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Oral *SS-14* DNA Vaccine is More Potent than Oral *SS-28* DNA Vaccine in Promoting Rat Lactation

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YGH and LX planned the experiment. YGH, JHY and LX executed the experiment and drafted the manuscript. QTZ, JHY, KL, YFH helped in laboratory work, statistical analysis and preparation of manuscript.

Key words
Somatostatin 14, Somatostatin 28, DNA vaccine, Lactation, Rats.

ABSTRACT

The study aimed at comparing the effect of oral somatostatin 14 (*SS-14*) and somatostatin 28 (*SS-28*) DNA vaccines in promoting lactation of rats. Fifteen female SD rats were randomly divided into three groups and were orally vaccinated, respectively *SS-14* (Group T1) and *SS-28* (Group T2) DNA vaccines fused *tPA* signal peptide and *CpG* adjuvant and delivered by attenuated *Salmonella choleraesuis* at weeks 0, 3 and 6 of the study, and rats in control group (Group C) was orally given empty vector vaccine. Blood samples were collected before primary immunization and at weeks 3, 5 and 7 after primary immunization and body weight of offspring were weighed at weeks 0, 2 and 4 after birth. Both *SS-14* and *SS-28* DNA vaccines induced humoral immune response, however, antibody response in T1 group were significantly stronger than that in T2 and C groups. Serum GH levels in T1 group was significantly higher than those in T2 and C groups, and serum PRL levels in T1 group was significantly higher than that in control group. Body weight of offsping rats in T1 group were significantly higher than that in control group at weeks 2 and 4 after birth, and body weight of offsping in T2 group were significantly higher than that in control group only at week 4. Oral *SS-14* DNA vaccine fused *tPA* signal peptide and *CpG* adjuvant and delivered by attenuated *Salmonella choleraesuis* was more effective than that oral *SS-28* DNA vaccine in promoting lactation of rats.

INTRODUCTION

Immunoneutralization against hormones have been considered as an effective method in improving animal growth and reproductive performance (Han *et al*., 2008; Liu *et al*., 2016; Dan *et al*., 2016). The active immunization against somatostatin (*SS*) has been proven to be very effective in improving animal growth performance (Wu *et al*., 2012; Liang *et al*., 2014). However, there have been only a few studies and controversy on the effect of active immunization against somatostatin on milk production. The active immunization against exogenous somatostatin can significantly increase milk production of mice or ewes by promoting the secretion of pituitary GH or PRL (Van Kessel *et al*., 1990; Bai *et al*., 2011). However, there are also some studies showing that immunization against somatostatin does not significantly promote animals' lactation (Yi *et al*., 1999; Kim *et al*., 2002).

In mammals, somatostatin is not species-specific and mainly contains two biologically active forms, *SS-14* and *SS-28* amino acids (Ding *et al*., 2014). *SS-28* is an N-terminally extended form of *SS-14* (Pradayrol *et al*., 1980). In the peripheral blood, the *SS-28,* but not *SS-14*, is the main form of somatostatin and it has a slower metabolic rate compared to *SS-14* (Patel *et al*., 1973; Tannenbaum *et al*., 1986). In addition, some studies showed that *SS-28* has a significantly longer acting than that *SS-14* in inhibiting spontaneous GH secretion (Tannenbaum *et al*., 1982, 1986). The principle of hormone immunoneutralization is that the antibody produced binds to endogenous hormone which results in that the endogenous hormone cannot bind to its receptor (Dan *et al*., 2016; Lei *et al*., 2017). In theory, when the amount of antibody is large enough, the immune effect produced by *SS-28* vaccine should be greater than that of *SS-14* vaccine, because that *SS-28* is the main form and has slower metabolic rate in the peripheral blood. Some studies have showed that the immunization against *SS-14* can improve animal lactation (Van Kessel *et al*., 1990; Bai *et al*., 2011), however, there are very few studies on the immunization effect against *SS-28* in enhancing

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animal lactation (Yi *et al*., 1999). Therefore, to further promote animal lactation performance, it is very necessary to compare the immunization effects of *SS-14* and *SS-28* on animal lactation.

