Urokinase-type plasminogen activator: a new target for male contraception?

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Urokinase-type plasminogen activator (uPA) is closely related to male reproduction. With the aim of investigating the possibility for uPA as a potential contraceptive target, in the present work, Kunming male mice were immunized by human uPA subcutaneous injection at three separate doses for 3 times. Then the potency of the anti-human uPA antibody in serum was analyzed, and mouse fertility was evaluated. Serum antibody titers for human uPA in immunized groups all reached 1:10,240 or higher levels by enzyme linked immunosorbent assay, and mating experiments revealed that pregnancy rates and the mean number of embryos implanted after mating declined obviously ($P < 0.05$) when compared with control groups. However, the mating capacity and reproductive organ weights had no obvious change, and histological analysis of the testes and epididymides also showed normal morphology for immunized male mice. Sperm function tests suggested that the sperm concentration, sperm viability, sperm motility, and in vitro fertilization rate for the cauda epididymis sperm in uPA-immunized groups were lower than those in the controls ($P < 0.05$). Together, these observations indicated that subcutaneous injection human uPA to the male mice could effectively reduce their fertility, and uPA could become a new target for immunocontraception in male contraceptive development.

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INTRODUCTION

Urokinase-type plasminogen activator (uPA), a member of the plasminogen activator (PA) system, is a kind of trypsin-like serine protease. uPA binds to a specific cell surface receptor and mediates proteolysis by activation of plasminogen or growth-factor. Directional proteolysis could play a role in physiological processes such as cell invasion, ovulation, embryo implantation, and wound healing.

Urokinase-type plasminogen activator is closely related to male reproduction. Many studies had defined that uPA mRNA were expressed in the Sertoli cells of mouse, rat, and monkey testis seminiferous epithelium, as well as in the epididymis, seminal vesicle, and prostate gland. Researches confirmed that uPA involved in the spermatogenesis and sperm maturation, venting, sperm motility, capacitation, the acrosome reaction, fertilization, and other male reproductive physiology process. The work by Zheng et al. showed uPA stimulated sperm motility, induced acrosome reaction and enhanced the sperm capacity to fertilize mature eggs in the rhesus monkey. The human seminal values were 15-fold higher than those found in the blood for uPA, which suggested a possible role of the fibrinolytic factor in seminal plasma. Furthermore, Huang et al. concluded that membrane-associated uPA on human spermatooza may be related directly to sperm motility and fertility. Together, these observations in males open the possibility that uPA might serve as a potential target for the development of methods for fertility regulation.

With the aim of exploring both the relevance of uPA for fertility and its potential use for the development of a contraceptive approach, in the present study, we attempted to induce immune response in male mouse by subcutaneous injection of human uPA in the assistant of adjuvant, and analyzed the changes of mouse fertility after immunization.

MATERIALS AND METHODS

Animals

Kunming mice (an outbred mouse strain), 8-week old with body weight of 30 ± 5 g, were obtained from the Animal Experiment Center of Hubei Provincial Center for Disease Control and Prevention. They were kept in a temperature-controlled room with 14 h/10 h light-dark cycles (dark starting at 20:00) and food and water were provided ad libitum. All protocols and experimental procedures for the use of animals in this study were performed in accordance with the National Institutes of Health Guiding Principles in the Care and Use of Animals.

Chemicals

All chemicals were of the purest analytical grade and were purchased from Amresco (Solon, OH, USA), unless otherwise indicated.

Treatment of mice

Male mice were divided into five groups at random as follows:

1. Blank control group: mice in this group were not treated with anything
2. Adjuvant control group: mice were subcutaneously injected with 100 μl physiological saline solution mixed with 100 μl Freund’s adjuvant (Santa Cruz Biotechnology, Inc., Texas, USA)
3. 20 μg uPA-immunized group: injection solution contained 20 μg uPA (extracted from human urine or nephridial tissue, Tianjin biochemical, pharmaceutical factory, Tianjin, China)
4. 40 μg uPA-immunized group: injection solution contained 40 μg uPA
5. 80 μg uPA-immunized group: injection solution contained 80 μg uPA.

