EFFICACY OF EGG SHELL-CALCIUM OXIDE AS NATURAL INACTIVATOR TO RABIES VIRUS FOR PREPARING RABIES VACCINE

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Abstract

This work was conducted to study the inactivation dynamics of Rabies Virus (RV) by calcinated eggshell calcium oxide (CaO) in comparison with Binary Ethyleneimine (BEI). Calcinated eggshell, of which the main component is calcium oxide, was evaluated in the form of powder as inactivator to rabies virus. The calcination process yielded calcium oxide with a purity of 97.92 % w/w. The results of X-R diffraction showed that the optimum calcination conditions for preparation of CaO from chicken eggshell is 900ºC for 1 hour with peak 2ɵ (2-Theta) at 32.3º, 37.4°, 54.0º, 64.3°and 67.5º. Complete virus inactivation was obtained with BEI (3% of 0.001M) after incubation for 3 hours. On the other hand, 10% Egg-CaO solution inactivated RV within 15 minutes. The safety and potency of CaO prepared inactivated rabies vaccines were evaluated in three groups of puppies. The inactivated rabies vaccine produced sufficient level of antibodies as measured by serum neutralization test (SNT) and was protected when challenged in mice. The obtained results revealed that no residual infectious virus was detected in Egg-CaO inactivated rabies vaccine that proved to be safe and effective as compared the same virus that inactivated with the classical inactivating agent.

Key words: Rabies vaccine, calcinated eggshell (Egg-CaO), binary ethylenimine, inactivators, XR- diffraction.

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 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). Research has shown that the vaccination of 70% of dogs should be sufficient to prevent epidemics and eliminate endemic rabies infections (6). Vaccines can be classified as live attenuated and inactivated. Currently, the recommended inactivating agent for this virus is beta propiolactone (BPL), which is a very expensive chemical and potentially carcinogenic (7). Other chemicals like formaldehyde and phenol not only inactivate the virus but also

adversely affect its antigenicity. Binary ethyleneimine (BEI), member of a group of alkylating substances "aziridines" reacts very little with proteins and therefore does not alter the antigenic components of the virus. BEI has an inactivation reaction that is more specific for the nucleic acid and it produces antigenically superior vaccine (8). Binary ethyleneimine (BEI) is very hazardous since it attacks nucleic acids and proteins. Thus, the current study is a trail to find an alternative inactivating agents which are not expensive, non-toxic and easily available. Disinfection effect of slaked lime is maintained for a long period if it is dry powder, but if it turns wet by rain, it becomes hardened (9). In addition, it was reported that when slaked lime was combined with carbon dioxide in the air or rain water, it became calcium carbonate (CaCO3) and the pH got lowered, bringing about the disappearance of disinfection effect (10). The egg and egg derivative consumption produce a great amount of residual shells which pose an environmental pollution as a result of microbial action. Chicken eggshell is household waste and its utilization is still relatively small. Eggshell containing calcium carbonate (94%), calcium phosphate (1%), organic compounds (4%), and magnesium carbonate (1%) (11). Wei, et al., illustrated that the high contents of calcium in eggshells can be converted as a CaO catalyst by calcinations process at temperature 900° C for 1 hour, where the reaction takes place as exothermic reaction and the decomposition of eggshell to produce CaO as a catalyst was run at various temperatures 600, 700, 800, 900, and 1000^{O}C, and then the characterization of CaO had done by X-R diffraction (12). The catalytic activities of calcium oxide obtained from natural sources (eggshell) were characterized and evaluated in the transesterification of vegetable oil. These catalysts are mainly composed of calcium carbonate, which is partially converted into CaO after calcination (900°C for 2 hours). Thammakarn, et al., reported that scallop shell powder solution at pH 12.3 did not inactivate avian influenza AIV (H7N1), however, at pH 13.0 it did. Therefore, aqueous solution above pH 12.5 seems to be able to inactivate the influenza viruses (13). The catalysts have some advantages, such as abundant occurrence, low cost, porous structure, and non-toxic (14).

The current study aims to prepare and evaluate the efficacy of a prepared inactivated rabies vaccine using eggshell calcium oxide (CaO) as inactivator in comparison with the classical inactivator binary ethylenimine (BEI) and assayed them in puppies.

