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Enhancement Of The Immune Response Of The Inactivated Rabies Vaccine Using Ginsenoside-Re

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ABSTRACT

To improve the efficacy of the inactivated rabies virus vaccine and vaccination of dogs and cats against rabies virus disease, ginsenoside Re was extracted from Panax ginsing used as adjuvant to rabies vaccine in different concentrations: 1.25, 2.50 and 5.00 mg/kg wt. This work included dogs and cats divided into eight groups of which five groups were vaccinated with inactivated rabies virus vaccine adjuvanted by ginsenoside Re. The 6th group was vaccinated with inactivated rabies vaccine adjuvanted by aluminum hydroxide gel (20%). On the other hand, the 7th group was inoculated by ginsenoside Re subcutaneously and the 8th group was left unvaccinated as control. It was revealed that ginsenoside Re is safe and did not show any clinical signs. Estimation of humoral immune response of vaccinated animals via indirect ELISA and serum neutralization test (SNT) revealed that vaccinated animals acquired rabies antibodies with high protective level by using ginsenoside Re as adjuvant. The obtained results were compared with those induced by the traditional gel vaccine. It was found that the use of Ginsenoside-Re could reduce the required protective dose and subsequently increase production rate. From these results, it could be concluded that ginsenoside-Re could be used as adjuvant to inactivated rabies virus vaccine.

INTRODUCTION

Rabies virus causes a fatal illness characterized by encephalopathy and generalized paresis *(1)*. It remains a significant threat to human and animal health throughout much of the world *(2,3)*. Domestic dogs play a main role in rabies transmission which accounts for more than 95% of human rabies cases*(4)*. Consequently, the vaccination of dogs against rabies is believed to be one of the most effective approaches for the control of the disease and its transmission to humans *(5,6)*. A research has shown that the vaccination of 70% of dogs should be sufficient to prevent epidemics and eliminate endemic rabies infections *(7)*. Most of the commercially available rabies vaccines currently in use for dog prophylaxis in Egypt are inactivated cell-culture vaccines. The inactivated rabies vaccine is generally safe and convenient for use and storage. In the development of suitable vaccine protocols and antigens, little attention has been paid to the potential of new vaccine adjuvant to enhance the host immune response. Yet in most cases, vaccines require the addition of an adjuvant to induce a protective and long lasting immune response *(8)*.In addition, rabies vaccines derived from cultured cells are costly to produce and in some cases, prohibitively expensive to purchase *(9)*.The root of Panax ginseng C.A. Meyer as a traditional Chinese medicine has been used for at least 2000 years *(10)*, and is believed to be a tonic to stimulate the body resistance against infections *(11)*. Ginseng saponins (ginsenosides) are believed to be the main pharmacologically active constituents of the plant. Ginsenoside Re is one of the constituents, isolated from the root as well as the stem and leaf of P. ginseng *(11,12)*. Interest in ginsenoside-Re for medical and veterinary vaccines, recently increased due to its many advantages, such as ready availability, low risk of side effects and toxicity. Studies of ginsenoside-Re suggest that it possesses abroad range of biological activities. Recent investigations on ginsenoside Re have demonstrated its adjuvant abilities to boost both cellular (Th1) and humeral (Th2) immune

response *(13,14)*. Adjuvant properties of ginseng saponins may be related to its activation of the innate immunity *(15)*. An optimal adjuvant should not only increase immunogenicity and effectiveness of a vaccine, but also should be cheap to produce, non toxic, biodegradable, biocompatible, immunologically inert, stable, and with long shelf life. It should promote not only humoral immunity, but also induce cellular immune response without inducing antibodies *(16,17)*.The present work aims to investigate the effect of Ginsenoside-Re as an adjuvant in order to improve rabies vaccines immunogenicity aiming to reduce the required protective dose.