Attenuated *choleraesuis* is an effective and low-cost tool for delivering *SS* DNA vaccines. In addition to being able to induce humoral and cellular immune responses, the DNA vaccine delivered by attenuated *choleraesuis* can also induce a long-lasting mucosal and systemic immune responses (Toussaint *et al*., 2013; Han *et al*., 2017; Chen *et al*., 2018). In addition, the DNA vaccine delivered by attenuated *choleraesuis* do not require protein antigen extraction and synthesis which is more convenient and cost effective (Lin *et al*., 2015; Liu *et al*., 2016). The oral *SS-14* DNA vaccine delivered by attenuated *Salmonella typhimurium* (CSO22) can significantly improve the lactation by enhancing the body weight of offspring mice at weeks 1 and 2 of lactation (Bai *et al*., 2011). However, the oral *SS* DNA vaccine contained a kanamycin antibiotic resistance gene which can cause the safety issue of antibiotic residue (Liang *et al*., 2014). In addition, the oral *SS* DNA vaccine by attenuated *Salmonella typhimurium* did not significantly enhance the weaning weight of offspring mice (week 3 of lactation) (Bai *et al*., 2011). *Salmonella choleraesuis* C500 with the *asd* and *crp* genes deleted has been widely used to construct non-antibiotic resistance DNA vaccines, in which the antibiotic resistance marker has been replaced by non-antibiotic resistance asd^+ balanced-lethal host-vector system which contains a complementing asd^+ expression plasmid (Han *et al*., 2014; Liang *et al*., 2014; Liu *et al*., 2016). Therefore, to further improve safety and efficiency of oral *SS* DNA vaccine in enhancing lactation, a non-antibiotic resistance and more effective oral *SS* DNA vaccines should be developed and applied.

To the best of our knowledge, the non-antibiotic resistance oral *SS-14* or *SS-28* DNA vaccines has not been applied to improve animals' lactation. Therefore, the study aims to compare the immunization effect of novel non-antibiotic resistance oral *SS-14* and *SS-28* DNA vaccines in enhancing lactation of female rats. The immunization effect of oral *SS* DNA vaccines against somatostatin 14 and 28 was evaluated by serum anti-somatostatin antibody, serum GH, PRL and IGF-1 levels, and offsping rats' weight of different lactation period.

MATERIALS AND METHODS

Vaccine construction

The *tPA* (tissue plasminogen activator signal peptide)-*CpG* (three 6 hexameric *CpG* motifs, 5′-TCGTCGTTTTGTCGTTTTGTCGTT -3′)-*HBsAg-S-2SS-14* (two copies of *SS-14* gene inserted into the hepatitis B surface antigen S gene)-*FLAG* (a protein label), *tPA-CpG-HBsAg-S-2SS-28* (two copies of *SS-28* gene inserted into *HBsAg-S* gene)-FLAG fusion genes and single *CpG* were synthesized chemically (Sangon Biotechnology Co., Ltd, Shanghai, China). The three genes were inserted separately into pVAX-asd plasmid (asd gene coding for aspartate-b-semialdehyde dehydrogenase), and then these recombinant plasmids (*pVAX-tPA-CpG-HBsAg-S-2SS-14-asd*, *pVAX-tPA-CpG-HBsAg-S-2SS-28-asd* and *pVAX-CpG-asd*) were electroporated, respectively into the attenuated *S. choleraesuis* C500 with the *asd* and *crp* genes deleted. These novel recombinant oral *SS-14* DNA vaccine C500 (*ptCS/2SS-14-asd*) and *SS-28* DNA vaccine C500 (*ptCS/2SS-28-asd*) (Fig. 1) were identified by PCR and sequencing.

