Immunocontraceptive Effect of DNA Vaccine Targeting Fertilin $\beta$ in Male Mice

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Introduction

Overpopulation of both human and selected groups of animals is recognized as a growing problem globally. Although many methods have been used in birth control, there is no ideal contraceptive which is safe, effective and widely acceptable. Sperm–egg binding and fusion are processes that play a key role in fertilization and production. Blocking the binding of sperm and egg has become a desirable target for immunocontraception. Sperm antigen such as fertilin has been considered to be very important in mediating the sperm–egg binding and fusion.1–3 Fertilin is a heterodimer made up of $\alpha$ and $\beta$ subunits. The $\beta$-subunit is considered as the functional unit. Moreover, fertilin peptides have been shown to inhibit the binding of sperm to oocytes and the fusion of the gametes.4–7 The key role played by fertilin is in the process of mammiferous fertilization in vivo, provided that sperm must be capable of undergoing the acrosome reaction, binding to the zona pellucida (ZP), and penetrating the ZP to fuse with the oolemma.4–7 Therefore, blocking the function of fertilin by immunocontraception may be of significant measurable benefit to population control in the future. In guinea pigs, fertilin $\beta$ subunit has a

Keywords
Antigen, contraception, DNA vaccine, fertilin $\beta$, immuno-adjuvant, sperm

Problem

In previous study, two eukaryotic expression plasmids pSG.SS.YL-F$_{\beta,ECD}$ and pSG.SS.C3d3.YL-F$_{\beta,ECD}$ were successfully constructed and transfected in HEK293 cells. Now, we want to evaluate the immunocontraceptive effect of these two DNA vaccines that target the extracellular domain (F$_{\beta,ECD}$) of sperm antigen fertilin $\beta$ subunit in Kunming male mice.

Method of study

DNA vaccines pSG.SS.YL-F$_{\beta,ECD}$ and pSG.SS.C3d3.YL-F$_{\beta,ECD}$ were injected into Kunming male mice three times at 0, 4, and 8 weeks, respectively. An antifertility effect was observed. Serum antibody and cytokines were also detected.

Results

Both vaccines significantly decreased both the pregnancy rate and the number of newborns. The serum levels of IL-2 and INF-$\gamma$ significantly decreased, whereas the levels of IL-4 and IL-10 significantly increased. Compared with pSG.SS.YL-F$_{\beta,ECD}$, pSG.SS.C3d3.YL-F$_{\beta,ECD}$ was more effective in birth control, and its specific F$_{\beta}$-IgG antibody titer in serum was significantly higher and longer.

Conclusion

The results indicate that both pSG.SS.YL-F$_{\beta,ECD}$ and pSG.SS.C3d3.YL-F$_{\beta,ECD}$ DNA vaccines are effective in birth control of mice. The immunocontraceptive effect of F$_{\beta,ECD}$ DNA vaccine in male mice is improved with the addition of immuno-adjuvant C3d3.
molecular mass of 44,000. Monoclonal antibodies against fertilin β subunit were found to inhibit fusion of guinea pig sperm with zona-free eggs, suggesting that fertilin has an important role in mammalian fertilization, and also homologs to these proteins have been isolated and cloned from the testis of humans and other species.

With the development of gene recombination technique, DNA vaccine targeting sperm antigen could potentially become the ideal immunocontraceptive. Fertilin monoclonal antibody PH-30 can inhibit sperm–oocyte membrane fusion during the course of fertilization in vitro and binding of un-ZP ovum and sperm in guinea pig. Fertilin beta extracellular domain (FβECD) is a region which is encoded from 1168 to 2058 base pairs of fertilin beta cDNA. Antigens often come with an immunoadjuvant to boost the immunity; C3d is a safe and effective immunoadjuvant that can fuse with hCG-beta to enhance contraceptive efficiency in vaccinated mice. In our previous study, we have constructed two DNA vaccines targeting sperm antigen FβECD, one of them expresses FβECD protein and the other expresses FβECD-C3d3 fusion protein. The objective of this study was to evaluate the immunocontraceptive effect of these two recombinant DNA vaccines in male mice. After the prototype of FβECD or FβECD-C3d3 DNA vaccine was injected into mice, the contraceptive rate, number of newborns, and specific antibody against FβECD antigen were measured, and also cellular immunity was determined by evaluating cytokine production.