MATERIAL AND METHODS

Rabies virus

BHK-21 cell culture adapted Evelyn Rokitincki Abelesth strain of rabies virus (ERA) of a titer 10^7 $TCID_{50}$/ml *(18)*, was supplied by the Department of Pet Animal Vaccine Research (DPAVR), Veterinary Serum and Vaccine Research Institute (VSVRI). Abbasia, Cairo. It was used for vaccine preparation, preparation of virus antigen for ELISA and serum neutralization test (SNT).

Challenge virus strain (CVS)

It is a fixed virus strain derived from the original Pasteur strain. It was propagated and fixed in mice brain. CVS was supplied kindly by Pasteur Institute, Paris, in a lyophilized form with a titer of 10^5 $MILD_{50}$/ml and used in the test of the National Institute of Health (NIH) during evaluation of the prepared vaccine formulae *(19, 20)*.

Adjuvant

Ginsenoside-Re

It was extracted from the stem and leaf of Panax ginseng C.A. Meyer, and supplied by Hongjiu Ginseng Industry Co., LTd.(Jilin,China) as a white powder with 98% purity and a molecular weight of 947. The ginsenoside-Re was first dissolved in dimethylsulfoxide (DMSO), diluted with physiological saline solution (1000μg/ml), and passed through a 0.22um filter. It was added as adjuvant using three concentrations to yield 1.25, 2.50 and 5.00 mg/ kg body weight *(21)*.

Aluminum hydroxide gel (2%)

It was supplied by Superfos Biosector a/s. Frydenlands Denmark. It was added as 20% to inactivated rabies virus suspension as adjuvant. It was found to be safe and immunogenic for dogs and cats *(20)*.

Virus inactivation by Binary Ethyleneimine (BEI)

The inactivation process was carried out *(22)*., where BEI was added at 37°c to the viral suspension as 3% concentration of 0.01M. The mixture was stirred continuously at 37°c for 3.5 hours *(23)*. Inactivation process was stopped by addition of cold sodium thiosulphate with a final concentration of 2%.

Vaccine Preparation

In order to prepare the virus suspension, it was preferable to replicate the virus at Multiplicity Of Infection (M.O.I), rate of 2:1 of virus/BHK-21 cells. The virus suspension was inactivated then divided into 6 parts, where 0.25, 0.50 and 0.75 ml of ginsenoside-Re were added to the inactivated virus suspension in 5 different manners as follow:

Part- a:0.25ml virus suspension + 0.25 ml ginsenoside-Re

Part-b:0.25ml virus suspension + 0.50 ml ginsenoside-Re

Part-c:0.25 ml virus suspension + 0.75 ml ginsenoside-Re

Part-d:0.50ml virus suspension + 0.25 ml ginsenoside-Re

Part-e:0.75ml virus suspension + 0.25 ml ginsenoside-Re

Aluminum hydroxide gel was added to the 6^{th} part as 20% adjuvant *(20)*.

Reference rabies vaccine

Delvac rabies vaccine was obtained from Mycofarm UK Limited Science Park, Milton Road; Cambridge CB44FR. It was used reference vaccine in application of NIH test. The vaccine containing cloned rabies virus

strain RIV/PTA/78/BHK clone 8 Batch 75894A.

Experimental animals

dogs and cats

Twenty four native breed dogs and twenty four native breed cats of about 3-4 months age were used in the present work and proved to be seronegative to rabies antibodies. They were apparently healthy free from external and internal parasites and housed under standard the hygienic measures in separate kennels receiving balanced diet and adequate water. All procedures related to the animals and their care conformed to the internationally accepted principles as found in the guidelines for keeping experimental animals.

Mice

Eighty Albino Swiss mice (18-22g) 3-4 weeks old were supplied by the Department of Pet Animal Vaccine Research, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo. These mice were used in the quality control tests of prepared rabies vaccine formulae, through application of the test of National Institute of Health (NIH) *(19,20)*.

Animal immunization

Each of dogs; cats and mice were divided into 8 groups (3 dogs or 3 cats/group) and (10 mice/ group) and subcutaneously injected *(21)*,as follow:

1st Group: was vaccinated with inactivated rabies vaccine (part-a), using a dose of 0.5 ml/animal.