Animals

Fifteen specific-pathogen-free (SPF) female SD rats aged seven weeks were purchased from Chongqing Academy of Chinese Materia Medica (Chongqing, China) and raised in the Southwest University Experimental Animals House (Chongqing, China) based on the guidelines of the Committee on the Care and Use of Laboratory Animals of China. One week after caging, these female rats were randomly divided into *SS-14* (Group T1), *SS-28* (Group T2) DNA vaccine groups and control group (Group C) for immunization and mating.

Immunization and sampling

All rats were orally pretreated with 1 mL sodium bicarbonate solution (7.5%) for 30 min before immunization, and then they were separately given orally with *SS-14*, *SS-28* DNA vaccines and empty vector vaccine at a dose of 5×10^9 CFU dissolved in 1 mL of sterile PBS. Subsequently, these rats were mated individually with adult male SD rats for one week. The immunization were boosted for twice at weeks 3 and 6 after primary immunization. Serum samples were obtained from eye socket before primary immunization (before mating) and at weeks 3 (first day after childbirth), 5 (week 2 after childbirth) and 7 (week 4 after childbirth) after primary immunization by centrifugation at 1157 × g for 10 min and stored at -20 °C for further use.

Detection of anti-SS antibodies

Specific anti-somatostatin antibodies were tested by an indirect enzyme-linked immunosorbent assay (ELISA) method (Bai *et al*., 2011). In brief, 96-well ELISA plates were coated with 100 ng/well of *SS-14* or *SS-28* antigen diluted in a bicarbonate buffer (pH 9.6) at 4°C for overnight. After washing three times with PBST (0.05% Tween-20 in a phosphate buffer saline),

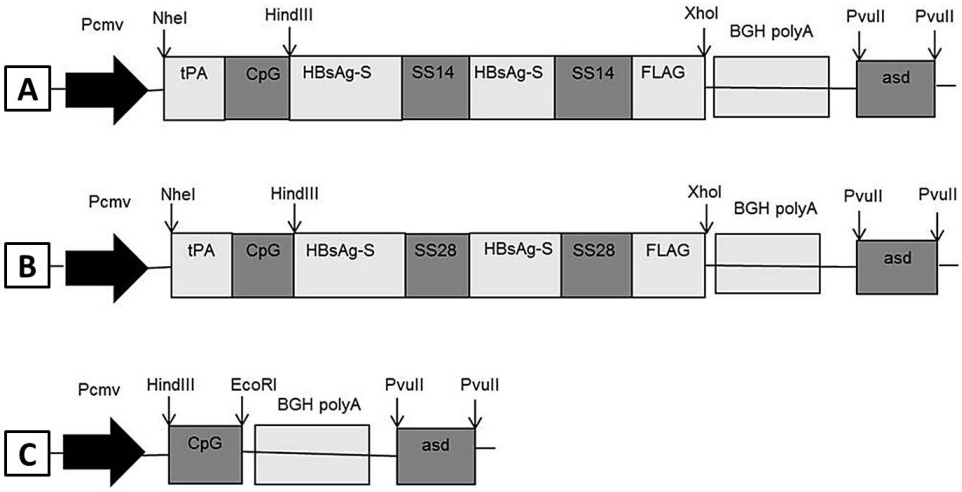

Fig. 1. Map of construction of oral somatostatin (*SS*) DNA vaccines encoding different lengths somatostatin and delivered by attenuated *Salmonella choleraesuis* C500. A, Map of construction of oral *SS-14* DNA vaccine fused *tPA* signal peptide and *CpG* adjuvant named C500 (*ptCS/2SS-14-asd*); B, Map of construction of oral *SS-28* DNA vaccine fused *tPA* signal peptide and *CpG* adjuvant named C500 (*ptCS/2SS-28-asd*); C, Map of construction of oral empty vector vaccine fused *CpG* adjuvant named C500 (*pCpG-asd*).