Urokinase-type plasminogen activator powder was dissolved in 100 μl physiological saline solution, and then emulsified with 100 μl Freund’s adjuvant in the three experimental groups. Mice were subcutaneously injected once per 2 weeks for three times. The first injection consisted of 100 μl of antigen emulsified with 100 μl of Freund complete adjuvant (Santa Cruz). For subsequent injections, Freund incomplete adjuvant (Santa Cruz) was used. The male mice were proved to have fertility by mating experiment before they were immunized.

Antibody potency test
In 1 week after the third immunization, six mice per group were anesthetized with 10% chloral hydrate and blood was obtained from the angular vein. Samples were let stand for 30 min at room temperature and centrifuged for 15 min at 500 g. Serum was separated from the pellet and stored at −20°C until use.

The potency of human uPA-specific antibody in serum in every group was determined via enzyme-linked immunosorbent assay. About 10 μg human uPA in 100 μl 0.1 mol l⁻¹ sodium bicarbonate/carbonate (pH 9.6), was coated in each well of a 96-well microtiter plate and incubated overnight at 4°C. The wells were washed 3 times with phosphate buffered saline (PBS) supplemented with 0.1% Tween 20 (Sigma-Aldrich Co., St. Louis, MO, USA). Nonspecific binding sites were blocked for 60 min at 37°C with a solution of 1% bovine serum albumin and 0.1% Tween 20 in PBS. Then the wells were washed as mentioned above. Each mouse sera diluted (four kinds of concentration, dilution ratio: 1:1280, 1:2560, 1:5120, 1:10,240 respectively) in 100 μl 0.1 mol l⁻¹ sodium bicarbonate/carbonate (pH 9.6) were placed in duplicate wells and incubated for 120 min at 37°C. The wells were then washed and incubated for 1 h at 37°C with anti-mouse IgG conjugated to horseradish peroxidase (Beijing Boss Biotechnology Company Limited, Beijing, China; 1:5000 in 0.1 mol l⁻¹ sodium bicarbonate/carbonate, pH 9.6). After washed, the substrate, tetramethylbenzidine (Sigma), was added at 0.1 mg ml⁻¹ in 0.025 mol l⁻¹ citric acid/0.01 mol l⁻¹ disodium hydrogen phosphate pH 5.0 and incubated for 30 min at 37°C. Absorbance at 450 nm was determined in a board type microplate reader (Bioss). When the OD450 values of uPA-immunized groups that can reflect the contents of human uPA-specific antibody in serum in every group indirectly, were detected, a new group, or PBS control group was added. For every diluted ratio, the OD450 average of uPA-immunized group subtracted that of blank control, and the OD450 average of adjuvant control subtracted that of blank control, then the ratio of the above former to the latter was OD450 average of uPA-immunized group subtracted that of blank control, and the OD450 average of adjuvant control subtracted that of blank control, then the ratio of the above former to the latter was OD450 average of uPA-immunized group subtracted that of blank control, and the OD450 average of adjuvant control subtracted that of blank control, then the ratio of the above former to the latter was OD450 average of uPA-immunized group subtracted that of blank control, and the OD450 average of adjuvant control subtracted that of blank control, then the ratio of the above former to the latter was OD450 average of uPA-immunized group subtracted that of blank control, and the OD450 average of adjuvant control subtracted that of blank control, then the ratio of the above former to the latter was OD450 average of uPA-immunized group subtracted that of blank control, and the OD450 average of adjuvant control subtracted that of blank control, then the ratio of the above former to the latter was.

Hematoxylin and eosin staining of the testis and epididymis
In 1 week after the third immunization, testes and epididymides of mice from every group were fixed in Bouin’s solution for 48 h. Tissues were then processed for paraffin embedding, and sectioning by routine methods. Deparaffinized sections were stained with hematoxylin and eosin solutions and examined by light microscopy.

Statistical analysis
Statistical analyses were conducted using the Statistical Package for Social Sciences program, Version 12.0 (SPSS Inc., Chicago, IL, USA). The Chi-squared test was used to compare mating rate, pregnancy rate, and IVF rate. Differences in means of OD450 value, live embryos, sperm count, viability, and motility were analyzed using one-way analysis of variance. P < 0.05 was considered as statistically significant.