MATERIALS AND METHODS

1. Rabies virus:

BHK-21 cell culture adapted Evelyn Rokitincki Abelesth strain of rabies virus (ERA) of a titer 10^7 $TCID_{50}$/ml**,** was supplied by the Department of Pet Animal Vaccine

Research, Veterinary Serum and Vaccine Research Institute (VSVRI). Abbasia, Cairo, and used for vaccine preparation and serum neutralization test (SNT).

2. Baby Hamster Kidney cell (BHK-21):

BHK-21 cell culture was used for preparation of rabies virus suspension and detection of rabies virus antibodies in vaccinated puppies by serum neutralization test (SNT).

3. 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.

4. Chicken eggshells:

Raw chicken eggshells were collected and washed with tap water until the egg white was completely removed and dried at room temperature. After that the eggshells were broken into small pieces and crushed in a porcelain mortar into a fine powder, and kept in desiccators at a room temperature.

5. inactivators:

5.1. Calcinated eggshell (CaO) powder:

The eggshell was rinsed several times with deionized water. Then, the eggshell was dried at 100°C in the dry oven. The dried eggshell was crushed and sieved to pass 60 meshes. An amount of 200g of the chicken eggshell was calcinated in a laboratory muffle furnace in Central Metallurgical Research and Development Institute (CMRDI). Tibin, Helwan, Cairo. The samples were calcined in an alumina crucible for 1, 3, and 5 hours at a heating rate of 10°C/min, at temperatures between 300 to 900°C for 1, 3and 5 hours. After calcination process completed a cold solid is obtained and down in air for a period of 20 minutes to avoid the hydrolysis, and stored in a desiccator for 24 hours. The structure of metal oxide was characterized using X-ray diffraction (XRD). Calcinated eggshell powder, was prepared in the suspensions of 3%, 5% and 10% (w/v) of Egg-CaO in redistilled water (dw_2) and then centrifuged at 12.000xg for 3 minutes, and the resulted supernatants were used as Egg-CaO solutions with pH at 12.7 (12).

5.2. Binary Ethyleneimine (BEI):

The inactivation process was carried out (8), 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. Inactivation process was stopped by addition of cold sodium thiosulphate with a final concentration of 2%.

6. Adjuvant:

6.1. Aluminum hydroxide gel (20%):

Rehydra Gel® LV. Aluminum hydroxide of low viscosity, was supplied by Chemtrade, New Jersey 07922. USA. It was added as 20% to inactivated rabies virus suspension as adjuvant (15 &16).

7. Vaccine Preparation:

In order to prepare the virus suspension, it was preferable to replicate the virus at a multiplicity of infection (M.O.I), rate of 2:1 of virus/BHK-21 cells (16). The virus suspension was divided into 2 parts. The first part was divided into 3 portions to be inactivated with 3%, 5% and 10% Egg-CaO (4:1), and incubated for indicated time (3 min to 1hr) at room temperature and then neutralized with 1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.2).The second part was inactivated with BEI. Aluminum hydroxide gel was added to the inactivated virus suspension as adjuvant.

8. Reference rabies vaccine:

Delvac rabies vaccine was obtained from Mycofarm UK Limited Science Park, Milton Road; Cambridge CB44FR. It was used as a reference vaccine in application of NIH test. The vaccine is containing cloned rabies virus strain RIV/PTA/78/BHK clone 8.

9. Calculation of Reduction Factor (RF):

Inactivation efficacy against rabies virus was determined using reduction factor (RF), which is calculated by the following equation: $RF=\log_{10}$ (amount of untreated intact virus/ml)-$\log_{10}$ (amount of treated virus with Egg-CaO/ml). Inactivation was considered to be satisfied when RF was ≥3 (13).

10. Experimental animals:

10.1. Dogs:

Twenty native breed puppies of about 3-4 months age were used in the present work and proved to be seronegative to rabies antibodies as screened by serum neutralization test (SNT). They were apparently health free from external and internal parasites and housed under hygienic measures in separate kennels receiving balanced diet and adequate water.

10.2. Mice:

Two hundred 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). All challenged mice were observed daily for two weeks for disease signs and survival.

11. Evaluation of prepared inactivated rabies vaccine:

11.1. Sterility test:

The inactivated virus was inoculated into blood agar and Sabouraud's dextrose agar to ensure the absence of bacterial and mycotic contamination, respectively.

11.2. Safety test:

In cell line: The inactivated virus suspensions were inoculated into BHK-21 cell monolayers to confirm total complete inactivation

In Mice: inoculated with inactivated rabies virus intracerebrally (I / C).