Materials and methods

DNA Amplification and Plasmid Construction

According to the mouse FβECD cDNA sequence, we selected the partial sequence of FβECD (amino acid 1168–2058 bp) as the sequence of the target gene. The primers used for PCR cloning were as follows:

- Upstream primer A: 5′-GAAGATCTGGAGGAGGAAGTGTGGGAA-3′
- Downstream primer B: 5′-CGGGATCCGTGGGAGAAGCTTG-3′

Primer A was inserted at BglII cutting site, whereas primer B was inserted at BamHI cutting site.

The amplification of the DNA fragment was performed using polymerase chain reaction (PCR) in accordance with the manufacturer’s instructions. The plasmid pYA3149-Fβ was presented by Dr Yanfeng Li. The plasmids pSG.SS.YL and pSG.SS.C3d3.YL, which contain signal peptide SS and tag YL were presented by Dr Pei-he Liang.

The purified Fβ was ligated into pSG.SS.YL and pSG.SS.C3d3.YL. Successful clones were confirmed by restriction mapping and sequencing. Fβ and FβC3d3 genes were expressed successfully in the HEK293 cell line. The plasmid purification kit and DNA purification kit were purchased from Omega Bio-Tek Inc (Norcross, GA, USA).

Immunization

In our previous study, plasmids pSG.SS.YL-FβECD and pSG.SS.C3d3.YL-FβECD were successfully constructed from pSG.SS.YL. After approval of the Animal Research Ethical Committee of the Third Military Medical University, 48-week-old Kunming male mice were randomized into two control groups and two experimental groups (n = 10). The mice, weighing 20–22 g, were purchased from the experimental animal center of the Third Military Medical University. Experimental groups were injected with either plasmid pSG.SS.YL-FβECD (YL-Fβ group) or pSG.SS.C3d3.YL-FβECD (C3d3-YL-Fβ group). Control groups were injected with either plasmid pSG.SS.YL (YL group) or PBS (PBS group). The amount of DNA was 100 pmol/100 μL and PBS was 0.01 M. Injections were scheduled at 0, 4, and 8 weeks. The injection site was the leg muscle. All injections were performed using the same technique and schedule.

Immunity Response in vivo

We harvested 15 μL of blood using the cutting tail method at the time points of 0, 2, 4, 6, and 8 weeks after the first vaccination. Specific serum antibody for F-beta was detected in YL-Fβ and C3d3-YL-Fβ groups using ELISA. The Th1-type cytokines IL-2 and INF-γ and Th2-type cytokines IL-4 and IL-10 were evaluated in all groups. All cytokine kits were purchased from R&D Systems Inc (Minneapolis, MN, USA) and all measurements were carried out by following the manufacturer’s instructions.

Antifertility Effect

Each of the immunized Kunming mouse was mated with a normal age-matched female mouse at the time points of 2, 4, 6, and 8 weeks after the immu-
nization. The male to female ratio was 1:1. Successful mating was confirmed by the presence of a vaginal plug of the female mouse. Contraception rate and number of litters of experimental groups were counted and compared with the controls after each time point of 2, 4, 6, and 8 weeks.

### Statistical Analysis

All the values are reported as the mean ± S.D. The statistical analysis was performed using spss 13.0 software (SPSS Inc., Chicago, IL, USA). The one-way anova was used to test for differences among at least three groups at different time points, and the Student’s t-test was used between two control and treatment groups.