these samples were blocked with 1% bovine serum albumin dissolved PBST at 37 °C for 1 h. Serum samples of tested rat were serially 2-fold diluted in PBST from 1:25 up to 1:3200, and then each well were added to 100 µl for incubating at 37 °C for 1 h. Negative control serum sample from preimmune rats were used. Specific *SS* bound antibodies were tested by adding horseradish peroxidase-labelled rabbit anti-rat IgG secondary antibodies (Abbkine, Inc., Redlands, CA, USA) diluted in PBST (1:5000) and incubated subsequently at 37 °C for 1 h. Tetramethylbenzidine substrate was used for the enzyme reaction by incubating the samples at 37 °C for 25 min. 2 MH_2SO_4 was applied to stop the reaction and the absorbance was detected at 450 nm by the Bio-Rad iMark Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA). End-point titers of these samples were determined as reciprocal of the highest serum dilution in which the absorbance was greater than the mean plus two standard deviations of negative control samples at the same dilution (Tannenbaum *et al*., 1982, 1986).

Detection of serum hormone levels

Serum GH, PRL and IGF-1 concentrations in rats were detected by radioimmunoassay (Beijing Sino-UK Institute of Biological Technology, Beijing, China). The intra-assay and inter-assay coefficients of variation were less than or equal to 15%, respectively.

Detection of lactation

One day after primary immunization, adult male rats were put into individually the cage to mate with the vaccinated female rats. Following parturition, the number of offspring rats of per litter was reduced to eight (four females and four males), and weaned after 4 week. Body weight of offspring rats from *SS-14*, *SS-28* DNA vaccine groups and control group was measured at weeks 0 (first day after childbirth), 2 and 4 after birth to compare lactation performance.

Statistical analysis

The differences between groups in terms of anti-*SS* antibody titers, serum GH, PRL and IGF-1 levels, and body weight of offspring rats were analyzed by one-way ANOVA and Duncan's test of SAS 8.1 analytical software (SAS Institute, Inc., Cary, NC, USA). $p < 0.05$ was considered as statistically significant and all the data was expressed as mean ± SD.

RESULTS

Vaccine designing and identification

Oral *SS* DNA vaccines encoding polypeptides of different lengths somatostatin was engineered which contained two copies of *SS-14* or *SS-28* gene, *tPA* signal peptide and *CpG* motifs (Fig. 1). The recombinant *SS-14* and *SS-28* DNA vaccines were identified by PCR (Fig. 2) and sequence analysis (data not shown). There was a 1086 bp amplified band in the electrophorogram after amplifying the *SS-28* DNA vaccine (Lane 1, Fig. 2), and there was a 1002 bp amplified band after amplifying the

SS-14 DNA vaccine (Lane 2, Fig. 2), suggesting that the recombinant oral *SS-14* and *SS-28* DNA vaccines was successfully developed.

