Assessments of immune response to vaccines of transmissible gastroenteritis virus inactivated by different agents

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Research

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Abstract

Background: Transmissible gastroenteritis virus (TGEV) could cause swine enteric infection, which is characterized by vomiting, severe diarrhea and dehydration, and the mortality in suckling piglets is often high up to 100%. Vaccination is an effective measure to control the disease caused by TGEV.

Methods: In this study, cell-cultured TGEV HN-2012 strain was inactivated by formaldehyde (FA), \( \beta \)-propiolactone (BPL) or binaryethylenimine (BEI), respectively. Then the inactivated TGEV vaccine was prepared with freund's adjuvant, and the immunization effects were evaluated in mice. The TGEV-specific IgG level was detected by ELISA.

Results: The results showed that the FA group (n=17) developed earlier and stronger TGEV-specific IgG level, while the BEI group could produce much longer-term IgG level. Lymphocyte proliferation test demonstrated that the BEI group showed a stronger inducibility of spleen lymphocyte proliferation. The BEI group got higher positive rates of CD4\(^+\) and CD8\(^+\) T lymphocyte subsets of peripheral blood lymphocyte than that of the FA and BPL groups through flow cytometry assay. The BPL group had the highest positive rate of CD4\(^+\)IFN-\(\gamma\)^+ T lymphocyte subset, while the positive rate of CD4\(^+\)IL-4^+ T lymphocyte subset was got the best in FA group. Moreover, there were no obvious pathological changes between the vaccinated mice and control group by the macroscopic and histopathological examination.

Conclusions: These results indicated that the three experimental groups both induced cellular and humoral immunities, and the FA group had better effect on humoral immunity, while the BEI group showed its excellent effect on cellular immunity.

Introduction

Transmissible gastroenteritis virus (TGEV) is an enveloped, positive, single-stranded RNA virus, which belongs to the \textit{Alphacoronavirus} genus, \textit{Coronaviridae} family. TGEV causes acute enteric disease of swine that characterized by vomiting, severe diarrhea and dehydration. The mortality of TGEV often reaches 100% in suckling piglets less than two weeks of age, and causes huge economic losses in pig industry around the world \cite{1,2}. Until now, there is no effective drug for treating TGEV infection, and in order to control the disease caused by TGEV, vaccination should be the effective measure \cite{3,4}.

To prevent TGEV infection, several vaccine technologies had been developed, including inactivated vaccine, attenuated vaccine, DNA vaccine, recombinant vaccine and vectored vaccine, even multiple vaccines which often combined with rotavirus and porcine epidemic diarrhea virus (PEDV) \cite{5,6}. For inactivated vaccines, viruses are completely inactivated with chemicals with an excellent safety, as well as good tolerance and few adverse reactions \cite{7}. Thus, the virus inactivation is a crucial step in production of vaccine, which need inhibit the replication of the virus without altering its antigenicity.

The commercial inactivated TGEV vaccines were mainly inactivated by formaldehyde (FA), which is a traditional inactivating agent that frequently used in many vaccines. FA mainly focuses on viral proteins
[8], and results in the alkylation of amino and affects the fusion ability of viruses. β-propiolactone (BPL) is another inactivating agent that mainly attacks nucleic acids, thus would not change the antigenic component of viruses [9]. In addition, some studies showed that BPL also could affect the viral proteins [10]. Comparing the two inactivating agents on Newcastle disease virus (NDV) or influenza virus indicated that the potencies of FA and BPL inactivated vaccines were different [11, 12]. In case of binary-ethylenimine (BEI), its inactivation mechanism is similar to BPL and reacts very little with viral proteins. However, some studies showed that BEI could better preserve the conformation and accessibility of viral epitopes than FA and BPL [10].

In the present study, three different inactivating agents (FA, BPL, BEI) were used to inactivate the TGEV HN-2012 strain. The immunogenicity of the inactivated TGEV vaccines in mice was evaluated, and these data may provide an assessment of different inactivating agents on vaccines.

