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Sperm Biology

Suppression of spermatogenesis by testosterone undecanoate-loaded injectable *in situ*-forming implants in adult male rats

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We have investigated the feasibility of administration of testosterone undecanoate (TU)-loaded injectable *in situ*-forming implant (ISFI) for contraception in adult male Sprague–Dawley rats. Male rats were treated with vehicle, TU-loaded ISFIs (540, 270 and 135 mg TU kg^{-1}) or TU injections (45 mg TU kg^{-1} every 30 days) for 120 days. Fertility tests served for determining infertility or restoration of fertility in treated rats. Serum testosterone concentration, epididymal sperm count, motility, morphology, and histology of the testis were monitored. The TU-loaded ISFIs increased serum testosterone levels in rats steadily without fluctuation over 3 months. One month after TU administration, the epididymal sperm count decreased significantly in all experimental groups. After 3 months, the animals treated with 270 and 135 mg kg^{-1} TU-loaded ISFIs were 100% infertile, and no implantation sites were produced in the mated females. However, some of males treated with 540 mg kg^{-1} ISFI or TU injections were still fertile but numbers of implantation sites were also significantly lower than control values. TU-loaded ISFI at an appropriate dose has potential as a long-acting male contraceptive drug that suppresses spermatogenesis consistently over a period of 3 months.

INTRODUCTION

Contraceptive development for men has lagged far behind that for women. Thus far, clinical practices are not provided with a safe, effective, reliable, and reversible male contraceptives.[1,2] Therefore, the study of contraceptives for men is becoming increasingly attractive in the field of reproductive medicine.

At present, all potential male hormonal contraceptives require an androgen for suppression of gonadotropins and spermatogenesis while maintaining androgenicity of healthy adult men. Traditionally, injectable testosterone (T) esters have been used for treatment, but they generate supra-normal T levels shortly after the 2–3 weekly injection interval. T levels then decline very rapidly, becoming subnormal during the days preceding the next injection. The rapid fluctuations in plasma T are subjectively experienced as disagreeable. These disadvantages hinder the acceptability of these regimens and highlight the need for long-acting preparations of T with more stable delivery kinetics. Testosterone undecanoate (TU; 17-hydroxy-4-andros-ten-3-one 17-undecanoate) is an unsaturated, aliphatic, fatty acid ester of testosterone that is used for the treatment of male hypogonadism and has been studied for use as a male contraceptive.[3,4] TU injection (a new injectable formulation of TU in tea seed oil) was first used in China for the treatment of hypogonadism and later also for contraception. A dose of 500–1000 mg TU injection at monthly intervals administered to normal Chinese men can sufficiently and reversibly suppress spermatogenesis without serious side-effects.[5]

However, pharmacokinetic studies of TU injection have found fluctuations in serum androgen concentrations are significant, which can lead to severe adverse reactions, such as mood swings or emotional instability.[6,7] It has been reported that TU administration is associated with supra-physiological peak values shortly after the injection and to sub-physiological levels in the days before the new injection.[6]

The *in situ*-forming implant (ISFI) can be defined as a liquid formulation generating a solid or semi-solid depot after subcutaneous injection. ISFI comprises a water-insoluble biodegradable polymer dissolved in a water-miscible, physiologically compatible solvent. Upon injection into an aqueous environment, the solvent diffuses into the surrounding aqueous environment while water diffuses into the polymer. Since the polymer is water-insoluble, it precipitates upon contact with the water and results in a solid polymeric implant. ISFIs have received increasing attention owing to distinct advantages. In particular, injections are less invasive than implants, and improved patient compliance can be expected, and controlled drug release for local or systemic applications can be attained. In our previous study, we prepared TU-loaded injectable *in situ*-forming implants (TU-loaded ISFIs) using a biodegradable polymer.[8,9] This injectable *in situ* polymer precipitation system comprises of poly (DL-lactide-ε-caprolactone) P(CL/DL-LA) dissolved in N-methyl-2-pyrrolidone (NMP), which is water-miscible and physiologically compatible. The release behavior and biocompatibility of TU-loaded ISFIs have also been evaluated; the polymer matrix is responsible for controlled release of drugs, and TU

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is released at a constant rate, following Higuchi kinetics both *in vitro* and *in vivo*.[8] The TU-loaded ISFI increases serum testosterone levels in rats over a period of 3 months without serious adverse effects, and the controlled release of TU from ISFIs can maintain a relatively stable serum concentration without large fluctuations.[8,9]

From the satisfactory sustained release behavior of TU-loaded ISFIs, it is of interest to study the effects on male fertility. The objective of this study was to investigate the antifertility potential of TU-loaded ISFIs for contraception in adult male rats, including a fertility test, serum testosterone concentration, epididymal sperm count, motility, morphology, and histology of the testis.