### Results

#### Observation of the Immunocontraception Effect

After vaccination, no significant change of shape or behavior could be observed in the mice. Compared with controls, the conception rates of YL-Fβ group and C3d3-YL-Fβ group decreased observably. In the PBS and YL group, the contraception rate was always 100%. In the YL-Fβ group, after immunization at time points 2, 4, 6, and 8 weeks, the contraception rate was 90, 60, 50, and 30%, respectively, and the average contraception rate was 57.5%. In the C3d3-YL-Fβ group, after immunization at time points 2, 4, 6, and 8 weeks, the contraception rate was 70, 20, 20, and 0%, respectively, and the average contraception rate was 27.5% (see Table I). The mean litter number of the same pregnancy reduced in YL-Fβ group and C3d3-YL-Fβ group as compared with that in the control groups. After immunization at 2, 4, 6, and 8 weeks, the mean litter number was 11.6 ± 2.2, 10.2 ± 3.3, 11.2 ± 2.6, and 11.4 ± 3.1 in PBS group, 10.0 ± 2.1, 11.2 ± 3.5, 11.3 ± 2.7, and 11.5 ± 2.7 in YL group, 10.7 ± 2.3, 6.2 ± 1.2, 4.1 ± 0.9, and 2.2 ± 0.3 in YL-Fβ group, and 9.6 ± 2.6, 2.5 ± 0.4, 2.3 ± 0.5, and 0 in C3d3-YL-Fβ group. In YL-Fβ group, the litter number showed significant decrease compared with that in the controls (P < 0.05). After immunization at 4, 6, and 8 weeks, the litter number reduced when compared with the control group. The litter number reduced by 39.2, 63.4, and 80.7% when compared with PBS group and by 44.6, 63.7, and 80.9% when compared with that in YL group. In C3d3-YL-Fβ group, after immunization at 4, 6, and 8 weeks, the litter number reduced by 75.5, 77.5, and 100% when compared with that in PBS group and reduced by 77.7, 79.6, and 100% compared with that in YL group. Compared with YL-Fβ group, C3d3-YL-Fβ group showed significant decrease in litter number after immunization at 4, 6, and 8 weeks by 59.7, 43.9, and 100% (P < 0.05). (see Table II).

#### Serum F-beta Antibody Titers

After first vaccination at the 2, 4, 6, and 8 weeks, YL-Fβ and C3d3-YL-Fβ groups showed positive antibody reaction, but not the YL and PBS groups. The average IgG titers at each time point in C3d3-YL-Fβ group were eight times higher than those in YL-Fβ group (see Table III). The contraception rate of mice was negatively correlated with serum Fβ antibody titers in both YL-Fβ and C3d3-YL-Fβ groups. The contraception rate decreased from 100% to 30%, whereas the Fβ antibody titer increased from 0 to 1/1280 after immunization in YL-Fβ group. The correlation between the two was significant (r = −0.9029, P = 0.0358) (see Fig. 1). The contraception rate decreased from 100 to 0%, whereas the Fβ antibody titer increased from 0 to 1/10,240 after

### Table I  Contraception Rate After Male Mice Immunization in Each Group

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total males per mating</th>
<th>Conception rate (%) after 2 weeks</th>
<th>Conception rate (%) after 4 weeks</th>
<th>Conception rate (%) after 6 weeks</th>
<th>Conception rate (%) after 8 weeks</th>
<th>Total conception rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS group</td>
<td>10</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>YL group</td>
<td>10</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>YL-Fβ group</td>
<td>10</td>
<td>90*</td>
<td>60*</td>
<td>50*</td>
<td>30*</td>
<td>57.5*</td>
</tr>
<tr>
<td>C3d3-YL-Fβ group</td>
<td>10</td>
<td>70*</td>
<td>20*</td>
<td>20*</td>
<td>0*</td>
<td>27.5*</td>
</tr>
</tbody>
</table>

Contraception rate at each time point after immunization; YL-Fβ group and C3d3-YL-Fβ group compared with PBS group *P < 0.05; YL-Fβ group and C3d3-YL-Fβ group compared with YL group 1P < 0.05; C3d3-YL-Fβ group compared with YL-Fβ group 1P < 0.05.
immunization in C3d3-YL-Fβ group, and the correlation was significant ($r = -0.9749$, $P = 0.0167$) (see Fig. 2). The results demonstrate that the higher the titer of Fβ antibody, the lower the contraception rate is. The contraception effect appears to be Fβ antibody titer dependent.