**Materials And Methods**

**Cells and virus**

Swine testis (ST) cells were maintained in Dulbecco's modified eagle medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco) at 37°C in a CO₂ incubator and used to serially passage and propagate TGEV. The monolayers of ST cells were maintained in DMEM with 0% FBS and TGEV were propagated by inoculating at a multiplicity of infection (MOI) of 0.1. The virulent TGEV used in our research was the passage 15 (P15) of TGEV HN-2012 strain that isolated and identified by our laboratory. The propagated TGEV culture was harvested when the cytopathic effect (CPE) was >80%, and the virus titer was determined to be 10^{-8.0} /0.1 mL. The cell debris were removed by 8 000 rpm centrifugation for 30 min at 4°C and then filtered by 0.2µL pore size filter.

**Inactivation protocols of TGEV**

Three inactivating agents were selected (FA, BPL and BEI) in our study. For FA inactivation agent, 40% FA (Sinopharm Chemical Reagent Co, Ltd) was used at a final concentration of 0.1%, 0.2%, 0.3% (v/v) respectively. Different concentrations of FA were incubated with TGEV at 37°C and collected after 6, 12, 18, 24, 30 and 36h, respectively. The reactions were stopped by adding sterilized 1M sodium thiosulfate (Sinopharm Chemical Reagent Co, Ltd) at the concentration 10 times of the FA final concentration. For BPL (Acros Organics, Geel, Belgium), three concentrations of 0.01%, 0.02%, 0.03% (v/v) were used as the final concentrations respectively. The three concentrations of BPL were incubated with TGEV at 4°C and were collected after 6, 12, 18, 24, 30 and 36h, respectively. The reactions were stopped by adding sterilized 1M sodium thiosulfate (Sinopharm Chemical Reagent Co, Ltd) at the concentration 10 times of the BPL final concentration. For BEI (Sigma-Aldrich, USA) was dissolved in 0.2 mol/L NaOH (Sinopharm Chemical Reagent Co, Ltd) to get the final concentration of 0.1 M of BEA. The solution was then incubated at 37°C for 1 h and BEI was formed. The three concentrations of 0.03%, 0.04%, 0.05% (v/v) of BEI were incubated with TGEV at 37°C and were collected after 6, 12, 18, 24, 30 and 36h, respectively. The reactions were stopped by adding sterilized 1M sodium thiosulfate (Sinopharm Chemical Reagent Co, Ltd) at the concentration 10 times of the BEI final concentration.
thiosulfate at the concentration of 10 times of the BEI final concentration. The experiments were performed 3 times.

**Tests of infectivity and sterility of TGEV**

In order to determine the infectivity of TGEV after three inactivation reagents treatment, inactivated TGEV was collected and cultured in ST cells for three passages. The CPE was observed and TCID$_{50}$ titers were calculated. Untreated TGEV was used as the positive control, and DMEM was used as the negative control. Sterility tests were conducted in common nutrient agar and ordinary broth. Bacterial growth was observed on the culture medium after 30 h at 37°C.

**Preparation of inactivated TGEV vaccine**

To produce the inactivated TGEV vaccine, the inactivated viral antigens were emulsified with Freund's complete adjuvant (Sigma-Aldrich, USA) at a ratio of 1:1 (v/v), and this vaccine was used for the first immunization of mice. For the second and the third immunization, the inactivated TGEV antigen was emulsified with Freund's incomplete adjuvant (Sigma-Aldrich, USA) at a ratio of 1:1 (v/v).

**Vaccines immunization of mice**

To detect the immunization effects of vaccines, sixty-eight female healthy 6-8-week-old BALB/c mice were purchased from the Henan Province Laboratory Animal Management Committee in China. Before injection, all mice were detected by the enzyme-linked immunosorbent assay (ELISA) kit (Wuhankeqian Animal Biological Products Co., Ltd.) to make sure the TGEV antibody was negative. The mice were then randomly divided into 4 groups (n = 17/group), and housed separately. Mice were subcutaneously injected into the backs with 200μL of TGEV vaccine inactivated with FA (group 1), 200 μL of TGEV vaccine inactivated with BPL (group 2), 200 μL of TGEV vaccine inactivated with BEI (group 3), and 200 μL PBS (group 4) as the negative control. In this research, vaccine injections were performed three times at two-week intervals. Animal experiments in this study were carried out in accordance with the Health guide for the care and use of Laboratory animals of Henan Agricultural University.