MATERIALS AND METHODS

Drugs and preparations

Testosterone undecanoate (TU) was obtained from Zhejiang Xianju Pharmaceutical Co., Ltd., (Taizhou, China). TU-loaded ISFIs were prepared as previously described.[8] Appropriate amounts of *P* (CL/DL-LA) were dissolved in NMP. The mixture was stirred periodically and kept at 37°C overnight until a clear solution was achieved. This solution was then capped and heated to 65°C to remove trapped air bubbles. TU was dispersed in the *P* (CL/DL-LA)/NMP solution. The mixture was agitated until complete dissolution of TU. TU injection was provided by Zhejiang Xianju Pharmaceutical Corp. This TU preparation was available in ampoules containing 250 mg of the ester in 2 ml tea seed oil.

Animals and treatment

Sprague–Dawley rats, males, and females weighed 200–220 g and 180–200 g, respectively, were obtained from Liaoning Changsheng Bio-Tech Co., Ltd., (Fushun, China). The animals were maintained at 20°C with free access to food and water. The rats were acclimatized for at least 1-week before the studies.

Adult male rats, aged approximately 90 days, were randomly divided into five groups. Animals in groups A, B, and C ($n = 30$) were given a single subcutaneous injection of TU-loaded ISFI into the midline dorsal area from 21 gauge needles (the doses were 540, 270, and 135 mg TU kg^{-1}, respectively). Males in group D ($n = 30$) received a single intramuscular injection of TU injection from a 200 µl micro-syringe (the dose of 45 mg kg^{-1} was given monthly, and the administration was continued for 3 months, providing a total dose in Group D of 135 mg kg^{-1} that was the same as that in group C). Group E was the control group ($n = 30$), receiving a single subcutaneous injection of blank ISFI. Because the TU-loaded ISFI increased serum testosterone levels in a period of 3 months, the duration of treatments in all groups was 3 months. Then the animals were permitted to recover. One month, 3 months, and 4 months (1-month following withdrawal) after first treatment, the animals were randomly chosen, 10 per group, for fertility testing. All measures taken for the rats were in accordance with the guidelines for the care and use of laboratory animals approved by the Chinese Council on Animal Care.

Fertility studies

Fertility tests were performed on days 30, 90 of treatment and 30 days after withdrawal of treatment. In short, two normally-cycling pro-estrous females were caged with one male, 10 males per group. Vaginal smears were evaluated in females during the mating period for the stage of estrous and the presence of spermatozoa. At the time of insemination, females were separated from the male, body weights were determined during gestation, and females were killed on gestation days 13 to 15. Implantation sites and corpora lutea were counted as measures of male

fertility. If the two females did not display sperm-positive vaginal smears 3 days after co-caging, another female was introduced to the male. If the female was still not observed with spermatozoa in its vaginal smear 3 days after co-caging, the three females were surgically examined together.

Body weight and weight of organs

The males were killed 6 days after mating in fertility studies. The body weights of rats were recorded, and the weight of the testes, epididymides, prostates, and seminal vesicles was determined at autopsy. The relative weight of reproductive organs was calculated by the formula: organ weight/body weight × 100.

Epididymal sperm count, motility, and morphology

About 100 mg cauda epididymidis was cut from left and right epididymis. Then the cauda epididymidis was weighed and minced in 15 ml Ringer's solution. Spermatozoa were allowed to swim out for 15 min at 37°C. An aliquot, after appropriate dilution, was counted in a hemocytometer (Qiujing Biochemical Instrument Factory, Shanghai, China, 0.1 mm deep, smallest grid 1/400 mm^2) at × 200 magnification. Three hundred sperm heads were counted, and results were expressed as number of spermatozoa per g of cauda epididymidis. The spermatozoa were classified as motile or immotile. Epididymal spermatozoa (300 per male) were randomly examined in a light microscope, and the percentage of motile cells was determined. Sperm morphology was also analyzed microscopically by viewing Hematoxylin-Eosin stained slides.

Histology

The left testis was fixed by immersion in Bouin's solution. After 1–2 h, the tunica albuginea was cut adjacent to the poles to give better fixation. After fixation for other 24 h and conventional histological processing, 5-µm-thick paraffin-embedded sections were stained with Hematoxylin and Eosin for light microscopic examination and morphometric studies. Apoptotic cells were recognized by a series of typical morphological features, such as shrinkage of the cell, fragmentation into membrane-bound apoptotic bodies and rapid phagocytosis by neighboring cells.[10,11] The minimum diameter of tubule profiles was measured.