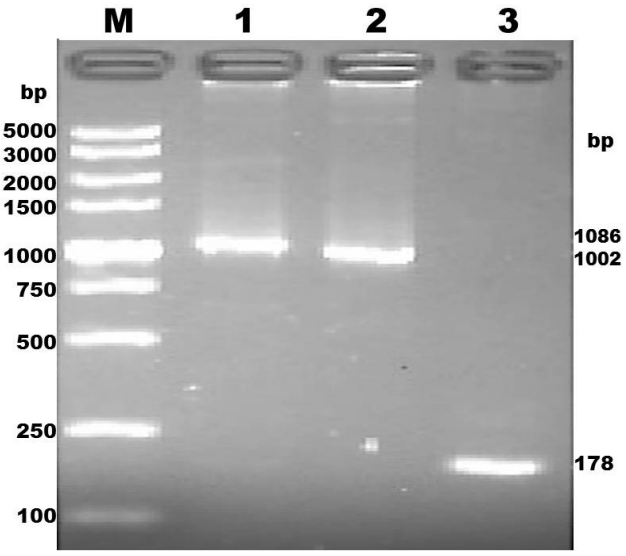

Fig. 2. Identification of recombinant *SS* DNA vaccines encoding different lengths somatostatin and empty vector vaccine by PCR. Lanes 1, 2 and 3, C500 (*ptCS/2SS-28-asd*), C500 (*ptCS/2SS-14-asd*) and C500 (*pCpG-asd*) recombinant DNA vaccines were amplified with *T7* and *BGH* primers; Lane M, DL5000 DNA marker. Three bands shown in Lanes 1, 2 and 3 are *tPA-CpG-HBsAg-S-2SS-28-FLAG* (1086 bp), *tPA-HBsAg-S-2SS-14-FLAG* (1002 bp) and *CpG* (178 bp) fragment, respectively.

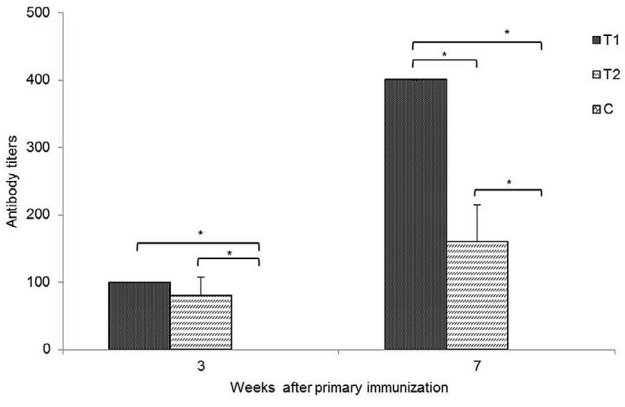

Fig. 3. Anti-*SS* antibodies titers in T1, T2 and C groups immunized with C500 (*ptCS/2SS-14-asd*), C500 (*ptCS/2SS-28-asd*) and C500 (*pCpG-asd*), respectively at weeks 3 (first day after childbirth) and 7 (week 4 after childbirth) after primary immunization. Vaccinations were respectively orally given at weeks 0, 3 and 6 weeks after primary immunization. Data are presented as means ± SD. *p < 0.05.

Anti-SS antibody response

SS-specific antibody titers in both *SS-14* and *SS-28* DNA vaccine groups were significantly higher than that in control group at weeks 3 and 7 after primary immunization (Fig. 3, $p < 0.05$). However, *SS*-specific antibody titers in *SS-14* DNA vaccine group were significantly higher than that in *SS-28* DNA vaccine group at week 7 after primary immunization (Fig. 3, $p < 0.05$).

