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ORIGINAL ARTICLE

Male Contraception

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.[1,2] Directional proteolysis could play a role in physiological processes such as cell invasion, ovulation, embryo implantation, and wound healing.[3,4]

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,[5–8] as well as in the epididymis, seminal vesicle, and prostate gland.[9,10] Researches confirmed that uPA involved in the spermatogenesis and sperm maturation,[6,7] venting,[11] sperm motility,[7,10] capacitation,[12] the acrosome reaction, fertilization,[13] and other male reproductive physiology process.[14] The work by Zheng *et al*.[15] 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.[16] Furthermore, Huang *et al*.[17] concluded that membrane-associated uPA on human spermatozoa 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 Biotechology, 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)

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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 Bioss 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 calculated for the judgment of antibody titer. When the ratio was > 2.1, the titer was assessed to positive at the diluted ratio, or negative for the ratio < 1.5.

Body and reproductive organ weights

In 1 week after the third immunization, 10 mice per group were treated by 10% chloral hydrate after they were weighed. Testis, epididymis, and seminal vesicle were removed, blotted free of blood and adhering tissues and weighed.

Mating experiments

After the third immunization, 12 mice per group were coupled with adult Kunming female mice (8-week old with body weight of 30 ± 5 g) by female/male ratio of 2:1. In the next morning, the vaginal plug or sperm in vaginal cells smear would be regarded as the evidence for successful mating. These female mice showing evidence of mating were removed to another cage. If male mice did not mate, they would continue to be housed with two females for 1 week. Pregnancy rate and the number of live embryos in pregnant females in every group were calculated on the 14th day after the successful mating.

Sperm function tests

In 1 week after the third immunization, sperm were collected by puncturing the cauda epididymides of mice and placed in a conical tube with 0.5 ml of *in vitro* fertilization (IVF) medium (Vitrolife Sweden AB, Kungsbacka, Sweden). The following indicators were detected. (1) Sperm concentration: according to the WHO guidelines,[18] 10 μl fixed sperm suspension was charged into a hemocytometer chamber, and the spermatozoa were counted under light microscope, and then sperm concentration was calculated. (2) Sperm viability: 10 μl sperm suspension was smeared and stained by 10 μl eosin Y for 10 s, and then the number of the nonstaining sperm in 200 sperm was calculated. (3) Sperm motility: 10 μl sperm suspension was smeared, and the number of the total motile sperm in 200 sperm was counted. (4) IVF rate: female mice were superovulated by i.p. injection of 10 IU of pregnant mare serum gonadotropin (Hangzhou Animal Medicine Factory, Hangzhou, China) followed by the administration of 10 IU of human chorionic gonadotropin (hCG, Hangzhou Animal Medicine Factory, Hangzhou, China) 48 h later. Oocyte-cumulus complexes were collected in M2 medium from mouse oviducts 14–16 h after hCG injection,[19] and transferred to drops of the IVF medium and fertilized *in vitro* in 35 mm culture dishes which were overlaid with mineral oil and pre-equilibrated overnight. After they had been incubated in the incubation (5% CO_2, 37°C) for 6 h, the eggs were washed and transferred to new drops. Two-cell embryos were observed 24 h after fertilization. Fertilization rate was counted in accordance with the number of two-cell embryos.

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 ($\bar{\chi}$±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±0.16	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	2.23±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, $\bar{\chi}$±s , *n*=10)

Groups	*Testis*	*Epididymis*	*Seminal vesicle*
Blank control	0.312±0.33	0.134±0.25	0.528±0.67
Adjuvant control	0.313±0.62	0.123±0.27	0.568±1.41
20 μg uPA	0.251±0.33	0.122±0.20	0.504±0.97
40 μg uPA	0.306±0.29	0.121±0.07	0.302±0.15
80 μg uPA	0.252±0.19	0.120±0.35	0.606±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 enclosed 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 immunocontraception.

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.[20] 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.[21] 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.[4] For this reason, we used human uPA as an antigen to immunize mice, which would not affect markedly mouse viability. In addition, the median lethal dose of uPA for the mouse by intravenous injection is 1 million IU kg^{-1}, or 8333 μg kg^{-1} according to drug instruction. The dosages used in this study (20 μg or 40 μg or 80 μg uPA) for subcutaneously injection to the mouse in our study were safe.

Table 4: Effect of uPA immumization on male mouse fertility

Groups	*Total number*	*Mating rate (%)*	*Pregnancy rate (%)*	*Number of live embryos ($\bar{\chi}$±s)*
Blank control	12	100.0	91.7	9.00±2.13
Adjuvant control	12	100.0	91.7	8.00±1.58
20 μg uPA	12	91.7	9.1**	0.81±2.59*
40 μg uPA	12	83.3	0**	0*
80 μg uPA	12	91.7	0**	0*

*P<0.05; **P<0.01 compared with the control groups. uPA: urokinase-type plasminogen activator

Table 5: Effect of human uPA immunization on sperm parameters and sperm function ($\bar{\chi}$±s, *n*=10)