Serum testosterone

The amount of testosterone in serum was determined as described before.[8] At predetermined time points, five rats in each group were randomly selected, and approximately 0.7 ml blood was collected from the lateral tail vein by serial bleeding from each animal. Serum was separated from the blood by centrifugation at 890 × *g* for 10 min and determined by electrochemiluminescence immunoassay (ECLIA). Standard commercial kits (Elecsys° Testosterone II, Roche Diagnostics, Mannheim, Germany) were used and the mean ± s.d. of the testosterone levels (ng ml^{-1}) is reported. The sensitivity of the assay was 0.04 ng ml^{-1} for testosterone. The intra- and inter-assay coefficients of variation were 5.3% and 5.4%, respectively. Samples, standards, and controls were analyzed in duplicate. When testosterone levels in samples exceeded the upper detection limit for the assay, the samples were diluted with blank serum and re-assayed.

Statistical analysis

Statistical analyses were performed using the SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) for Windows software package. For all experiments, the statistical analysis was first performed for all groups using a one-way ANOVA to determine statistical significant variance between the groups. Statistical significance between all groups at each time point was then calculated from the LSD *t*-test. The Kruskal–Wallis

test followed by *post hoc* Mann–Whitney analysis test was used when the data distribution was skewed. Proportions were compared using the Fisher exact test. $P < 0.05$ was considered as statistically significant.

RESULTS

Effects of TU-loaded in situ-forming implants on the male reproductive system of rats

Body and organ weights

Mean body weights of animals were not statistically different between groups in the first 3 months after TU treatment (**Figure 1**), but after 4 months, the group treated with 540 mg kg^{-1} TU-loaded ISFI (group A) showed statistically lower body weights than the control group E ($P < 0.05$).

The effect of different doses of TU-loaded ISFI on the male reproductive organ weights is shown in **Table 1**. One month after administration, testicular weights of groups B, C, and D were significantly lower than the control value (group E), but there was no differences between high-dose TU group A and control group E. None of the other organ weights in the TU-treated groups were changed from the control group in the first month. The weights of
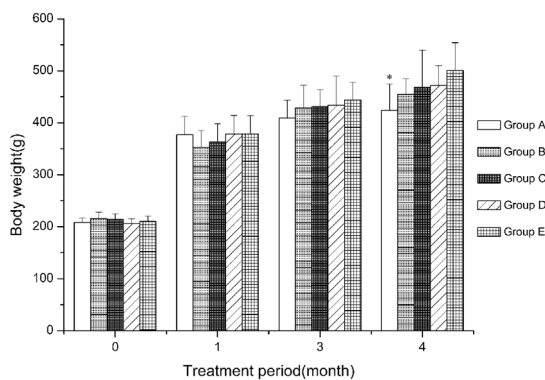

Figure 1: Changes in body weight of treated rats at various times after treatment ($n = 10$). *$P < 0.05$, compared with the placebo group E.

the testes and epididymides were decreased markedly 3 months after the administration of TU in each group. After 1-month following withdrawal, the weights of the testes and epididymides began to recover, particularly in groups B and C. No other changes were found.

Fertility test

The occurrence of mating was assessed by the presence of seminal plugs and sperm-positive vaginal smears in all mated females. One month after treatment, the percentage of sperm-positive females remained within the range of 90 to 100 for all TU-treated groups with the exception of the control group E in which only 65% females were pregnant or sperm-positive (**Table 2**). After 3 months, the animals in groups B and C were 100% infertile, and no implantation sites were produced in the mated females. One in 10 males of group D did not lose fertility. In the high-dose group A, 70% of males still were fertile, and the implantation rate was also lower than the control value ($P < 0.05$). One month after withdrawal of treatment, the percentage of males with recovered fertility was 30 in group B and 40 in group C. Instead of recovering fertility, the animals in groups A and D were unexpectedly 100% infertile.

Epididymal sperm count, motility, and morphology

The epididymal sperm count decreased significantly at 3 months after TU administration in all experimental groups (**Table 3**). After 1-month withdrawal, the sperm count had partly recovered. However, the sperm count of group A was still low. In the first month, the sperm motility slightly rose in animals treated with middle-dose TU-loaded ISFI (group B, $P < 0.05$). After 3 months, the sperm motility of all groups dropped significantly ($P < 0.05$) and was completely lost in groups B and C. The sperm count of TU-treated groups also decreased severely. It is interesting to note that the sperm count and motility of the high-dose group A declined continuously at 4 months.