RESULTS
Analysis for antibody potency
Compared with control groups, human uPA-specific antibody level in three doses of uPA-immunized groups remarkably increased (P < 0.05). The antibody levels showed a downward trend with the increase of immunization dose, but they had no statistical difference (P > 0.05) among three groups (Table 1). The ratios of OD450 for every immune group were all higher than 2.1 (Table 2), which suggested that the titers of human uPA-specific polyclonal antibody in immune serum all reached 1:10,240 or higher levels.

Analysis for body and reproductive organ weights
As shown in Table 3, no difference was observed among these groups for testis, epididymis and seminal vesicle weights (P > 0.05).
Table 1: OD450 values of antiserum against human uPA by ELISA (x±s, n=6)

| Groups            | 1:1280 | 1:2560 | 1:5120 | 1:10 240 |
|-------------------|--------|--------|--------|----------|
| PBS control       | 0.20±0.01 | 0.25±0.01 | 0.20±0.01 | 0.23±0.03 |
| Blank control     | 0.31±0.01 | 0.21±0.01 | 0.21±0.01 | 0.22±0.03 |
| Adjuvant control  | 0.70±0.44 | 0.46±0.46 | 0.34±0.19 | 0.27±0.07 |
| 20 μg uPA         | 2.33±0.37* | 1.73±0.33* | 1.71±0.36* | 1.41±0.64* |
| 40 μg uPA         | 2.11±0.35* | 1.52±0.55* | 1.41±0.58* | 1.08±0.49* |
| 80 μg uPA         | 2.01±0.30* | 1.35±0.42* | 1.18±0.35* | 0.89±0.26* |

- *P<0.05 compared with the control groups. uPA: urokinase-type plasminogen activator; PBS: phosphate buffered saline; ELISA: enzyme linked immunosorbent assay.

Table 2: The titers of antiserum against human uPA in immunized groups

| Groups            | 1:1280 | 1:2560 | 1:5120 | 1:10 240 |
|-------------------|--------|--------|--------|----------|
| 20 μg uPA         | + (4.9) | + (5.9) | + (11.4) | + (27.5) |
| 40 μg uPA         | + (4.6) | + (5.1) | + (9.2)  | + (19.9) |
| 80 μg uPA         | + (4.3) | + (4.4) | + (7.4)  | + (15.4) |

- uPA: urokinase-type plasminogen activator.

Table 3: Effect of immunization on reproductive organ weights (mg per 100 g of body weight, x±s, n=10)

| Groups            | Testis | Epididymis | Seminal vesicle |
|-------------------|--------|------------|----------------|
| Blank control     | 0.31±0.33 | 0.13±0.25 | 0.52±0.67 |
| Adjuvant control  | 0.31±0.62 | 0.12±0.27 | 0.56±1.41 |
| 20 μg uPA         | 0.25±0.33 | 0.12±0.20 | 0.50±0.97 |
| 40 μg uPA         | 0.30±0.29 | 0.12±0.07 | 0.30±0.15 |
| 80 μg uPA         | 0.25±0.19 | 0.12±0.35 | 0.60±1.21 |

- uPA: urokinase-type plasminogen activator.

Changes of capacity in mating and fertility
As presented in Table 4, there was no significant difference in the mating rate between the immunized groups and the control groups (P > 0.05). However, pregnancy rate and the average number of live embryos in the 20 μg uPA-immunized group obviously decreased (P < 0.05). Moreover, no mouse got pregnancy in both the 40 μg uPA and the 80 μg uPA-immunized groups. These indexes were not different between the blank control and the adjuvant control (P > 0.05).

Changes in sperm function
By comparison to those in the control groups, sperm concentration, sperm viability, sperm motility and IVF rates in three doses of uPA-immunized groups had a remarkable decline (P < 0.05) (Table 5).

Testis and epididymis histological features
Histological analyses of the testes and the epididymides by light microscopy showed normal seminiferous and epididymal tubules in all of uPA-immunized mice. Spermatogonia, spermatocytes, and spermatids were systematically arranged in the seminiferous tubules, and the germ cells were encased by Sertoli cells (Figure 1). Epithelial cells arranged orderly, and sperm was found in the epididymal tubules (Figure 2). No signals of leukocyte infiltration were observed.