**Detection of TGEV-specific IgG by indirect ELISA**

Blood samples were randomly collected from 5 mice in each group at 0, 1, 2, 3, 4, 5, 6, 7 and 8 week after the initial immunization, and placed at 37°C for 1 h. After centrifugation at 3500 rpm for 10 min, the serum from each mouse was collected. IgG was detected by TGEV antibody-IgG ELISA kit (Wuhankeqian Animal Biological Products Co., Ltd.). All the steps in the kits were followed, and the optical density (OD) 450 was measured. When OD$_{450}$ values of the experimental groups were greater than or equal to 2.1 times of the values of the control group, the samples considered to be positive. Values $\leq 0.05$ were excluded.

**Detection of CD4$^+$, CD8$^+$ T lymphocytes**
300 μL of blood samples were randomly collected from eyeballs of 3 mice in each group at 21 day post-inoculation (dpi) and 35 dpi of the first immunization, respectively. The positive rates of CD4+, CD8+ T lymphocyte subsets were analyzed by flow cytometry. Briefly, 3 mL volume of red blood cell lysis buffer (Solarbio) was added to each sample to crack red blood cells totally. Then the samples were washed and re-suspended with DMEM to 1×10^6 cells/mL. Cells were transferred to 48-well plates with 100μL volume of each well, 2μL of cell activation cocktail (Bio legend) and 1μL of BrefeldinA (Bio legend) were added to each sample and incubated at 37°C for 6h. After washed and re-suspended with PBS, samples were incubated with specific fluorescent antibodies (Bio legend) of Brilliant Violet 510 (BV510) conjugated anti-mouse CD3 antibody (0.4 μg/sample), PerCP/Cyanine 5.5 conjugated anti-mouse CD4 antibody (0.2 μg/sample), and fluorescein isothiocyanate (FITC) conjugated anti-mouse CD8a antibody (1 μg/sample) for 30 min at room temperature in the dark according to the manufacturer’s guidelines. All samples were stained in triplicate. The samples were analyzed by flow cytometry with a BD FACS Canto plus (BD Biosciences, US). Data were analyzed using Canto diva software (BD Biosciences, US).

**Detection of CD4+IFN-γ+, CD4+IL-4+ T lymphocytes**

To further determine the levels of CD4+IFN-γ+, CD4+IL-4+ T lymphocyte subsets, cells treated in 2.7 were fixed with 500 μL of 4% FA for 20 min in the dark, ruptured with 1 mL of Permeabilization Wash Buffer (Bio legend). Then the cells were stained with allophycocyanin (APC) conjugated anti-mouse IFN-γ antibody (0.8 μg/sample), phycoerythrin (PE) conjugated anti-mouse IL-4 antibody (0.2 μg/sample) (Biolegend) for 30 min. All samples were stained in triplicate. These samples were analyzed by flow cytometry, and data were analyzed.

**Lymphocyte proliferation assay**

At 14, 21 and 35 dpi, lymphocytes were isolated randomly from the spleens of three mice in each group. The protocol of lymphocyte isolation from spleen was modified from the previous study [13]. And the lymphocytes were re-suspended in DMEM and adjusted to 1.0×10^6 /mL. Cells were cultured in 96-well flat-bottom plates for 100μL per well, and stimulated with concanavalin A (ConA, Sigma) with the final concentration of 50 μg/mL and 20μL of inactivated TGEV antigen (1×10^5 TCID_{50}/mL) in each well, respectively. DMEM was used as a negative control. All treatments were in triplicate. The plates were incubated at 37°C for 20 h, and then added methylthiazoltetrazolium (MTT) (5 mg/mL) with 10 μL/well, and incubated the cells further at 37°C for 4 h. 100 μL of 10% dimethyl sulfoxide (DMSO, Solarbio) was added to stop the reaction. The OD$_{492}$ value of the solution was determined. The stimulation index (SI) was calculated with the following formula: SI = (OD sample well – OD blank well)/(OD negative well – OD blank well).