Sperm morphology was classified as normal or abnormal. The abnormality was classified into a variety of head and tail abnormalities, such as blunt hook, banana-head, amorphous, pin-head, two-head, two-tail, small head, and bent tail. In the early days of treatment, no significant deviation in the percentage of normal spermatozoa was noted among any ISFI group and control. For the TU injection group,

Table 1: Changes in reproductive organ weights (g) of rats at various times after treatment (mean±s.d., *n*=10)

	Groups				
	Control (E)	*High-dose TU-ISFI (A)*	*Middle-dose TU-ISFI (B)*	*Low-dose TU-ISFI (C)*	*TU injection (D)*
1-month after treatment					
Testis	2.96±0.48	2.76±0.48#	2.47±0.28*	2.31±0.33*	2.21±0.63*
Epididymides	1.36±0.22	1.27±0.19	1.38±0.18	1.30±0.25	1.18±0.14
Prostate	0.32±0.05	0.30±0.04	0.29±0.05	0.35±0.15	0.32±0.15
Seminal vesicle	1.96±0.37	2.28±0.66	2.34±0.56	2.36±0.70	1.98±0.47
3 months after treatment					
Testis	3.52±0.14	1.79±0.26*,#	1.82±0.50*,#	1.98±0.69*,#	1.11±0.47*,#
Epididymides	1.59±0.06	1.19±0.12*	1.11±0.15*	1.18±0.10*	1.11±0.10*
Prostate	0.35±0.13	0.32±0.05	0.35±0.07	0.35±0.09	0.36±0.09
Seminal vesicle	2.35±0.31	2.21±0.56	2.52±0.46	2.18±0.37	2.57±0.49
4 months after treatment (1-month following withdrawal)					
Testis	3.73±0.33	1.76±0.48*	2.34±1.02*,#	2.42±0.79*,#	1.54±0.54*
Epididymides	1.89±0.22	1.24±0.04*	1.84±0.62#	1.39±0.31*	1.42±0.28*
Prostate	0.49±0.12	0.40±0.06	0.38±0.16	0.48±0.13	0.43±0.15
Seminal vesicle	2.92±0.51	2.51±0.25	3.23±0.58	3.16±0.72	2.86±0.62

*$P<0.05$, compared with control group E; #$P<0.05$, compared with TU injection group D. s.d.: standard deviation; TU-ISFI: testosterone undecanoate-loaded injectable *in situ*-forming implants; TU: testosterone undecanoate

Table 2: Results of mating trials (mean±s.d.)

	Pregnant/ mated females	Number of corpora lutea in mated females	Number of implantation sites in pregnant females
1-month after treatment			
High-dose TU-ISFI (A)	11/12*	12.1±3.9	14.7±1.4
Middle-dose TU-ISFI (B)	13/14*	13.4±3.0	14.7±1.7
Low-dose TU-ISFI (C)	15/16*	12.4±2.9	14.8±2.5
TU injection (D)	17/18*	13.5±2.5	14.5±3.9
Control (E)	13/20	12.4±2.7	13.4±4.0
3 months after treatment			
High-dose TU-ISFI (A)	7/14#	13.5±2.1	7.6±4.9*
Middle-dose TU-ISFI (B)	0/16*	10.4±3.1	-
Low-dose TU-ISFI (C)	0/16*	11.7±3.6	-
TU injection (D)	2/20*	12.9±3.6	10.0±4.2
Control (E)	12/20	12.7±2.2	13.0±1.8
4 months after treatment, (1-month following withdrawal)			
High-dose TU-ISFI (A)	0/13*	13.0±1.5	-
Middle-dose TU-ISFI (B)	3/14*,#	12.4±2.4	11.3±0.6
Low-dose TU-ISFI (C)	6/18*,#	13.1±3.0	15.5±2.7*
TU injection (D)	0/24*	12.9±2.3	-
Control (E)	18/19	12.8±2.1	12.7±1.9

*$P<0.05$, compared with control group E; #$P<0.05$, compared with TU injection group D. s.d.: standard deviation; TU-ISFI: testosterone undecanoate-loaded injectable *in situ*-forming implants; TU: testosterone undecanoate

Table 3: The effect of TU-ISFI on epididymal sperm count, motility, and morphology (mean±s.d., $n=10$)

