle (Fig. 3A, C, D). On the other hand, the expression level of connexin 43 mRNA was increased after 72 hr of decidual induction (Fig. 3B).

DISCUSSION

The blood level of sex steroid hormone is controlled by negative feedback in hypothalamus-pituitary-ovary axis. Therefore, exogenous steroid hormone works as a mediator of negative feedback and disturb the physiological homeostasis. Nandrolone is a helper in getting muscle mass and healing anemia but its usage is very restricted because it has severe side effects in CNS and reproductive system. It was demonstrated that nandrolone could advance and retard pubertal onset in females, enhance and diminish libido in both sexes, and suppress reproductive competence in both sexes (Clark & Henderson, 2003; Clark et al., 2006). Estrus cycle is controlled by the gonadotrophins and the steroid hormones. Exogenous androgen and estrogen cause to disturb estrus cycle. However, interestingly the tS-C did not cause of disturbance of the rhythmical estrus cycle. It showed a possibility that hormonal complex tS-H did not much give effect on negative feedback of estrogen or testosterone in female mouse. It is suggested that tS-C can be applied to the artificial treatment for some diseases. However, it is need more information about safety in female.

Stimulating of blastocyst induce the stroma cell differentiation. This is happened only in the prepared endometrium for implantation. Stroma cells surrounded the blastocyst immediately differentiated into polyploid decidual cells, forming an avascular primary decidual zone. The surrounding stroma cells of primary decidua differentiate into well-vascularized secondary decidual zone. Typically stroma cell is enlarged into a larger, spherical cell. It is associated with the accumulation of glycogen and increased expression of prolactin and others (Brosens et al., 1999; Gellersen et al., 2007; Mori et al., 2011).

To evaluate the possible effects of tS-C in uterine responsibility to the decidual stimulation, in here the artificial decidualization method was employed because the pivotal roles of sex steroid hormones are well known in this progress (Ledford et al., 1976). Artificial deciduas were induced both in vehicle and tS-C and the phenotypes were similar between them. One of the histological characters of decidualizing cell is large and spherical cell. The histological characters were not different between the vehicle and the tS-C. In addition, the expression of prolactin was detected in the decidua of tS-C and vehicle. Those means that tS-C did not disturb the uterine responsibility to the decidualization. Therefore it is suggested that tS-C can be applied in pregnant female if can be used as a medicine.

In the artificial decidual model, the decidua developed after stimulation and the sizes of deciduas increased dramatically until 72 hr after stimulation and then slowly decrease. Therefore it is need to know the specific genes expression during these periods. Alkaline phosphatase is a molecular marker of decidua (Witte et al., 1991). Its expression patterns were similar until 72 hr after stimulation but after then its level was decreased in tS-C. Conexin 43 is also highly expressed in deciduas (Grümmer et al., 1994, 1996). Its specific mRNA expression was increase until 48 hr after stimulation both in the vehicle and tS-C. The expression patterns were similar between them. The expression of Doc-1 is detected in the decidua and is suggested as a marker of decidualization. Its specific mRNA expressions were similar between the vehicle and the tS-C until 72 hr after stimulation. After then its level decreased in tS-C. CTLA-2beta is expressed specifically in the decidua surrounding embryo (Cheon et al., 2004). It also can be used as a decidua marker. Its specific mRNA ex-

pressions were increased until 72 hr both in vehicle and tS-C after stimulation, but its level was decreased in tS-C. On summary the expression patterns of decidua markers was similar until 72 hr post induction but after then those were showed different patterns by markers and by treatment. It could be explained by the differentiation of deciduoma, interestingly the chronic changes of structure and functions were observed during the process (Ledford et al., 1976). Those results clearly showed that the tS-C did not disturb the uterine sensitivity to the decidual stimulation and the progress of decidual differentiation of stroma cell.

On summary, the tS-C did not disturb the rhythmic estrus cycle. tS-C pretreated uterus could be response normally to the decidual responses. It also revealed that tS-C did not inhibit the progress of stroma differentiation to the deciduas. The molecular markers of decidua were expressed with similar patterned with that of the vehicle. Therefore, it is suggested that tS-C is apply to the female without disturbing the pregnancy.

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