2nd Group: was vaccinated with inactivated rabies virus vaccine (part-b), using a dose of 0.75ml/animal.

3rd Group: was vaccinated with inactivated rabies virus vaccine (part-c), using a dose of 1ml/animal.

4th Group: was vaccinated with inactivated rabies virus vaccine (part-d), using a dose of 0.75 ml/animal.

5th Group: was vaccinated with inactivated rabies virus vaccine (part-e), using a dose of 1ml/animal.

6th Group: was vaccinated with inactivated rabies virus vaccine adjuvanted with aluminum hydroxide gel, using a dose of 2ml/animal *(22)*.

7th Group: was inoculated with ginsenoside-Re only, S/C using the mentioned doses used in vaccine adjuvant as safe test.

8th Group: was kept as unvaccinated animal control.

Three weeks post-vaccination, blood samples from each group were collected weekly, and then serum was separated to estimate rabies serum neutralizing antibodies.

Enzyme Linked Immunosorbent Assay (ELISA)

Serum samples were analyzed to measure the rabies specific antibodies using a commercially available ELISA kit (Zoeits Inc., USA) according to the instructions *(23)*.

National Institute of Health (NIH) test:

According to *(22)*, NIH was carried out using the volumetric method to determine the antigenic value with application of this equation

$$AV = \frac{\text{ED50 of reference vaccine}}{\text{ED50 of test vaccine}}$$

Serum neutralization test (SNT)

It was carried out *(24)*, and used to screen previous experimental animals to rabies antibodies. The antibody titer was calculated as the reciprocal of the final serum dilution which neutralized and inhibited the cytopathic effect(CPE) of 100 $TCID_{50}$ of rabies virus.

Statistical analysis

Data were expressed as mean." F ;" calculated. One-way ANOVA was used to compare the parameters between groups. P-values of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Table 1. Rabies antibody ELISA optical density in sera of vaccinated dogs

Animal groups	ELISA optical density/weeks post-vaccination						
	0WPV*	1WPV	2WPV	4WPV	8WPV	12WPV	16WPV
1	0.120	0.260	0.350	0.310	0.223	0.320	0.318
2	0.101	0.331	0.271	0.320	0.430	0.326	0.322
3	0.021	0.250	0.293	0.339	0.337	0.332	0.435
4	0.110	0.233	0.265	0.420	0.329	0.322	0.325
5	0.111	0.244	0.300	0.439	0.341	0.534	0.431
6	0.154	0.240	0.290	0.435	0.336	0.333	0.530
7	0.069	0.168	0.111	0.142	0.078	0.104	0.0190
8	0.090	0.063	0.156	0.140	0.012	0.005	0.0158

Optical density(OD) obtained with the positive control is ≥ 0.300 and the negative control is <0.50×OD Positive control.
WPV*= week post-vaccination
Group-1: received 0.25 RV+0.25 Re (1.250mg) in 0.5ml.
Group-2: received 0.25 RV+0.50 Re (2.50mg) in 0.75ml.
Group-3: received 0.25 RV+0.75 Re (5.00mg) in1ml.
Group-4: received 0.50 RV+0.25 Re (2.50mg) in 0.75ml.
Group-5: received 0.75 RV+0.25 Re (5.00mg) in1ml.
Group-6: received 1.6ml RV+0.4 ml aluminum hydroxide gel(20%)in 2ml.
Group-7: received 0.25, 0.5& 0.75ml (2.50 & 2.50, 5.00mg Re) as safety test
Group-8: unvaccinated animal as control.