	Epididymal sperm count ($\times10^6\,g^{-1}$)	Motile sperm (%)	Normal morphology sperm (%)
1-month after treatment			
High-dose TU-ISFI (A)	27.7±4.5*	88.9±13.7	82.6±8.9
Middle-dose TU-ISFI (B)	52.9±16.7*	92.4±8.0*,#	87.4±9.1
Low-dose TU-ISFI (C)	61.9±23.2*	85.6±13.5	89.5±7.4
TU injection (D)	41.3±15.6*	74.7±19.2	57.4±9.9*
Control (E)	105.4±13.5	73.6±8.1	83.3±5.6
3 months after treatment			
High-dose TU-ISFI (A)	19.0±14.3*,#	8.0±12.2*	59.8±8.3*,#
Middle-dose TU-ISFI (B)	5.9±4.1*	0.0±0.0*	42.1±7.5*
Low-dose TU-ISFI (C)	5.2±7.0*	0.0±0.0*	19.8±3.4*
TU injection (D)	2.1±2.5*	16.5±7.38*	27.5±11.1*
Control (E)	105.9±33.4	75.9±11.1	88.9±6.6
4 months after treatment (1-month following withdrawal)			
High-dose TU-ISFI (A)	9.8±7.4*	0.0±0.0*	54.2±10.3*,#
Middle-dose TU-ISFI (B)	18.4±12.8*	16.3±8.5*	76.2±7.1#
Low-dose TU-ISFI (C)	30.1±8.5*	20.0±18.8*	72.4±7.5#
TU injection (D)	7.2±9.8*	2.7±7.58*	17.4±9.9*
Control (E)	107.4±10.1	80.7±13.9	81.5±9.4

*$P<0.05$, compared with control group E; #$P<0.05$, compared with TU injection group D. s.d.: standard deviation; TU-ISFI: testosterone undecanoate-loaded injectable *in situ*-forming implants; TU: testosterone undecanoate

the percentage of normal spermatozoa was lower than that in control animals ($P < 0.05$). In response to 3 months of TU treatment, there was a significant decrease in the percentage normal morphology sperm in all groups compared with that of controls. The abnormal spermatozoa were mainly banana-head, bent tail and decapitated. A large number of apoptotic and exfoliated spermatocytes and spermatids were also found in the epididymal semen. After 1-month withdrawal, there were

no differences between groups B, C, and control group E in sperm morphology.

Histological

Figure 2 shows the morphological appearance of seminiferous tubules from high-dose TU-ISFI-treated (a), middle-dose TU-ISFI-treated (b), low-dose TU-ISFI-treated (c), and TU injection treated (d) rats. Testicular weights were almost unaffected 1-month after TU treatment but were significantly decreased after 3 months, by approximately 49.1% (group A), 48.3% (group B), 43.4% (group C), and 68.5% (group D). Similarly, the tubule diameter was unchanged after 1-month but was reduced significantly after 3 months, by approximately 30.4% (group A), 14.8% (group B), 13.3% (group C), and 41.5% (group D).

One month after treatment, TU had no significant effect on the changes of morphological appearance of seminiferous tubules and spermatogenesis from the controls. Somewhat differently, degeneration or apoptosis of spermatocytes and spermatids was observed in a few tubules of group D. Three months after TU treatment, more obvious changes were observed as follows (**Figure 3**): (i) a looser arrangement of spermatogenic cells, abnormally large empty spaces formed between lines, bundles or groups of spermatids and spermatocytes (often radial spaces running toward the tubule lumen) in tubule profiles; (ii) the seminiferous tubules began to atrophy, the edge of tubules became irregular and inter-tubular spaces also increased which contained atrophic Leydig cells; (iii) only few elongated and elongating spermatids existed in the seminiferous epithelium, and degenerating round spermatids and spermatocytes were observed in the lumen; (iv) severe spermatogenic damage was observed in some tubules, in which the lumen became empty with only Sertoli cells, few spermatogenic cells and degenerating spermatocytes in the seminiferous epithelium; (v) in some cases, formation of multinuclear giant cells were noticed.

One month after withdrawal of treatment (the fourth month after treatment), the majority of seminiferous tubules had partly recovered spermatogenesis in groups B and C, but in groups A and D, some seminiferous tubules were still atrophic.

Serum testosterone

The serum concentration profiles of testosterone are illustrated in **Figure 4**. There was a clear dose-response relationship between the injected TU doses and serum T levels. Subcutaneous injections of 540, 270, and 135 mg kg^{-1} TU-ISFI in uncastrated male rats resulted in increased mean serum testosterone (T) levels from <5 ng ml^{-1} to more than 38.8, 18.5, and 12.7 ng ml^{-1} in 28 days, respectively. Thereafter, serum T levels decreased progressively and reached the normal level for adult rats by day 90 in group B and C. For the TU injection group D, the serum testosterone increased much more quickly than TU-loaded ISFI groups. The maximum (Cmax) serum T concentrations of group D after the first, second, and third injections were 21.8, 29.9, and 34.3 ng ml^{-1}, respectively. Rapid fluctuations in plasma T still existed.