In dogs: Five puppies were injected with double doses of each prepared vaccines, S/C in different sites and observed for 15 days for development of any clinical signs or local reaction.

11.3. Potency:

The potency of the prepared vaccine formulae was evaluated using National Institute of Health (NIH) test. The mice were divided into four groups (50 mice / each) and injected intraprotenial with fivefold dilutions (1/5 to 1/50). Two doses of each vaccine were given to each mice group two weeks apart. Mice were challenged with CVS virus strain at three weeks after the last immunization, using a dose of 0.03 ml, intracerebrally. All challenged mice were observed daily for two weeks for disease signs and survival. NIH was carried out according to (17), using the volumetric method to evaluate the antigenic value as following equation:

$$\text{Antigenic value (AV)} = \frac{ED_{50} \text{ of reference vaccine}}{ED_{50} \text{ of test vaccine}}$$

12. Animal immunization:

Puppies were divided into three groups (5 animals / each) and subcutaneously injected as follow:

1st Group: was vaccinated with inactivated rabies virus vaccine with 10 % Egg-CaO.

2nd Group: was vaccinated with inactivated rabies virus vaccine with BEI.

3rd Group: unvaccinated as animal control.

Blood was collected from the vaccinated and non-vaccinated puppies weekly for 4 weeks post vaccination then every month up to 4 months (16 weeks). Serum was separated to estimate rabies serum neutralizing antibodies.

13. Serum neutralization test (SNT):

It was carried out (18), and used to follow up the induced rabies antibodies in vaccinated animals. The antibody titer was calculated as the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 $TCID_{50}$ of rabies virus (19).

RESULTS AND DISCUSSION

Table 1. XRD data of CaO and Ca (OH) $_2$ from chicken eggshell calcinated at various temperatures

Caption	Legend	Angle	d value	Intensity	Intensity %
		2-Theta (2 ɵ)	Angstrom	Count	%
d=4.90294	Ca(oH)2	18.078	4.90294	31.7	6.7
d=3.09716	Ca(oH)2	28.803	3.09716	22.7	4.8
d=2.76700	CaO	32.328	2.767	175	37.1
d=2.61590	Ca(oH)2	34.251	2.6159	50.8	10.8
d=2.39758	CaO	37.481	2.39758	471	100
d=1.92488	Ca(oH)2	47.179	1.92488	20.7	4.4
d=1.79209	Ca(oH)2	50.914	1.79209	19.5	4.1
d=1.69619	CaO	54.019	1.69619	195	41.4
d=1.44740	CaO	64.308	1.4474	57.8	12.3
d=1.38585	CaO	67.536	1.38585	52.8	11.2

Started XRD: 4.00 - End: 70.000-Step 0.020º – step time: 0.4s – Temp: 25°C (Room) Time started: 0s – 2 Theta: 4.000 – Theta: 2.00