Table 2. Statistical analysis of results of rabies antibody ELISA optical density in sera of vaccinated dogs

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P value*	*F crit*
Between Groups	0.535764	7	0.076538	7.288774	6E-06	2.207436
Within Groups	0.504037	48	0.010501			
Total	1.0398	55				

Table 3. Rabies antibody ELISA optical density in sera of vaccinated cats

Animal Groups	ELISA optical density/weeks post-vaccination						
	0WPV*	1WPV	2WPV	4WPV	8WPV	12WPV	16WPV
1	0.079	0.222	0.245	0.290	0.321	0.319	0.217
2	0.098	0.230	0.265	0.310	0.318	0.325	0.321
3	0.193	0.295	0.330	0.325	0.330	0.329	0.335
4	0.010	0.230	0.261	0.317	0.324	0.320	0.325
5	0.108	0.344	0.325	0.333	0.339	0.342	0.334
6	0.100	0.300	0.333	0.335	0.332	0.339	0.337
7	0.132	0.005	0.023	0.122	0.065	0.097	0.068
8	0.055	0.040	0.014	0.090	0.076	0.053	0.100

Optical density(OD) obtained with the positive control is ≥ 0.300 and the negative control is < 0.50 × OD Positive control.
WPV*= week post-vaccination
Group-1: received 0.25 RV+0.25 Re (1.250mg) in 0.5ml.
Group-2: received 0.25 RV+0.50 Re (2.50mg) in 0.75ml.
Group-3: received 0.25 RV+0.75 Re (5.00mg) in 1ml.
Group-4: received 0.50 RV+0.25 Re (2.50mg) in 0.75ml.
Group-5: received 0.75 RV+0.25 Re (5.00mg) in 1ml.
Group-6: received 1.6 ml+0.4 ml.(aluminum hydroxide gel(20%)in 2ml.
Group-7: received 0.25, 0.5& 0.75ml (2.50, 2.50& 5.00mg Re) as safety test.
Group-8: unvaccinated animal as control.

Table 4. Statistical analysis of results of rabies antibody ELISA optical density in sera of vaccinated cats

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.49414	7	0.070591	11.80851	1.25E-08	2.207436
Within Groups	0.286944	48	0.005978			
Total	0.781084	55				