DISCUSSION

A number of clinical approaches to male hormonal contraception with T esters given alone or in combination with additional gonadotrophin-suppressive agents have been investigated.[12,13] However, to attain physiological mean T levels, it is necessary to accept fluctuations in circulating T levels between injections, sometimes causing unwanted side-effects. These disadvantages have hindered the acceptability of these regimens and highlighted the need for long-acting preparations of T with more stable delivery kinetics. TU injection is a long-acting injectable preparation based on tea seed oil as vehicle and recently
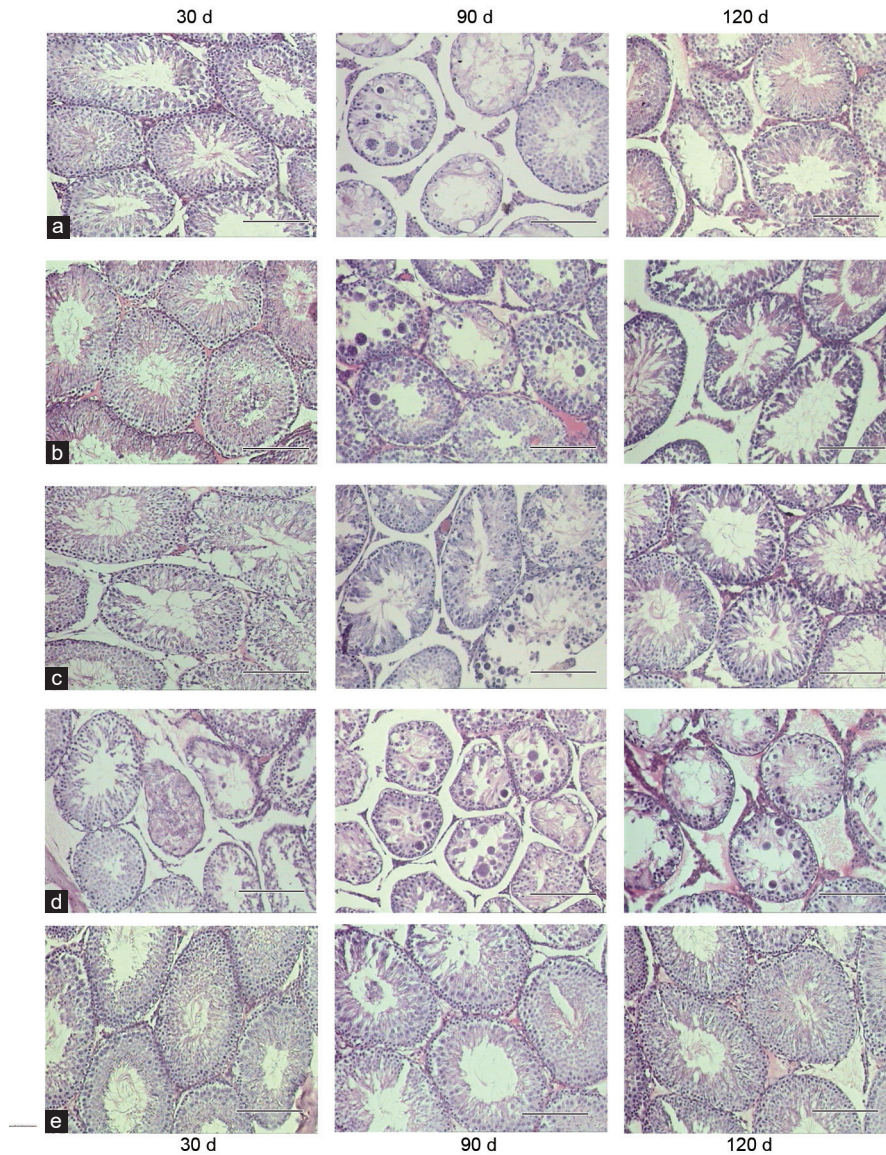
Figure 2: Testicular sections from high-dose TU-loaded ISFIs treated (**a**), middle-dose TU-loaded ISFIs treated (**b**), low-dose TU-loaded ISFIs treated (**c**), TU injections treated (**d**) and control (**e**) rats after 30, 90, and 120 days. Scale bar = 0.1 mm.
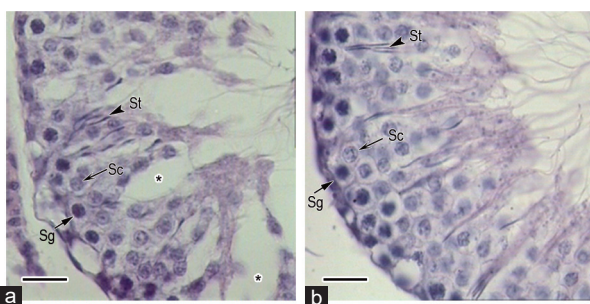