DISCUSSION
In this study, we demonstrated that male mice were induced to produce the antibody against human uPA by subcutaneous injection. The fertility of uPA-immunized mice had a sharp decline, suggesting that uPA could be a new target for the male immuncontraception.

There are two types of PAs, tissue-type PA (tPA) and uPA which are products of two separate genes with different genomic organization and chromosome localization. Despite the apparent differences in their basic biological functions, functional redundancy between uPA and tPA has been demonstrated in various physiological settings using gene-deficient mice. Hence, mice with single deficiencies of uPA by and large exhibit normal phenotype with respect to growth, reproduction and survival though uPA involves in the immune response, tissue remodeling and wound healing by regulating cell migration, adhesion and proliferation.

Our results reveal that the pregnancy rates and the mean number of embryos of the females mated with uPA immunized males declined obviously (P < 0.05). Notably, there was no detectable pregnancy in both the 40 μg uPA and the 80 μg uPA-immunized groups. The loss of pregnancy could be due to the loss of sperm functions (Table 5), the reason for which could be that polyclonal antisem against human uPA could cause a cross reaction with mouse endogenous uPA molecules because amino acid sequence homology with the uPA as a new target for male immuncontraception.
protein between human and mouse is 71% consensus.\textsuperscript{22} uPA involved in spermatogenesis and sperm maturation,\textsuperscript{6,7} sperm motility,\textsuperscript{12,17} capacitation,\textsuperscript{10} and fertilization.\textsuperscript{13} Therefore, normal physiological function of endogenous uPA in reproduction system of the immunized male mice could be disturbed, which could result in fertility decline. It is unlikely that the impaired sperm functions is caused by a change of testosterone level because mating capacity and reproductive weights of testis, epididymis, and seminal vesicles in uPA-immunized mice remained comparable to the controls, as well as histological features of testes and epididymis.

The sperm from the cauda epididymis in the 40 μg uPA and the 80 μg uPA-immunized groups could fertilize mature oocytes in vitro although the percentage of motile sperm was low, indicating that those sperm were cable of capacitation and fertilization. The lack of pregnancy in the above two groups could result from impaired sperm functions. We suspect that low sperm motility is caused by the attack of uPA antibody. The injection of human uPA antigen to male mice did not affect spermatogenesis in the testis because of blood-testis barrier, which was confirmed by testis histological analysis. However, Sperm from testis must mature in the epididymis, and can be a target of the antibody against human uPA in the epididymis. The epididymis is vulnerable to infiltration of various leukocytes as immunologically privileged organs\textsuperscript{23} and cannot protect sperm from the attack by the immune system. Second, seminal plasma ejaculated could also contain the antibody against human uPA, which further increased sperm damage. The antibody in the seminal plasma could also hinder uPA functions in the female genital tract during the mating at the same time to contribute to the infertility.

CONCLUSION
Our results indicated that subcutaneous injection human uPA to the male mice could effectively reduce their fertility. These observations support the possibility of uPA as a potential target for male immunocontraception.

Figure 1: Testicular sections from urokinase-type plasminogen activator (uPA)-immunized mice subjected to histological examination (H and E). (a) Control mice; (b) 20 μg uPA-immunized mice; (c) 40 μg uPA-immunized mice; (d) 80 μg uPA-immunized mice. Scale bars = 20 μm.

Figure 2: Mouse epidymidal sections subjected to histological examination (H and E). (a) Control mice; (b) 20 μg urokinase-type plasminogen activator (uPA)-immunized mice; (c) 40 μg uPA-immunized mice; (d) 80 μg uPA-immunized mice. Scale bars = 50 μm.

AUTHOR CONTRIBUTIONS
LZ, CLX, and YQ conceived and designed the research study. YQ and YH carried out the experiments. HGL and LH analyzed the data. YQ drafted the manuscript. LZ revised the manuscript. All authors discussed the results and implications, and reviewed and approved the final manuscript.

COMPETING INTERESTS
All authors declare that there are no competing interests.