Fig. (1): Rabies antibody ELISA optical density in sera of vaccinated dogs

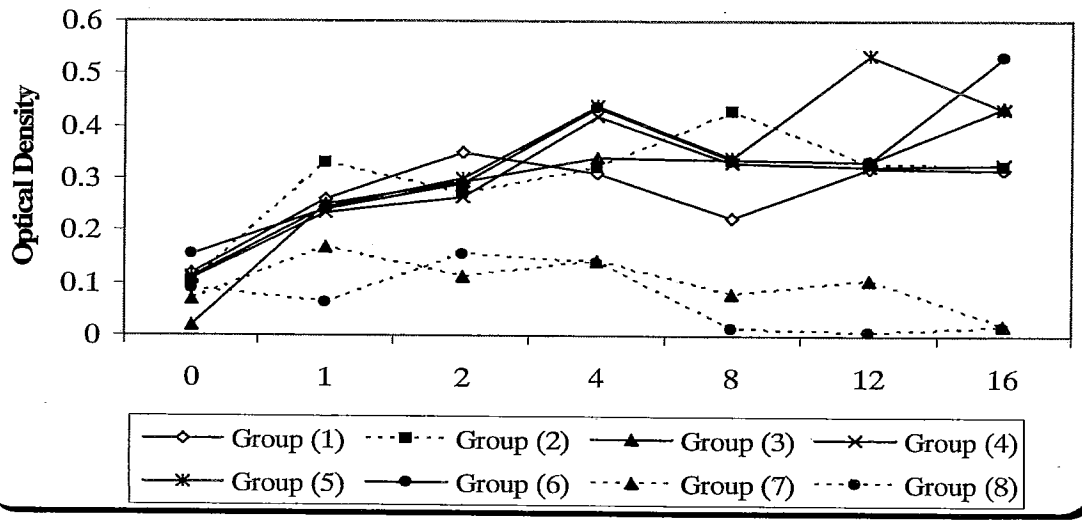

Fig. (2): Rabies antibody ELISA optical density in sera of vaccinated cats

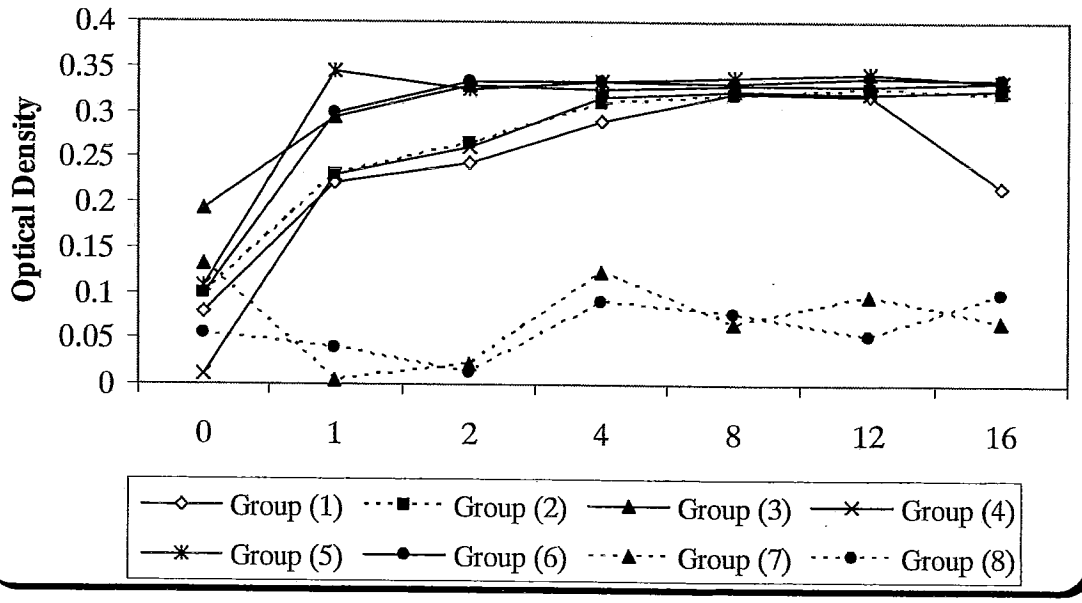

Table 5. Mean titers of rabies serum neutralizing antibodies in sera of vaccinated dog

Animal Groups	Mean rabies serum neutralizing antibody titer* /weeks post-vaccination						
	0WPV**	1WPV	2WPV	4WPV	8WPV	12WPV	16WPV
1	0.00	4	4	8	16	16	32
2	0.00	4	16	16	32	64	64
3	0.00	8	32	64	128	128	128
4	0.00	4	8	16	16	32	64
5	0.00	8	32	64	64	128	128
6	0.00	4	16	16	32	64	128
7	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00	0.00	0.00	0.00

*Antibody titer= the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 $TCID_{50}$ of rabies virus
WPV**= week post-vaccination
Group-1: received 0.25 RV+0.25 Re (1.250mg) in 0.5ml.
Group-2: received 0.25 RV+0.50 Re (2.50mg) in 0.75ml.
Group-3: received 0.25 RV+0.75 Re (5.00mg) in 1ml.
Group-4: received 0.50 RV+0.25 Re (2.50mg) in 0.75ml.
Group-5: received 0.75 RV+0.25 Re (5.00mg) in 1ml.
Group-6: received 1.6 ml+0.4 ml aluminum hydroxide gel(20%)=2ml.
Group-7: received 0.25, 0.5& 0.75(2.50 & 2.50, 5.00mg/kg Re) as safety test
Group-8: unvaccinated animal as control.