Figure 3: Higher magnified views of portions of tubules from rats that treated with low-dose TU-loaded ISFI (group C) for 90 days (**a**) and control animals showing normal spermatogenesis (**b**). St: spermatids; Sc: spermatocytes; Sg: spermatogenous cells; *: empty space (crack) between spermatogenic cells. Scale bar = 0.02 mm.

synthesized and commercially available in China. It has been confirmed that a dose of 500–1000 mg TU, injection at monthly intervals

administered to normal Chinese men can sufficiently and reversibly suppress spermatogenesis without serious side-effects[3,5] although fluctuations in serum androgen concentrations still exist. In this study, the control-release of TU in tea seed oil solution was not efficient enough to provide stable T concentrations, and animals in the TU injection group suffered from marked fluctuations in the concentrations of serum T. The TU-loaded ISFI is a system based on the solvent removal precipitation of the water-insoluble *P* (CL/DL-LA), and the polymer matrix is responsible for controlled release of the drug. The polymer matrix was more efficient than the oil vehicle of the TU injection. The preparation releases TU at a constant rate following Higuchi kinetics both *in vitro* and *in vivo*.[8] Serum concentration curves of testosterone started smoothly then decreased progressively without large fluctuation. In this study, we have investigated further the antifertility potential of TU-loaded ISFI for contraception in adult male rats.

As discussed previously,[8] the investigation demonstrates that *P* (CL/DL-LA) ISFI can sustain TU delivery for a prolonged
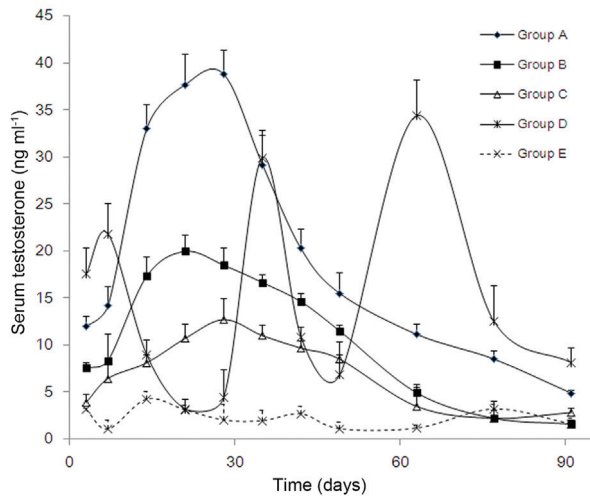
Figure 4: Mean serum T levels in each treatment dose group and in the placebo group during treatment and recovery periods (*n* = 5).

period (about 90 days) and induce high serum testosterone levels to suppress spermatogenesis. The present data demonstrated that the administration of TU caused a pronounced decline in the number of epididymal spermatozoa 30 days after treatment, and severe suppression of spermatogenesis was found after continued treatment for another 60 days in each group. In groups providing 270 and 135 mg TU kg^{-1} every 3 months, spermatogenesis had partially recovered 1-month after TU withdrawal. It is interesting that ISFI groups showed a remarkable increase in sperm motility 30 days after treatment that decreased quickly. Exogenous T suppresses both hypothalamic GnRH and pituitary FSH and LH production, resulting in a depletion of intra-testicular T.[14] These lead to a suppression of spermatogenesis, but the existing spermatozoa are not affected. Testicular spermatozoa have to pass through the rete cavity and epididymis to undergo the process of maturation that involves acquisition of forward motility and fertilizing capacity. Testosterone enters the epididymis with the testicular fluid and is also supplied via the bloodstream.[15] Stimulation by androgens is essential for the synthesis and secretion of a number of proteins by the epithelial cells of the epididymis, which are vital for sperm maturation and sperm storage. For rats, the duration of the seminiferous epithelium cycle has been determined to be 10 days. But the entire process of spermatogenesis takes 48 days including the process of maturation.[16] The time lag and high T concentration in the epididymis may have led to the initial transient increase of sperm motility. Meanwhile, the initial higher mating rate may be due to the stimulation of sexual behavior after treatment of TU.[17]