Groups	*Sperm concentration ($\times 10^9$ l^{-1})*	*Sperm viability (%)*	*Sperm motility (%)*	*IVF rate (%)*
Blank control	6.2±0.9	63.1±12.6	52.5±12.7	84.21
Adjuvant control	4.7±1.0	63.5±13.6	62.5±8.4	67.39
20 μg uPA	2.6±2.7*	38.2±24.5*	29.1±15.1*	16.82**
40 μg uPA	1.9±1.2*	30.4±8.5*	20.0±9.9*	25.00**
80 μg uPA	2.3±1.5*	31.3±10.4*	18.6±7.3*	25.29**

The data of sperm concentration, sperm viability and sperm motility came from 10 mice and were presented as $\bar{\chi}$±s. The sperm used in IVF came from six mice per group and the number of oocytes in each group varied from 46 to 107. *P<0.05 compared with the control groups; **P<0.01 compared with the control groups. uPA: urokinase-type plasminogen activator; IVF: *in vitro* fertilization

Human uPA can induce an immune response in male mice, and anti-human uPA antibody level in the three doses of uPA-immunized groups remarkably increased in our study ($P < 0.05$). The reason is that the human uPA is a heterologous antigen and has the immunogenicity to the mouse. The uPA powder for injection with the purity of more than 99% is isolated from healthy human urine, and it is a mixture of two proteins, 33kDa and 54kDa uPAs. More than 90% of the uPA powder consists of the 54kDa uPA, which is a good candidate for antigen because of its multiple epitopes and stable chemical structure. Furthermore, we find that the levels of antibody show a downward trend with the increase of immune dose, but the differences is not statistically significant ($P > 0.05$), which suggests that the phenomenon of immune tolerance would be induced if we continued to increase the dosage of the antigen.

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 antiserum against human uPA could cause a cross reaction with mouse endogenous uPA molecules because amino acid sequence homology with the uPA

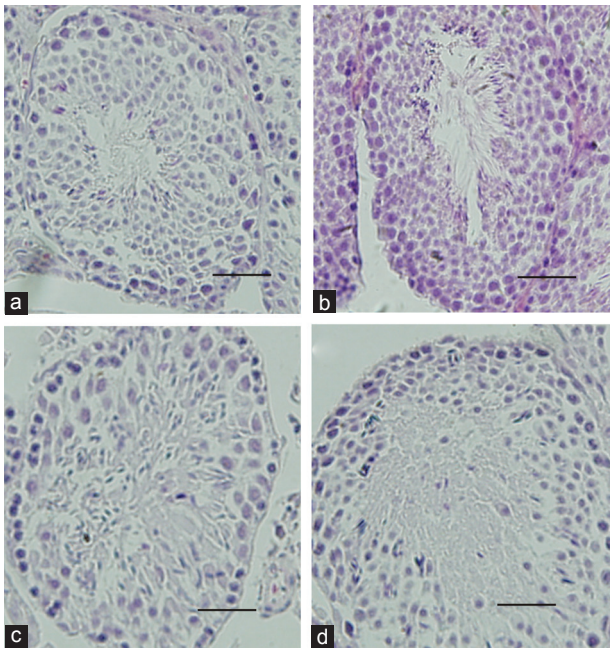

Figure 1: Testicular sections from urokinase-type plasminogen activator (uPA)-immunized mice subjected to histological examination (H and E). (**a**) Control mice; (**b**) 20 μg uPA-immunized mice; (**c**) 40 μg uPA-immunized mice; (**d**) 80 μg uPA-immunized mice. Scale bars = 20 μm.

protein between human and mouse is 71% consensus.[22] uPA involved in spermatogenesis and sperm maturation,[6,7] sperm motility,[7,17] capacitation,[12] and fertilization.[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 epidymidis 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 epidymidis, and can be a target of the antibody against human uPA in the epidymidis. The epididymis is vulnerable to infiltration of various leukocytes as immunologically privileged organs[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.

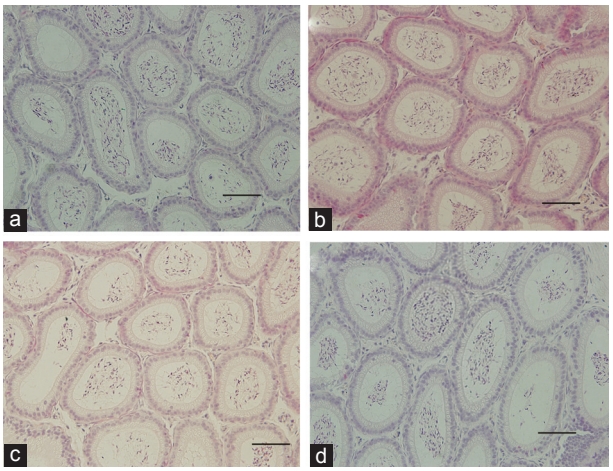

Figure 2: Mouse epididymal sections subjected to histological examination (H and E). (**a**) Control mice; (**b**) 20 μg urokinase-type plasminogen activator (uPA)-immunized mice; (**c**) 40 μg uPA-immunized mice; (**d**) 80 μg uPA-immunized mice. Scale bars = 50 μm.

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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