**Gross pathology and histopathology**

At 35 dpi, 3 mice in each group were randomly selected and killed. The major organs which including heart, liver, spleen, lung, kidney, small intestines (jejunum and ileum) and muscles of back that injected
with vaccine were examined grossly, and then fixed by 10% formalin for 48 h. To evaluate whether the vaccine had adverse reactions to mice, these fixed tissues were stained with Mayer’s H.E for histopathological examination.

**Statistical analysis**

Data of the three experimental groups and control group were evaluated by SPSS 17.0 software, and error bars represented standard deviations. The results were unpaired two-way analysis of variance (ANOVA). The results were expressed as mean ± standard deviation (SD), with $P$-values<0.05, $P$-values<0.01 and $P$-values<0.001 considered to be statistically high, significantly high and extremely high, respectively.

**Results**

**Inactivation of TGEV with FA, BPL and BEI**

In order to compare the effects of three different inactivating agents, TGEV HN-2012 strain was divided into independent batches. The different concentrations of FA, BPL and BEI were treated with TGEV at different time, and the untreated virus was used as a positive control. TCID$_{50}$ titers were calculated on ST cells for three passages to determine the infectivity of TGEV after inactivation. As shown in Table.1, three final concentrations of 0.1%, 0.2% and 0.3% of FA inactivation agent were used and all of them could completely inactivate the TGEV at 18 h. For BPL inactivation agent, both of the final concentrations of 0.01% and 0.02% could inactivate the TGEV at 12 h, while the final concentration of 0.03% could inactivate the TGEV at 6 h. In case of BEI, the final concentration of 0.03% could inactivate the TGEV at 12h. And another two final concentrations of 0.04% and 0.05% could inactivate the TGEV at 6 h. Based on these results, we chose the final concentration of 0.2% of FA with 24 h inactivation of TGEV, the final concentration of 0.03% of BPL with 18h inactivation of TGEV, and the final concentration of 0.04% of BEI with 12h inactivation of TGEV to carry out the follow-up experiments, respectively. Sterility tests were conducted in common nutrient agar and ordinary broth at 37°C for 30 h, and no bacterial growth was observed on the culture medium.

**Detection of TGEV-specific IgG by ELISA**

TGEV-specific IgG antibody in serum was detected by ELISA in each group at weekly intervals. As shown in Fig.1, FA group, BPL group and BEI group all produced IgG antibody after immunization when compared with the PBS control group. The TGEV-specific IgG antibody increased significantly after three weeks of the first immunization. The level of TGEV-specific IgG antibody in FA group peaked at 49 dpi. In BPL group, the IgG antibody titer reached the highest level at 35 dpi. In the BEI group, the IgG antibody titer peaked at 56 dpi. Above all, the FA group produced earlier and stronger IgG than that of BEI and BPL groups, while the BEI group could produce much longer-term IgG than that of FA and BPL groups.

**Analysis of CD4$^+$, CD8$^+$T lymphocytes**
At 21 dpi and 35 dpi of the first immunization, blood samples were randomly collected from mice in each group, respectively. The positive rates of CD4⁺, CD8⁺ T lymphocyte subsets were analyzed by flow cytometry (Fig.2). The results showed that the positive rates of CD4⁺ T lymphocyte subsets in FA group, BPL group and BEI group were 44.4±2.902, 44.3±3.661 and 46.3±1.178%, respectively, which a little higher than that of the control group (P>0.05) at 21 dpi. At 35 dpi, the positive rate of CD4⁺ T lymphocyte subset in BEI group was 55.3±9.874% and reached the highest level (*P<0.05) (Fig.2A). The positive rates of CD8⁺ T lymphocyte subsets in FA group, BPL group and BEI group were also higher than the control group, and the BEI group reached the highest compared to other groups (Fig.2B). At 21 dpi, the positive rate of CD8⁺ T lymphocyte subset was 21.3±5.084% in BEI group. And at 35 dpi, the value was 24.93±7.239% in BEI group, which had a significant difference compare with the control group (*P<0.05).