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REFERENCES
1. Ferraris GM, Sidenius N. Urokinase plasminogen activator receptor: a functional integrator of extracellular proteolysis, cell adhesion, and signal transduction. Semin Thromb Hemost 2013; 39: 347–55.
2. Rabbani SA, Mazar AP. The role of the plasminogen activation system in angiogenesis and metastasis. Surg Oncol Clin N Am 2001; 10: 393–415.
3. Vassalli JD, Sappino AP, Belin D. The plasminogen activator/plasmin system. J Clin Invest 1991; 88: 1067–72.
4. Del Rosso M, Margheri F, Serrati S, Chilli A, Laurenzana A, et al. The urokinase receptor system, a key regulator at the intersection between inflammation, immunity, and coagulation. Curr Pharm Des 2011; 17: 1924–43.
5. Liu YX, Du Q, Liu K, Fu GQ. Hormonal regulation of plasminogen activator in rat and mouse seminiferous epithelium. Biol Signals 1995; 4: 232–40.
6. Vihko KK, Penttilä TL, Parvinen M, Belin D. Regulation of urokinase- and tissue-type plasminogen activator gene expression in the rat seminiferous epithelium. Mol Endocrinol 1989; 3: 52–9.
7. Zhang T, Zhou HM, Liu YX. Expression of plasminogen activator and inhibitor, urokinase receptor and inhibit subunits in rhesus monkey testes. Mol Hum Reprod 1997; 3: 223–31.
8. Huang DH, Zhao H, Tian YH, Li HG, Ding XF, et al. Gene expression changes of urokinase plasminogen activator and urokinase receptor in rat testes at postnatal stages. Asian J Androl 2007; 9: 679–83.
9. Zhang T, Guo CX, Hu ZY, Liu YX. Localization of plasminogen activator and inhibitor, LH and androgen receptors and inhibit subunits in monkey epididymis. Mol Hum Reprod 1997; 3: 945–52.
10. Huarte J, Belin D, Bosco D, Sappino AP, Vassalli JD. Plasminogen activator and mouse spermatozoa: urokinase synthesis in the male genital tract and binding of the enzyme to the sperm cell surface. J Cell Biol 1987; 104: 1281–9.
11. Lacruix M, Fritz IB. The control of the synthesis and secretion of plasminogen activator by rat sertoli cells in culture. Mol Cell Endocrinol 1982; 26: 247–58.
12. Taitzoglou IA, Chapman DA, Killian GJ. Induction of the acrosome reaction in bull spermatozoa with plasmin. Andrologia 2003; 35: 112–6.
13. Huarte J, Vassalli JD, Belin D, Sakkas D. Involvement of the plasminogen activator/ plasmin proteolytic cascade in fertilization. Dev Biol 1993; 157: 539–46.
14. Liu YX. Involvement of plasminogen activator and plasminogen activator inhibitor
type 1 in spermatogenesis, sperm capacitation, and fertilization. *Semin Thromb Hemost* 2007; 33: 29–40.

15 Zheng P, Zou RJ, Liu YX. Source of plasminogen activator in rhesus monkey semen and its possible role in sperm capacitation. *Sheng Li Xue Bao* 2001; 53: 45–50.

16 Van Dreden P, González J, Poirot C. Human seminal fibrinolytic activity: specific determinations of tissue plasminogen activator and urokinase. *Andrologia* 1991; 23: 29–33.

17 Huang X, Xia W, Xiong C, Xiao D, Shen J, et al. Studies on the relationship between urokinase plasminogen activator (uPA) and human sperm motility. *J Tongji Med Univ* 1997; 17: 213–7.

18 WHO. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. p. 32–55. Available from: http://www.who.int/reproductivehealth/publications/infertility/9789241547789/en.

19 Quinn P, Barros C, Whittingham DG. Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *J Reprod Fertil* 1982; 66: 161–8.

20 Collen D, Lijnen HR. Basic and clinical aspects of fibrinolysis and thrombolysis. *Blood* 1991; 78: 3114–24.

21 Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, et al. Physiological consequences of loss of plasminogen activator gene function in mice. *Nature* 1994; 368: 419–24.

22 Degen SJ, Heckel JL, Reich E, Degen JL. The murine urokinase-type plasminogen activator gene. *Biochemistry* 1987; 26: 8270–9.

23 Itoh M, Terayama H, Naito M, Ogawa Y, Tainosho S. Tissue microcircumstances for leukocytic infiltration into the testis and epididymis in mice. *J Reprod Immunol* 2005; 67: 57–67.