### Cytokines Expression of IL-2, INF-γ, IL-4, and IL-10

In the two control groups, the IL-2, INF-γ, IL-4, and IL-10 cytokine levels showed no significant change after PBS or plasmid pSG.SS.YL injection. Compared with the control groups, the Th1-type cytokines IL-2 (see Table IV) and INF-γ (see Table V) showed a reducing trend in YL-Fβ and C3d3-YL-Fβ groups. C3d3-YL-Fβ group expressed less Th1-type cytokines than YL-Fβ group. Compared with the PBS group, the average expression of IL-2 in the YL-Fβ group decreased by 21.9, 28.8, 31.8 and 39.2% after immunization at 2, 4, 6, and 8 weeks. Compared with the PBS group, the average expression of IL-2 in the C3d3-YL-Fβ group decreased by 37.2, 42.6, 44.2, and 54%. Compared with the YL group, the average expression of IL-2 in the YL-Fβ group decreased by 19.6, 18.8, 31.8, and 40% after immunization at 2, 4, 6, and 8 weeks. In the C3d3-YL-Fβ group, it decreased by 35.3, 42.1, 44, and 54.6%.

![Fig. 1](image1.png)

**Fig. 1** In YL-Fβ group, the contraception rate had a negative relation to Fβ antibody titer, $r = -0.9209$, $P = 0.0358$.

![Fig. 2](image2.png)

**Fig. 2** In C3d3-YL-Fβ group, the contraception rate had a negative relation to Fβ antibody titer, $r = -0.9747$, $P = 0.0167$. 

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Compared with the YL-Fβ group, the average expression of IL-2 in the C3d3-YL-Fβ group decreased by 19.5, 19.3, 18.2, and 24.3% after immunization at 2, 4, 6, and 8 weeks.

Compared with PBS group, the average expression of INF-γ in YL-Fβ group decreased by 18.9, 19.6, 31.6, and 38.7% after immunization at 2, 4, 6, and 8 weeks. In C3d3-YL-Fβ group, it decreased by 34.1, 40.2, 50.8, and 50.4%. Compared with YL group, the average expression of INF-γ in YL-Fβ group decreased by 19.2, 20, 31, and 38.2% at each time point after immunization. In C3d3-YL-Fβ group, it decreased by 34.2, 40.5, 50.4, and 49.9%. Compared with YL-Fβ group, the average expression of INF-γ in C3d3-YL-Fβ group decreased by 18.8, 25.6, 28.2, and 19% at each time point after immunization.

The Th2-type cytokines IL-4 (see Table VI) and IL-10 (see Table VII) showed an increasing trend in YL-Fβ and C3d3-YL-Fβ groups compared with that in the control groups. The C3d3-YL-Fβ group expressed more Th2-type cytokines than the YL-Fβ group. Compared with PBS group, the average expression of IL-4 in YL-Fβ group increased by 14.2, 27.9, 35.5, and 39.8% at each time point after immunization. In C3d3-YL-Fβ group, it increased by 35.1, 47.5, 56.8, and 61.3%. Compared with YL group, the average expression of IL-4 in YL-Fβ group increased by 14.4, 27.4, 34.5, and 38.8% at each time point after immunization.
time point after immunization. In C3d3-YL-F_p group, it increased by 35.3, 46.9, 55.6 and, 60.1%. Compared with YL-F_p group, the average expression of IL-10 in C3d3-YL-F_p group increased by 18.3, 15.3, 15.7, and 15.4% at each time point after immunization.

Compared with PBS group, the average expression of IL-10 in YL-F_p group increased by 33.5, 53.5, 85.5, and 101.7% at each time point after immunization. In C3d3-YL-F_p group, it increased by 70.2, 91, 122.6, and 140.2%. Compared with YL group, the average expression of IL-10 in YL-F_p group increased by 32.9, 52.8, 87.1, and 100% at each time point after immunization. In C3d3-YL-F_p group, it increased by 69.5, 90.2, 124.5, and 138.1%. Compared with YL-F_p group, the average expression of IL-10 in C3d3-YL-F_p group increased by 27.5, 24.5, 20, and 19.1% at each time point after immunization.