**Analysis of CD4⁺IFN-γ⁺, CD4⁺IL-4⁺ T lymphocytes**

At the 21 and 35 dpi of the first immunization, blood samples were randomly collected from mice in each group, respectively. The positive rates of CD4⁺IFN-γ⁺ T lymphocyte subsets were higher in FA group and BPL group than the control group at 21 dpi, while the BEI group was a little lower than the control group (Fig.3A). At 35 dpi, all experiment groups were higher than the control group. Moreover, the BPL group was the highest both at 21 dpi and 35 dpi with the percentages of 2.77±0.45 and 3.75±0.25%, respectively. For the CD4⁺IL-4⁺ T lymphocyte subsets (Fig.3B), the FA group was the highest group with the percentages of 2.5±1.406%, and had a significant difference with the control group at 35 dpi (*P<0.05).

**Result of spleen lymphocyte proliferation**

Spleens of three mice in each group were collected at 14, 21 and 35 dpi of the first immunization, respectively. The effects of spleen lymphocyte proliferation were analyzed by MTT assay and the data shown in Fig.4. The results indicated that at 14 dpi, the three experimental groups stimulated with inactivated TGEV antigen had no significant differences compared with control group. At 21 dpi and 35 dpi, the stimulating effects of FA group, BPL group and BEI group all increased significantly when compared with the negative control group (**P<0.001). In the entire immune program, the SI values of the BEI group were higher than other two experimental groups, which indicated that the BEI group showed a stronger inducibility of spleen lymphocyte proliferation.

**Macroscopic and histopathological examination of the vaccinated mice**

Three mice were randomly selected in each group and euthanized at 35 dpi for macroscopic and histopathological examination. The macroscopic examination results showed that the collected tissues, including heart, liver, spleen, lung, kidney, small intestines (jejunum and ileum) and muscles of back from the mice injected with vaccine were no change compare with the control group. These tissues were also investigated through H.E staining assay, and no obvious pathological changes were found in the vaccinated mice.
Discussion

TGEV targets the villous and crypt enterocytes of the small intestine and induces severe watery diarrhea and results in high mortality in pigs less than two weeks of age [14]. The administration of vaccines is an important way for preventing and controlling the diseases in animals. In recent years, TGEV had decreased in many countries of the world. However, TGEV is still outbreak in swine farms in some Asia countries such as China and Korea. At present, different types of vaccines are available. DNA vaccines of TGEV were constructed and showed well immunogenicity of humoral, mucosal, and cellular immunities in piglets [15-18]. Inactivated TGEV vaccines with adjuvants of CpG DNA or nano silicon were enhanced at the humoral and cellular immune responses [3, 19]. Among these vaccines, commercial vaccines, including inactivated and attenuated vaccines, have been widely used in China, and the inactivated vaccine is the better choice for its excellent safety and has been given higher priority in the pig industry.

Inactivation procedures should not affect the immunogenicity of the viral antigen. In vitro potency of FA inactivated NDV vaccine was lower than that of BPL inactivated NDV [11]. Inactivated influenza vaccine with BPL resulted in undetectable infectivity levels, while FA-treated virus retained very low infectious titers. BPL inactivated influenza virus induced higher levels of activation of TLR7 than that of FA-inactivated virus [12]. Since the mode of action of FA, BPL and BEI is different, in this study, the three inactivating agents were prepared to inactivate the TGEV HN-2012 strain, and the effects of the three inactivating agents were assessed by TGEV-specific IgG, the positive rates of CD4+, CD8+ T lymphocyte subsets, CD4+IFN-γ+, CD4+IL-4+ T lymphocyte subsets, lymphocyte proliferation and the histopathological examination. Inactivation protocols were optimized by different concentrations and time lengths of FA, BPL and BEI, and the immunogenicity and safety of the vaccines were tested in mice. The commercial inactivated vaccines of TGEV were mainly inactivated by FA, and for the purpose to compare the three inactivated agents, we did not set commercial inactivated TGEV vaccines as a control group.