Portlandite, syn – Ca(OH)$_2$ –y : 10.42% - dx by: 1- wl

Lime – CaO –y: 97.92%

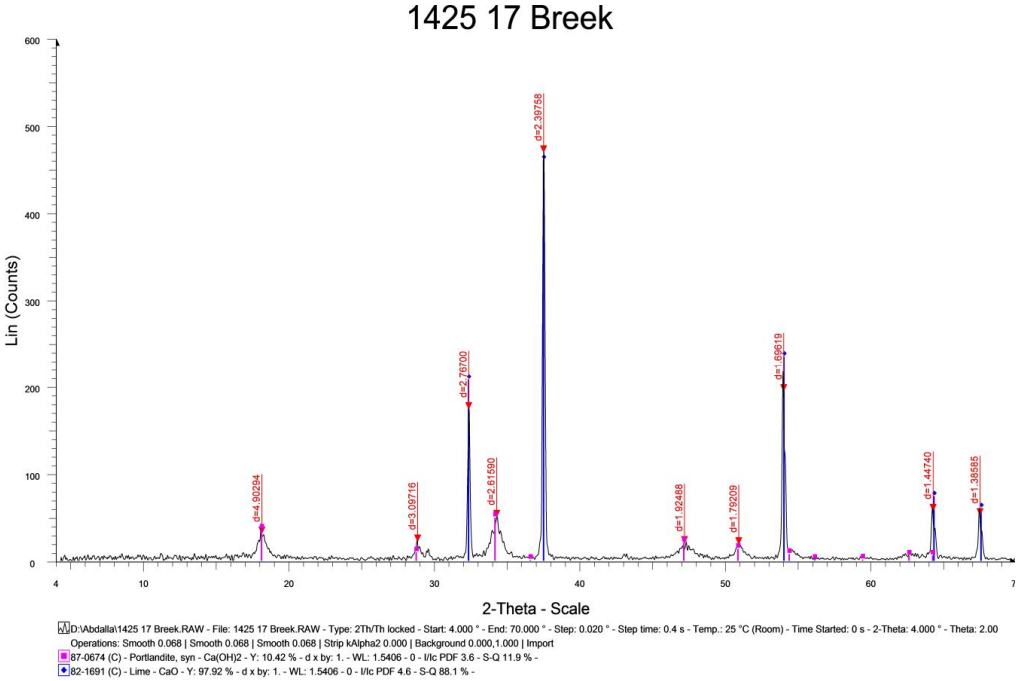

Fig. 1. XRD peak patterns and the phase transformation of chicken eggshells calcinated from 300° C to 900° C for 1h.

Table 2. Inactivation of rabies virus using calcinated eggshell (CaO) at 25ºC

Periods of virus inactivation process/ min.	Virus titer (Log_{10} $TCID_{50}$/ml) with different concentrations of Egg-CaO solutions (pH 7.2)		
	(3%)	(5%)	(10%)
Pre-inactivation	7	7	7
3	6.6	5.7	5.6
10	5.1	4.5	1.4
→15	4.5	3.6	→0.0
20	3.5	2.5	0.0
30	2.1	0.0	0.0
45	0.0	0.0	0.0
60	0.0	0.0	0.0
RF	>5.00	>5.25	>5.92

Reduction factor (RF) =log_{10} (amount of untreated intact virus/ml) – log_{10} (amount of treated virus with Egg- CaO/ml).Inactivation was considered to be satisfied when RF was ≥3.

.→: Time of complete inactivation.

Fig. (2): Inactivation of rabies virus using calcinated eggshell (CaO) at 25ºC.

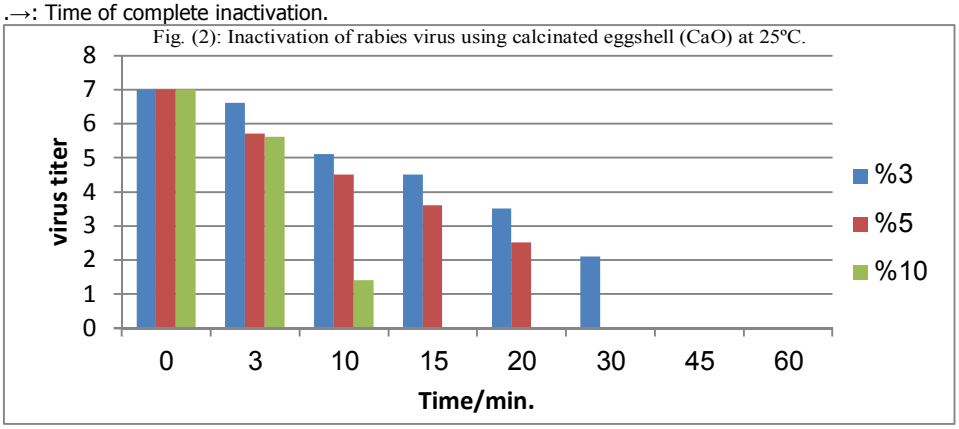

Table 3. Inactivation of rabies virus using Binary Ethyleneimine (BEI) at 37ºC

Periods of virus inactivation process/ hours	Virus titer ($Log_{10}TCID_{50}$/ml) with different molarities of BEI		
	(0.001)	(0.002)	(0.003)
Pre-inactivation	7	7	7
1	6.7	6.6	4.8
2	6.5	6.2	3.5
→3	6.0	5.4	→0.0
4	5.7	4.6	0.0
5	4.8	0.0	0.0
6	4.7	0.0	0.0
7	0.0	0.0	0.0
RF	> 2.3	>2.4	>3.5

Reduction factor (RF) = log_{10} (amount of untreated intact virus/ml) – log_{10} (amount of treated virus with BEI/ml).Inactivation was considered to be satisfied when RF was ≥3

→: Time of complete inactivation.

Fig. (3): Inactivation of rabies virus using Binary Ethylenimine (BEI) at 37ºC.