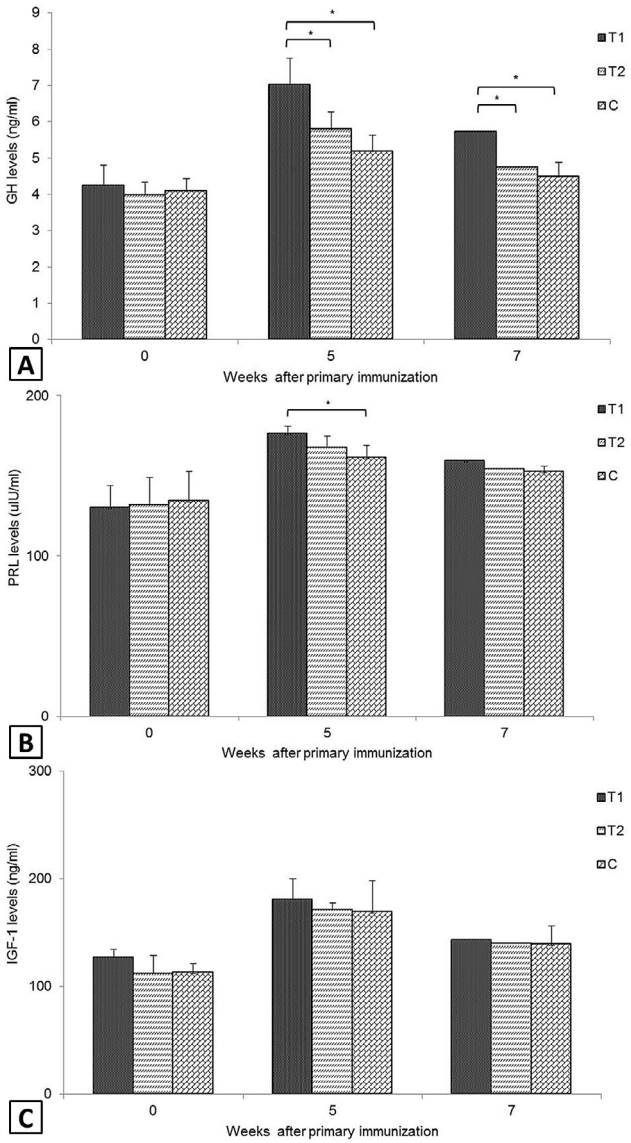

Fig. 4. Serum GH (A), PRL (B) and IGF-1 (C) concentrations (ng/mL, uIU/mL and ng/mL, respectively) in T1, T2 and C groups immunized with C500 (*ptCS/2SS-14-asd*), C500 (*ptCS/2SS-28-asd*) and C500 (*pCpG-asd*), respectively at weeks 0 (before mating), 5 (week 2 after childbirth) and 7 (week 4 after childbirth) after primary immunization. Data are presented as means ± SD. *p<0.05.

Serum GH, PRL and IGF-1 levels

Serum GH concentrations in *SS-14* DNA vaccine group were significantly higher than that in *SS-28* DNA vaccine group and control group at weeks 5 and 7 after primary immunization (Fig. 4A, $p < 0.05$). However, no significant difference was observed on serum GH concentrations between *SS-28* DNA vaccine group and control group at weeks 0, 5 and 7 after the primary immunization (Fig. 4A, $p > 0.05$).

Serum PRL concentrations in *SS-14* DNA vaccine group were significantly higher than that in control group at week 5 after primary immunization (Fig. 4B, $p < 0.05$). However, there was no significant difference on serum PRL concentrations between *SS-28* DNA vaccine group and control group at weeks 0, 5 and 7 after the primary immunization (Fig. 4B, $p > 0.05$).

There was no significant difference on serum IGF-1 concentrations between *SS-14* DNA vaccine group and *SS-28* DNA vaccine group or control group at weeks 0, 5 and 7 after the primary immunization (Fig. 4C, $p > 0.05$). In addition, there was also no significant difference on serum IGF-1 concentrations between *SS-28* DNA vaccine group and control group at weeks 0, 5 and 7 after the primary immunization (Fig. 4C, $p > 0.05$).

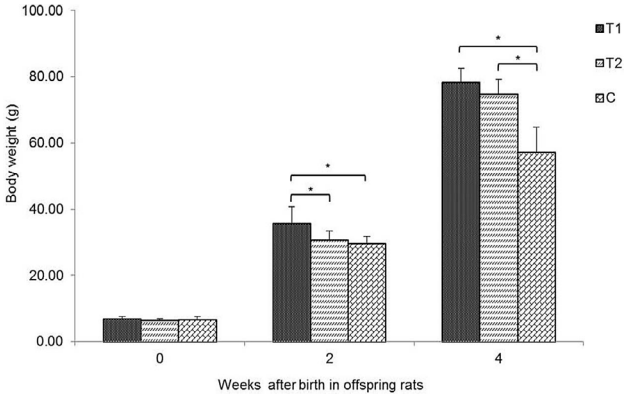

Fig. 5. Body weight (g) of offspring rats in T1, T2 and C groups at weeks 0 (first day after childbirth), 2 (week 2 after childbirth) and 4 (week 4 after childbirth) after birth. Data are presented as means ± SD. *$p < 0.05$.