It is clear that the level of IgG antibody is an important indicator to evaluate the effect of the vaccine. In our study, three experimental groups induced specific TGEV-IgG antibodies in serum of mice after immunization. These results showed that the TGEV inactivated vaccines induced humoral immunity effectively and efficiently. The specific IgG antibody induced by the vaccines were higher than those in the control group at 14 dpi (P>0.05). At 21 dpi, the differences were statistically significant to control group (***P<0.001), and the IgG levels of TGEV antibodies in three experimental groups increased between 14-56 dpi. In addition, the values of the FA group peaked at 49 dpi and decreased thereafter, the values of the BPL group peaked at 35 dpi and then decreased, and the values of the BEI group peaked at 56 dpi. These results showed that the FA and BEI groups were better in eliciting humoral immune response to TGEV than the BPL group.

Inactivated vaccine can induce T lymphocyte proliferation within the body, and the change of T lymphocyte ratios could reflect the state of cellular immune response. In this study, spleen-derived lymphocytes from immunized mice clearly showed that immunization with vaccine significantly induced T cell proliferation. T lymphocyte proliferation induced by the BEI group was higher than other two
experimental groups, but no significant differences ($P>0.05$) were observed among them. At 21 dpi and 35 dpi, the SI values of all three experimental groups were significant higher than the control group ($*P<0.05$). The results confirmed that the experimental groups were able to induce cellular responses.

T lymphocytes are important effector cells for protection against virus infection. Previous studies showed that T cells have protection effects in animals [20, 21]. CD4$^{+}$, CD8$^{+}$ T lymphocyte subsets are critical to produce the immunity to virus and were analyzed in this study. The numbers of CD4$^{+}$ and CD8$^{+}$ T lymphocytes increased in the peripheral blood of mice from experimental groups, and higher at 35 dpi than that of the 21 dpi. The BEI group was the highest group with the significant difference ($*P<0.05$) compared to the control group. In addition, these results were consistent with the TGEV-specific IgG level and T lymphocyte proliferation which induced by the BEI group were higher than other two groups.

Th cells are differentiated into Th1 and Th2 lymphocyte subsets. Th1 cells play an important role in regulating cellular immune responses and mostly influenced by IFN-γ, while Th2 cells activate the humoral and mucosal immunities that mainly controlled by IL-4 [22, 23]. In this study, we detected higher level of CD4$^{+}$IFN-γ$^{+}$, CD4$^{+}$IL-4$^{+}$ T lymphocyte subsets in TGEV vaccine groups, and the results indicated that inactivated TGEV vaccine could activate Th1 and Th2 immune responses. The higher positive rate of CD4$^{+}$IFN-γ$^{+}$ T lymphocyte subset was observed in the BPL group at 35 dpi, and the higher positive rate of CD4$^{+}$IL-4$^{+}$ T lymphocyte subset was observed in the FA group at 35 dpi, however, the differences between the three experimental groups were not significant ($P>0.05$). The differences among the potencies of FA, BPL and BEI inactivated vaccines are most likely related to the fact that protein is the primary target of FA, nucleic acid is the mainly target of BEI, while both of protein and nucleic acid are attacked by BPL [24,25].

**Conclusions**

In summary, to our knowledge, this is the first study to evaluate the immunogenicity of TGEV vaccine inactivated by three different inactivating agents. Our results showed that the FA group had better effects on humoral immunity, while the BEI group showed its excellent effect on cellular immunity. Considering the effects of both humoral and cellular immunities, BEI might be the better inactivating agent to TGEV compared with FA and BPL.

**Declarations**

**Ethics approval and consent to participate**

All experimental procedures were reviewed and approved by the Henan Agriculture University Animal Care and Use Committee (license number SCXK (Henan) 2013-0001).