Table 6. Mean titers of rabies serum neutralizing antibodies in sera of vaccinated cats

Animal Groups	Mean rabies serum neutralizing antibody titer* /weeks post-vaccination						
	0WPV**	1WPV	2WPV	4WPV	8WPV	12WPV	16WPV
1	0.00	4	4	4	8	16	64
2	0.00	4	8	16	16	32	64
3	0.00	4	16	64	64	128	128
4	0.00	4	16	16	32	64	64
5	0.00	8	16	64	64	128	128
6	0.00	8	8	16	32	64	128
7	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00	0.00	0.00	0.00

*Antibody titer= the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 $TCID_{50}$ of rabies virus
WPV**= week post-vaccination
Group-1: received 0.25RV+0.25Re (1.250mg) in 0.5ml.
Group-2: received 0.25 RV+0.50 Re (2.50mg) in 0.75ml.
Group-3: received 0.25RV+0.75Re (5.00mg) in 1ml.
Group-4: received 0.50 RV+0.25 Re (2.50mg) in 0.75ml.
Group-5: received 0.75 RV+0.25 Re (5.00mg) in 1ml.
Group-6: received 1.6 ml+0.4 ml. aluminum hydroxide gel(20%)in 2ml.
Group-7: received 0.25, 0.5& 0.75(2.50 & 2.50, 5.00mg Re) as safety test
Group-8: unvaccinated animal as control.

Table 7.Relative Potency of tested rabies virus vaccine formulae are determined by National Institute of Health test(NIH)

Tested vaccine formulae	Adjuvant	Antigenic value
1st group		1.0
2nd group		1.7
3rd group	Ginsenoside-Re	2.4
4th group		1.9
5th group		2.6
6th group	Aluminum hydroxide gel(20%)	2.3

N.B: The recommended antigenic value (AV) of the rabies vaccine should not be less than 0.3

Group-1: received 0.25RV+0.25Re (1.250mg) in 0.5ml.

Group-2: received 0.25 RV+0.50 Re (2.50mg) in 0.75ml.

Group-3: received 0.25RV+0.75Re (5.00mg) in 1ml.

Group-4: received 0.50 RV+0.25 Re (2.50mg) in 0.75ml.

Group-5: received 0.75 RV+0.25 Re (5.00mg) in 1ml.

Group-6: received 1.6 ml+0.4 ml. aluminum hydroxide gel(20%)in 2ml.

Rabies remains a very important public health problem in Egypt and vaccination is believed to be the most efficacious and valuable tool in the prevention of the disease. Serum rabies antibodies were measured using ELISA kit to determine the adjuvant effect of ginsenoside-Re on humoral immune response. The obtained results as demonstrated in Table (1 and 2) and Figure (1), showed that the 1st group, exhibited significantly lower specific anti-rabies antibody of optical density (OD) value (0.318) in 16th week post-vaccination than others groups. However, antibody (OD) value (0.430), were significantly higher in the 2nd group at 8th week and, the 4th group, induced no significant difference between this and the anti-rabies antibody (OD) value (0.420), but in the 4th week. The highest antibody (OD) value (0.435) was reported in the 3rd group. These obtained results found in the 3rd group at 16th week post-vaccination. The 5th group was showed rabies antibody(OD) value (0.534), in the 12th week, then decline into (OD) value (0.431), in the 16th week post-vaccination. The 6th group, vaccinated with Aluminum hydroxide gel (20%) as traditional adjuvant showed nearly the same antibody (OD) value (0.530), in the 16th week as in the 3rd and 5th groups ***(19)***. The results of the present study illustrated that in Table (3 and 4) and Figure (2) , the vaccinated cats, in the 1st group, exhibited lower rabies antibody (OD) value (0.321), in the 8th week. On the other hand, the 2nd and 4th groups exhibited the same antibody (OD) value (0.325), in the 12th week and 16th week post vaccination. But, the 3rd and 5th groups exhibited significantly higher antibody (OD) value (0.335 and 0.342), in the 16th week and 12th week, respectively. These obtained results were compared with the 6th group which showed antibody (OD) value (0.339), in the 12th week. It was illustrated that Tables (2 and 4), indicated, there is a significant difference at $P \geq 0.05$. The obtained results demonstrated in Tables (5 and 6), using SNT showed that vaccinated dogs and cats exhibited the same rabies antibody titer (128), in the 3rd ,5th and 6th groups at 16th week post-vaccination. The results of the present study illustrated that rabies vaccine adjuvanted with ginsenoside-Re produced a high protective level similar to Aluminum hydroxide gel ***(13,15)***. Similar results indicat that, the use of Ginsenoside-Re could reduce the required protective dose to half and subsequently increase production rate ***(15)***. On the other side, in the 7th group injected with ginsenoside-Re purely (1.25, 2.50 and 5.00 mg/kg Bw/dose), showed no side effect indicated that ginsenoside-Re is safe. The obtained results in Table (7), revealed that NIH test carried out on mice revealed the highest potent rabies vaccine adjuvant with ginsenoside-Re (5.00mg/kg BW) as the 3rd and 5th groups inducing antigenic