Effect of immunization on lactation

There were no significant difference on birth weight of offspring rats among groups at first day after childbirth. Body weight of offspring in *SS-14* DNA vaccine group was significantly higher than that in control group at weeks 2 and 4 after childbirth, and was significantly higher than that in *SS-28* DNA vaccine group at week 2 after childbirth (Fig. 5, $p < 0.05$). However, body weight of offspring rats in *SS-28* DNA vaccine group was significantly higher than that in control group only at week 4 after childbirth (Fig. 5, $p < 0.05$).

DISCUSSION

Long-term active immunoneutralization of somatostatin is a potential method of improving animal lactation performance. GH/IGF-I axis and PRL play a vital role in the development of the mammary gland and animal lactation and they are mainly regulated by GH-releasing hormone and somatostatin (García-Tornadú *et al*., 2006; Villa-Osaba *et al*., 2016; Lékó *et al*., 2017). Somatostatin exerts an inhibitory effect on the secretion of pituitary GH and PRL and consequently animal lactation (Vale *et al*., 1974; Luque *et al*., 2016). Somatostatin is mainly consisted of two biologically active components somatostatin-14 and somatostatin-28 (Ding *et al*., 2014). Although somatostatin-14 DNA vaccine delivered by attenuated *Salmonella typhimurium* improved the mice lactation performance, the oral somatostatin DNA vaccine used a kanamycin antibiotic resistance gene as a selection marker which can cause the safety issue of antibiotic residue (Liang *et al*., 2014), and the humoral response induced by the oral somatostatin DNA vaccine is still weak (Bai *et al*., 2011). Somatostatin-28 has a significantly longer acting than that somatostatin-14 in inhibiting spontaneous GH secretion (Tannenbaum *et al*., 1982, 1986), however, there are very few studies on the lactation-promoting effect against *SS-28* DNA vaccine. *tPA* signal peptide and *CpG* adjuvant and can effectively improve the DNA vaccines' immunogenicity (Wang *et al*., 2011; Li *et al*., 2016), however, they have not been widely used in the construction of oral somatostatin DNA vaccines. Therefore, to improve the safety and efficacy of somatostatin DNA vaccine in enhancing animal lactation, we developed the novel non-antibiotic resistance *SS-14* and *SS-28* DNA vaccines fused *tPA* signal peptide and *CpG* adjuvant and and delivered by attenuated *S. choleraesuis* C500 deleted the *asd* and *crp* genes.

In this study, the novel oral *SS-14* DNA vaccine delivered by attenuated *S. choleraesuis* C500 induced stronger humoral immune response and subsequently more effective stimulation of GH and PRL secretion than that oral *SS-28* DNA vaccine. Both the anti-*SS* antibody titers in the oral *SS-14* and *SS-28* DNA vaccines was significantly higher than that in control group. In the study of lactation promotion, previous studies showed that immunization of only *SS-14* vaccine can induce humoral immune response in lactation studies (Yi *et al*., 1999; Bai *et al*., 2011). However, our results indicated that the immunization of both oral *SS-14* DNA vaccine and *SS-28* DNA vaccine can induce a strong humoral immune response. The anti-