Discussion

Population explosion is a growing problem in both developed and developing countries. The world population may approach 10 billion by year 2050. Still, there is no birth control method which is safe, simple and effective enough to address this growing problem. This invites the development of new and effective enough to address this growing problem. This invites the development of new and interesting solutions in the next decades.17

Immunocontraception is a promising technique for population control in both humans and rodents, which induces specific antibodies to inhibit sperm–oocyte interaction.18 Contraceptive vaccines may provide viable and valuable alternatives to the currently available methods of contraception. The molecules that are being explored for contraceptive vaccine development target gamete production, gamete function, or gamete outcome.19 The contraceptive vaccines targeting gamete function include anti-sperm and anti-oocyte ZP vaccines. Recombinant DNA technologies were employed to search for sperm antigens as useful candidate antigens for contraceptive vaccines. Fertilin, a sperm antigen, was found on the plasma membrane of mammalian sperm. The integrin of fertilin alpha 6 beta 1 as a cell–cell adhesion receptor has been reported to mediate sperm–egg binding.20 Sperm from mice lacking fertilin beta were shown to be deficient in sperm–egg membrane adhesion, sperm–egg fusion, migration from the uterus into the oviduct, and binding to the egg zona pellucida. Fertilin beta knock-out male mice had greatly reduced fertility.21 Interestingly, fertilin beta-deficient sperm lacked fertilin alpha, and showed a greatly reduced amount of cyritestin, whereas cyritestin-deficient ones lacked fertilin alpha and showed reduced fertilin beta expression compared with the wild-type sperm.21 Although fertilin alpha-deficient mice model, and their associated levels of fertilin beta and cyritestin expression, have not been reported, these three ADAMs appear to play important roles in the sperm–oocyte binding, but not in the fusion.22 Therefore, fertilin beta could be the candidate antigen for immunoon contraception. We showed that a sperm surface antigen located in the fertilin beta extracellular domain (F_b ECD) was taken as the trigger of humoral immunity. The results shown in our experiments, after introducing the recombinant plasmids pSG.SS.C3d3.YL-F_b ECD or pSG.SS.YL-F_b ECD into mice, are that (1) the specific antibody targeting F_b ECD could be expressed in vivo, (2) anti-F_b ECD antibody would be produced after mice had been immunized with the recombinant plasmids, and (3) the contraception rate had a negative correlation with the specific antibody titer.

Furthermore, we introduced a molecule C3d, a complement C fragment, which could combine with the specific fertilin beta antigen to enhance humoral immunity. C3d has been used as an immunoadjuvant for more than a decade. A major function of

### Table VII IL-10 Concentration (mean ± S.D.) in Each Group (pg/mL, n = 10)

<table>
<thead>
<tr>
<th>Group</th>
<th>2w</th>
<th>4w</th>
<th>6w</th>
<th>8w</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS group</td>
<td>24.5 ± 2.2</td>
<td>24.5 ± 2.2</td>
<td>23.5 ± 2.6</td>
<td>23.4 ± 2.3</td>
</tr>
<tr>
<td>YL group</td>
<td>24.6 ± 2.1</td>
<td>24.6 ± 2.3</td>
<td>23.3 ± 2.8</td>
<td>23.6 ± 2.2</td>
</tr>
<tr>
<td>YL-F_p group</td>
<td>32.7 ± 2.7*</td>
<td>37.6 ± 2.9*</td>
<td>43.6 ± 3.8*</td>
<td>47.2 ± 3.8*</td>
</tr>
<tr>
<td>C3d3-YL-F_p group</td>
<td>41.7 ± 3.5*</td>
<td>46.8 ± 3.8*</td>
<td>52.3 ± 4.3*</td>
<td>56.2 ± 5.5*</td>
</tr>
</tbody>
</table>