Although there was a clear dose-response relationship between the injected doses and serum T levels, spermatogenesis was not suppressed in a TU dose-dependent manner after 3 months treatment. A pronounced difference was that a large dose (540 mg kg^{-1} every 3 months) of TU did not significantly suppress spermatogenesis, which is consistent with previous studies indicating that large dose of testosterone can maintain or restore spermatogenesis in hypophysectomized or intact rats.[18–21] These results have led to the hypothesis that high levels of exogenous androgens or residual androgen production after withdrawal of LH and FSH may allow spermatogenesis to proceed and bring about contraceptive failure. Another possibility is that androgens at sufficient levels within the testes will support spermatogenesis. No quantitative relationship between

sperm concentration and intra-testicular androgen levels has been demonstrated after prolonged gonadotropin inhibition.[22,23] High doses of testosterone are associated with large increases in serum testosterone and as a result, the levels of intra-testicular exogenous testosterone increase. The present results suggest that high levels of intra-testicular testosterone might be sufficient to maintain semen parameters and fertility despite inhibition of gonadotrophin secretion.

The results of the fertility tests, which demonstrated that all the males treated for 3 months in groups B and C were infertile, whereas 90% in group D, indicate that 270 or 135 mg kg^{-1} TU-loaded ISFIs suppressed the output of spermatozoa more effectively than TU injections. This indication is also supported by the motility of spermatozoa that was dramatically decreased in the rats from groups B and C compared with that of group D. On the contrary, the percentage of abnormal spermatozoa increased in groups B and C, and 1-month after withdrawal of treatment, unlike infertile rats in group D, the suppression of spermatogenesis in rats of groups B and C was quickly reversed. The controlled-release TU-loaded ISFI could maintain relatively stable serum concentrations without fluctuation throughout the treatment period. Conversely, when rats are treated with TU injection monthly, the serum testosterone concentrations rises quickly, and the supra-physiological peaks are observed with large fluctuations according to the timing of TU injection re-administration.[6,7,24] Consequently, it can be seen that the dosage form of controlled-release TU-loaded ISFI at an appropriate dose, reducing the intra-testicular testosterone concentration mildly and stably, could be better than that of TU injection. And 4 months after treatment, same rats in groups B and C had restored fertility. On the other hand, the serum TU concentrations were still high in the rats treated with high-dose TU-loaded ISFIs and TU injections after 3 months. Therefore, all of the animals in groups A and D were still infertile after 4 months.

The morphology of testicular sections in the present study revealed that appreciable damage to spermatogenesis occurred after 3 months of treatment. Detachment of spermatocytes and spermatids became evident, with apparent radial spaces being formed between the germ cells. Only few elongated and elongating spermatids existed in the seminiferous epithelium and degenerating round spermatids and spermatogenic cells being observed in the lumen. These changes indicate impairment in meiosis (development of spermatids from spermatocytes) and spermatogenesis (transformation of elongated spermatids from round spermatids).[25] Spermatid degeneration observed in the present study or apoptosis observed previously[26] was likely another factor in the reduction of spermatid numbers. The apoptotic and exfoliated spermatocytes and spermatids in the epididymal semen may be also associated with abundant apoptosis.[27,28] The analysis showed that almost half the epididymal spermatozoa after TU-loaded ISFI treatment appeared morphologically abnormal, and the percentage of motile spermatozoa was only 0%~8% of the control. It has been found that after gonadotrophin withdrawal, the spermatogenic process is primarily suppressed at both its starting point (conversion from type A to type B spermatogonia) and end point (releasing of elongated spermatids, i.e. spermiation).[29,30] In our investigation, we presumed that there were two predominant lesions induced by TU treatment. The first, a reduced number of spermatogonia entering into the cycle (and subsequently to the number of all germ cell types), and the second, the loss of round spermatids (degeneration or sloughing) leading to the failure of spermiogenesis. With the limited conditions, the apoptosis of germ cells is not investigated in this paper. How TU administration influences germ cell apoptosis need further research, and the relationship between TU administration dosages,

intra-testicular testosterone concentration and contraceptive effect also need to be further studied.

CONCLUSION

The present findings suggest that TU-loaded *in situ*-forming implants at an appropriate TU dose have potential as a long-acting male contraceptive drug and consistent suppression of spermatogenesis that can be maintained for a period of 3 months. The control release of TU from ISFI could maintain a relatively stable serum concentration without marked fluctuation, and all of animals lost their fertility when treated with TU-loaded ISFIs (270 and 135 mg kg^{-1} every 3 months). And the fertility of males can be quickly and easily recovered after withdrawal.

AUTHOR CONTRIBUTIONS

XWZ designed the experiment, wrote and revised the paper. CZ and PW carried out the investigation of serum testosterone and participated in the fertility studies. WZ, DY, SM, and JG carried out the fertility studies and sperm parameters. DHL designed and carried out the experiment, and revised the paper. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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