**Consent to publication**

All authors have reviewed the final version of the manuscript and approve it for publication.
Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors’ contributions

Fujie Zhao performed the experiments and analyzed the results. Lintao Liu participated in performing the experiments. Menglong Xu participated in correcting the manuscript. Xiangli Shu participated in correcting the manuscript. Lanlan Zheng Wrote and edited the draft. Zhanyong Wei designed and funded the study.

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Not applicable.

Abbreviations

TGEV, transmissible gastroenteritis virus; FA, formaldehyde; BPL, β-propiolactone; BEI, binary-ethylenimine; PEDV, porcine epidemic diarrhea virus; NDV, Newcastle disease virus; ST, Swine testis; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; P, passage; CPE, cytopathic effect; BEA, 2-bromo-ethylamine HBr; ELISA, enzyme-linked immunosorbent assay; OD, optical density; dpi, day post-inoculation; BV510, Brilliant Violet 510; FITC, fluorescein isothiocyanate; APC, allophycocyanin; PE, phycoerythrin; ConA, concanavalin A; MTT, methylthiazoltetrazolium; DMSO, dimethyl sulfoxide; SI, stimulation index; SD, standard deviation.

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**Tables**

Table1. Inactivation results of TGEV with FA, BPL and BEI
| Inactivating agents | Final concentration (v/v) | Time length of inactivation (hour) | Temperature of inactivation (°C) |
|---------------------|---------------------------|-----------------------------------|----------------------------------|
|                     |                           | 6 | 12 | 18 | 24 | 30 | 36 |                  |
| FA                  | 0.1%                      | - | - | +  | +  | +  | +  | 37                |
|                     | 0.2%                      | - | - | +  | +  | +  | +  |                  |
|                     | 0.3%                      | - | - | +  | +  | +  | +  |                  |
| BPL                 | 0.01%                     | - | + | +  | +  | +  | +  | 4                 |
|                     | 0.02%                     | - | + | +  | +  | +  | +  |                  |
|                     | 0.03%                     | + | + | +  | +  | +  | +  |                  |
| BEI                 | 0.03%                     | - | + | +  | +  | +  | +  | 37                |
|                     | 0.04%                     | + | + | +  | +  | +  | +  |                  |
|                     | 0.05%                     | + | + | +  | +  | +  | +  |                  |

- The TGEV was still alive with infectivity; +The TGEV was inactivated thoroughly.

**Figures**
Figure 1

Detection of TGEV-specific IgG in mice sera. Mice sera were collected weekly after the first immunization and TGEV-specific IgG was detected by ELISA kit (n=5). Bars represent the mean (±standard deviation) of three replicates per treatment in one experiment.

Figure 2

The positive rates of CD4+ and CD8+ T lymphocyte subsets were analyzed by flow cytometry. At 21 day post-inoculation (dpi) and 35 dpi, blood samples were collected from mice (n=3). A. The positive rates of CD4+ T lymphocyte subset. B. The positive rates of CD8+ T lymphocyte subset. Bars represent the mean (±standard deviation) of three replicates per treatment in one experiment. Statistical significance was indicated by * P<0.05 (significant) compared with control group.
Figure 3

The positive rates of CD4+IFN-γ+ and CD4+IL-4+ T lymphocyte subsets analyzed by flow cytometry. At 21 dpi and 35 dpi, blood samples were collected from mice (n=3). A. The positive rates of CD4+IFN-γ+ T lymphocyte subset. B. The positive rates of CD4+IL-4+ T lymphocyte subset. Bars represent the mean (±standard deviation) of three replicates per treatment in one experiment. Statistical significance was indicated by *P<0.05 (significant) compared with control group.

![Graph showing proliferation results](image)

Figure 4

The proliferation result of spleen lymphocyte by MTT assay. Spleens of three mice in each group were collected at 14, 21 and 35 dpi, respectively (n=3). Lymphocytes were obtained and stimulated with inactivated TGEV antigen at 37°C for 24 h. Con A was used as the positive control, and the DMEM was used as the negative control. Bars represent the mean (±standard deviation) of three replicates per treatment in one experiment. Statistical significance was indicated by ***P<0.001 (extremely significant) compared with the negative control group.