value of 2.4 and 2.6 respectively, (must not be less than 0.3). But, in the 6th group, the rabies vaccine adjuvanted with aluminum hydroxide gel induced antigenic value of 2.3 ***(19,20)***. The present study demonstrated that ginsenoside Re acted as an adjuvant enhanced the immune response to the inactivated rabies virus vaccine in dogs and cats was in agreement with that reported before ***(11,14)***. The present obtained results revealed that addition of ginsenoside-Re to rabies vaccine led to a dose dependent increase of serum antibody levels. The serum antibody response elicited by the rabies vaccine was also found to be dose dependent. Our results suggest that ginsenoside-Re could be used to reduce the dose and help to increase vaccine production ***(25)***. Moreover, it could be suggested that the co-administration of ginsenoside-Re enhanced the maintenance of long lasting serum antibody titers to the rabies vaccine. In light of the fact that a yearly prophylactic shot of inactivated rabies vaccines is currently being used to immunize dogs and cats to control rabies in Egypt, a longer duration of protective antibody titers is preferable to make the rabies control program more effective.

These findings indicated that ginsenoside-Re may be a promising adjuvant to the veterinary rabies vaccine by providing higher antibody levels and long lasting protection.

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الملخص العربى

رفع الكفاءة المناعية للقاح السعار المثبط باستخدام جينسوسيد Re

محمد بريك شندى

معهد بحوث الأمصال واللقاحات البيطرية - العباسية – القاهرة - ص . ب : ١٣١

أملا لرفع كفاءة لقاح السعار المثبط لتحصين القطط والكلاب ضد مرض السعار تم استخدام مادة الجينسوسيد Re المستخلصة من نبات Panax Ginseng كمساعد للقاح بتركيزات مختلفة : (5.00, 2.50 , 1.25 ملى جرام/ كيلو من وزن الجسم) حيث أجرى تحصين خمس مجموعات من القطط والكلاب بلقاح السعار المثبط مع مادة جينسوسيد Re كمساعد للقاح بالإضافة إلى تحصين مجموعه سادسة بلقاح السعار المثبط مع مادة الهيدراجل والمستخدم حاليا كمساعد للقاح للمقارنة بين كفاءة المادتين . تم حقن القطط والكلاب بمجموعه سابعة تحت الجلد بمادة الجينسوسيد Re لتحديد مدى سلامة تلك المادة بينما تركت مجموعة ثامنة دون تحضين كضابط للتجربة هذا ولم تظهر الحيوانات المحصنة أو المحقونة بمادة الجينسوسيد Re حتى جرعة ٥ ملى جرام/ كيلو جرام من وزن الجسم أية أعراض مرضية مشيرة إلى سلامة استخدام تلك المادة. وبإجراء استبيان المستويات المناعية المتكونة فى أمصال الحيوانات المحصنة بكل من اختبار المصل المتعادل والإنزيم المد مص المناعي أوضحت النتائج أن القطط والكلاب المحصنة تكتسب أجساما مناعية نوعية للسعار ذات مستويات وقائية في حالة استخدام مادة الجينسوسيد Re كمساعد للقاح السعار المثبط مماثلة لحالة استخدام الجيل إلا أنه يقلل الجرعة المستخدمة للنصف مما يؤدى إلى تقليل تكلفة وزيادة الإنتاج. وعلى ذلك يوصى باستخدام مادة الجينسوسيد Re كمساعد للقاح السعار المثبط.