SS antibody titers in oral *SS-14* DNA vaccine group was significantly higher than that in oral *SS-28* DNA vaccine group and control group. The result indicated that oral *SS-14* DNA vaccine induced stronger humoral immune response than oral *SS-28* DNA vaccine. The antibody titer in oral *SS-14* and *SS-28* DNA vaccines delivered by attenuated *S. choleraesuis* C500 was significantly higher than that in control group from day after childbirth to week 4 after childbirth, however, Bai *et al*. (2011) study showed that the antibody titer in oral *SS-14* DNA vaccine delivered by attenuated *Salmonella typhimurium* significantly higher than that in control group only from week 2 after the booster immunization to the day after childbirth, and there was no significant difference in antibody titer between oral *SS-14* DNA vaccine group and control group at week 3 after childbirth. The result indicated that novle oral *SS-14* and *SS-28* DNA vaccines fused *tPA* signal peptide and *CpG* adjuvant and delivered by attenuated *S. choleraesuis* C500 can induced stronger immune response than that oral *SS-14* DNA vaccine delivered by attenuated *Salmonella typhimurium*. At weeks 5 and 7 after primary immunization, the GH concentration in the *SS-14* DNA vaccine group was significantly higher than that in the *SS-28* DNA vaccine group and control group, however, there was no significant difference in GH concentration between oral *SS-28* DNA vaccine group and control group. Meanwhile, the PRL concentration in the *SS-14* DNA vaccine group was significantly higher than that in control group, however, there was no significant difference in PRL concentration between oral *SS-28* DNA vaccine group and control group. Previous studies also showed that immunization of *SS-14* DNA vaccine can stimulate the secretion of GH and PRL by the immunoneutralization of anti-*SS* antibody induced (Hugues *et al*., 1986; Bai *et al*., 2011; Liang *et al*., 2014). These results indicated that immunization of the novel oral *SS-14* DNA vaccine may stimulate the secretion of GH and PRL more effectively than that novel oral *SS-28* DNA vaccine. Interestingly, there was no significant difference in IGF-1 concentration between oral *SS-14* DNA vaccine and control group or between oral *SS-28* DNA vaccine and control group, which may be because that the GH levels produced was not high enough or the shoter half-life of plasma IGF-1 (Guler *et al*., 1989; Carro *et al*., 2000). However, lower GH levels still can promote the mammary development and lactation by the binding to the GH receptor or PRL receptor (Ng *et al*., 1997; Xu *et al*., 2013).

Immunization of novel *SS-14* DNA vaccine improved the weight of offspring rat during lactation more effectively than that *SS-28* DNA vaccine. The body weight of offspring in *SS-14* DNA vaccine was significantly higher than that in *SS-28* DNA vaccine group and control group at week 2 after childbirth which was in middle stage of lactation, and the body weight of offspring in *SS-14* DNA vaccine was still significantly higher than that in control group at week 4 after childbirth which was at weaning (late stage of lactation). Bai *et al*. (2011) study showed that oral *SS-14* DNA vaccine delivered by attenuated *Salmonella typhimurium* can significantly improve the weight of offspring mice in the middle stage of lactation, however, our studies showed that oral *SS-14* DNA vaccine can significantly improve the weight of offspring rats in the middle and late stage of lactation, which indicated that oral *SS-14* DNA vaccine fused *tPA* signal peptide and *CpG* adjuvant and delivered by attenuated *S. choleraesuis* C500 can promote animal lactation more permanently than that oral *SS-14* DNA vaccine delivered by attenuated *Salmonella typhimurium*. Interestingly, the body weight of offspring rats in *SS-28* DNA vaccine group was significantly higher than that in control group at weaning, however, there was no significant difference in the GH and PRL levels between the *SS-28* DNA vaccine group and control group. In the late stage of lactation, the offspring need to maintain the growth through diet and lactation (Daneshvar *et al*., 2015). Therefore, the significant difference of the body weight of offspring rats between *SS-28* DNA vaccine group and control group may be due to the differences of individual feeding and nutrition of the offspring rats at weaning. These results indicated the oral *SS-14* DNA vaccine fused *tPA* signal peptide and *CpG* adjuvant and delivered by attenuated *S. choleraesuis* C500 was more effective than that *SS-28* DNA vaccine in promoting lactation of rats.

CONCLUSION

The oral *SS-14* DNA vaccine fused *tPA* signal peptide and *CpG* adjuvant and and delivered by attenuated *S. choleraesuis* C500 can induce stronger humoral immune response and subsequently promote rat lactation more effectively than that oral *SS-28* DNA vaccine. Further studies should be conducted to study the effect molecular mechanism and of oral *SS* DNA vaccine on milk composition.

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Statement of conflict of interest

The authors declare no conflict of interest.

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