At different week point, YL-F_p group and C3d3-YL-F_p group compared with PBS group *P < 0.05; YL-F_p group and C3d3-YL-F_p group compared with YL group 1P < 0.05; C3d3-YL-F_p group compared with PBS group *P < 0.05.
complement is the opsonization of antigen–immune complexes. This is partially mediated by the covalent attachment of cleavage fragments of C3 and C4 to the surface of pathogens, which links the innate and the adaptive immune responses by targeting the immune complex to specific complement receptors CD35 and CD21. Simultaneously, engagement of B-cell antigen receptor (BCR) and CD21/CD19 by antigen-C3d complex tremendously decreases the BCR/antigen affinity threshold, which significantly prolongs BCR attachment in lipid rafts, and induces stronger B-cell activation than antigen alone. Thus, immunized mice with recombinant hen egg lysozyme containing C3d3 molecule have surprisingly enhanced antibody formation and immunity response. The efficacy of DNA vaccines dramatically increased after fusion of some immunogens with 3Cd3 molecular adjuvant, the encoding antigens from different pathogens such as influenza, HIV, and pseudorabies virus. In our experiment, after the fusion of C3d3 to fertilin β, the specific Fβ antibody in C3d3-YL-Fβ group was eight times higher than that in YL-Fβ group. In addition, the cytokines expression was much likely to shift from Th1 type to Th2 type. Th cells are a subgroup of lymphocytes that activate and modulate other immune cells by releasing cytokines. Th1 cells are characterized by the production of IFN-γ, IL-2, and TNF-β. Th2 cells produce IL-4, IL-5, IL-9, and IL-13. During development and maturation of the immune system, both genetic factors and environmental stimuli influence the direction of differentiation of Th1 and Th2 subsets from naïve CD4 T lymphocytes. Naive CD4 cells develop into Th1 cells in response to microbial activation of antigen-presenting cells (APC) under the influence of IL-12. Differentiated Th1 cells secrete IFN-γ to fight intracellular pathogens and eliminate cancer cells. Th2 differentiation occurs in response to environmental allergens through APC under the influence of IL-4. Furthermore, Th1 and Th2 cells can be mutually regulated. Pregnancy was proposed as a Th2 bias state. IL-4 and IL-10 were demonstrated to be spontaneously produced from murine fetoplacental tissues, but not from maternal splenocytes. The exposure of peripheral blood mononuclear cells to dydrogesterone in women undergoing pre-term delivery resulted in a significant inhibition in the production of the pro-inflammatory cytokines IFN-gamma and TNF-alpha. This also significantly increased the levels of the anti-inflammatory cytokine IL-4, resulting in a substantial shift in the ratio of Th1/Th2 cytokines. The antigen-specific Th2 humoral immune response bias of the hCGbeta DNA vaccine might be improved greatly after the fusion of gene C3d3 to hCGbeta. Expression of both Th1 cytokines (IL-2 and IFN-gamma) and Th2 cytokines (IL-4 and IL-10) was enhanced in DNA vaccinated mice compared with that in the controls, with a bias towards Th1 response. The TH1/TH2 shift appears to be caused primarily by a decrease in cellular IFN-γ synthesis in TH1 lymphocytes. IL-2 and INF-γ expression decreased in YL-Fβ and C3d3-YL-Fβ groups, whereas IL-4 and IL-10 expression increased in them. A Th1 to Th2 shift in cytokine bias was accomplished by inhibiting pro-inflammatory cytokine production and increasing anti-inflammatory cytokine production. Furthermore, higher IgG titer and stronger Th2 humoral immunity showed that Fβ-ECD-C3d3 was a greater immuno-stimulus in Kunming mice.

To elucidate the antifertility effect of pSG.SS.C3d3.YL-Fβ.ECD and pSG.SS.YL-Fβ.ECD DNA vaccines with 3C3d molecular adjuvant, our experiment shows no evidence of any side-effects to the recipients. The recombinant vaccines pSG.SS.C3d3.YL-Fβ.ECD and pSG.SS.YL-Fβ.ECD result in a significant reduction of fertility, not far from completely satisfactory. The 27.5% pregnancy rate in C3d3-YL-Fβ group still needs further endeavor to consummate. In conclusion, the recombinant DNA vaccines pSG.SS.C3d3.YL-Fβ.ECD and pSG.SS.YL-Fβ.ECD are effective in contraception of Kunming mice, and the adjuvant booster C3d3 is safe, simple, and potent.

Acknowledgments

We thank Dr Peilhe Liang for presenting plasmids pSG.SS.YL and pSG.SS.C3d3.YL.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.
References