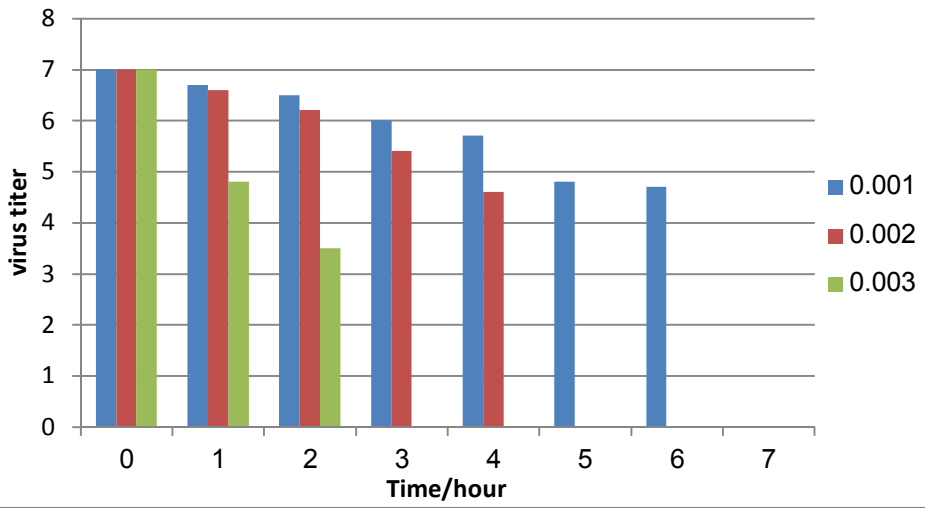

Table 4. Antigenic values of the prepared rabies vaccine formulae

Tested rabies vaccine formulae	Inactivators	Adjuvant	Antigenic value
1st group	10% Egg-CaO	Aluminum hydroxide gel	3.00
2nd group	Binary Ethylenimine (BEI		2.4

Group-1: vaccinated with inactivated rabies virus vaccine with 10 % Egg-CaO
Group-2: vaccinated with inactivated rabies virus vaccine with BEI
NB: The antigenic value (AV) of the vaccine must not be less than 0.3

Table 5. Titers of rabies serum neutralizing antibodies in sera of vaccinated dogs

Animal Groups	Rabies serum neutralizing antibody titer° /weeks post-vaccination						
	♦0WPV	1WPV	2WPV	4WPV	8WPV	12WPV	16WPV
1	0.00	16	32	64	64	128	128
2	0.00	8	16	32	32	64	128
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Group-1: was vaccinated with inactivated rabies virus vaccine with 10% Egg-CaO.
Group-2: was vaccinated with inactivated rabies virus vaccine with BEI.
Group-3: unvaccinated animal as control.
♦WPV= week post-vaccination. Antibody titer° = the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 $TCID_{50}$ of rabies virus.
N.B: The protective level of rabies antibody titer not less than (32).

Rabies remains a very important public health problem in Egypt and vaccination is believed to be the most efficacious and valuable tool in the disease control. Different chemical and physical agents have been used to inactivate rabies virus for vaccine production as beta-propiolactone (20, 21 & 22), and binary ethylenimine (8) are the most widely used for this purpose. It is suggested that it is necessary to develop the calcination method to change the egg shell which is industrial waste, into a valuable 'Egg-CaO. The conversion of raw chicken eggshells which consist of mainly calcium carbonate ($CaCO_3$) to calcium oxide (CaO) depends on the thermal treatment. The

calcium carbonate (CaCO3) has been converted into calcium oxide (CaO) at decomposition temperature of 900 ºC for 1 hour is approximately 97.92 % w/w (12).

The experimental work showed that X-ray diffraction spectra of the samples were obtained with a 2 ɵ scan range of 4°–80°. The XRD peaks of the calcined chicken eggshells can be seen at 2 ɵ equal to 32.328 (175), 37.481 (471), 54.019 (195), 64.308 (57.8) and 67.536 (52.8) (23), as shown in Table (1) & Figure (1). The safety test of egg-CaO powder was performed in BHK-cells which did not show cytopathic effect (CPE) confirming complete virus inactivation and when inoculated into mice and puppies, no any abnormal clinical signs were determined is in agreement with (24). It was found that neutralization of the pH of CaO solutions with 1M HEPES did not result in virus titer reduction in comparison with the positive control. It was found that titration of the neutralized samples in BHK-cells showed that egg- CaO shell solutions at pH 12.3 did not inactivate avian influenza (AIV) strain (H7N1), however, at pH 13.0 it did. Therefore, aqueous solution above pH 12.5 seems to be able to inactivate the influenza viruses (13). Thus, the pH got 12.3, bringing about the disappearance of disinfection effect of rabies virus (10). The virucidal effects of 3%, 5% and 10% egg-CaO solutions against rabies virus are summarized in Table (2) & Figure (2), as rabies virus was inactivated within 45, 30 and 15 minutes incubation, respectively. In this study, Egg-CaO was successfully used to inactivate rabies virus. Table (3) & Figure (3), illustrated that the complete inactivation process of rabies virus using BEI of 0.001, 0.002 and 0.003M concentrations at 37˚C were obtained after 7, 5 and 3 hours, respectively. The obtained results revealed that 0.003M concentration of BEI inactivated rabies virus within 3 hours of exposure and reduction factor (RF) about > 3.5. Thus this indicates that inactivation of virus was considered to be satisfied when RF was ≥3 (13), without affecting its antigenicity or immunogenicity, similar findings were stated before (25).

The obtained results of NIH test (table, 4) revealed that rabies vaccine with 10% Egg-CaO had the highest antigenic value adjuvant with aluminum hydroxide gel (3.00) in (12). The rabies vaccine inactivated with binary ethyleneimine had an antigenic value of 2.4 (17).

The demonstrated results in table (5) showed that vaccination of dogs with the prepared inactivated rabies vaccine formulae evoked different levels of specific rabies antibodies as measured by SNT. The protective level of neutralizing antibody titers against rabies virus was (32) as reported by (26). The level of rabies serum neutralizing antibody titers in group-1 (received CaO vaccine) were higher than that group -2 (BEI vaccine) during the first 4 weeks after vaccination as 64 and 32, respectively and remained at good levels till the end of experiment.

Depending on the obtained results it could be concluded that, the calcinated eggshell (Egg-CaO)–inactivated rabies virus proved to be safe, potent and effective and could be preferable than the same virus inactivated with BEI avoiding the possible public health hazard. Thus, it could be recommended to using Egg-CaO as inactivating agent

for rabies virus vaccine saving time and costs of inactivation process and at the same time its safety and potency.

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كفاءة أوكسيد الكالسيوم المستخلص من قشر البيض
كمثبط طبيعى لفيروس السعار فى تحضير
لقاح السعار

محمد بريك شندى،أكرم فؤاد سليمان، همت سليمان الامام، عطيات محمد قطب

معهد بحوث الامصال واللقاحات البيطرية – العباسية– القاهرة– ص ب:١٣١

هذه الدراسة تهدف الى تطوير و انتاج لقاح السعار وذلك بأستخدام مادة أوكسيد الكالسيوم المستخلص من قشر بيض الدجاج كمادة مثبطة لفيروس السعار بالمقارنة بمادة البينارى ايثيلين أمين، حيث أن الحصول على مادة أوكسيد الكالسيوم تتم عند معالجة قشر البيض حرارياعند درجة حرارة ٩٠٠ درجة مئوية لمدة ساعة٠ تم التعرف علي هذه المادة باستخدام أشعة أكس٠ وقد اجريت هذه التجربة علي فيرو س السعار بتثبيطه بمادة أوكسيد الكالسيوم عند تركيز(١٠%) تماما لمدة خمسة عشرة دقيقة بينما تم تثبيط للفيروس باستخدام مادة البينارى ايثيلين امين عند (٠,٠٠٣ مول) لمدة ٣ ساعات دون ان يؤثر ذلك على القوة المناعية للفيروس حيث تم استخدام ثلاث مجموعات من الاجراو عمر من ٣ الى ٤ أشهر لتقييم السلامة والقوة المناعية للقاح السعار المثبط المحضر واظهرت هذه النتائج انه لا يوجد أى بقايا معدية للفيروس وذلك عند استخدام لقاح السعار المثبط بمادة أوكسيد الكالسيوم الذى ثبت أنه أمن وفعال بالمقارنة بالمادة المعتاد تثبيط الفيروس بها (البينارى ايثيلين امين)٠ وهكذا فإن طريقة التثبيط البديلة بمادة أوكسيد الكالسيوم قادرة على الحفاظ على سلامة البروتين الفيروسى التى تكون ضرورية لتحسين فاعلية لقاح السعارالمثبط الذى ينتج مستوى كاف من الاجسام المضادة التى تم قياسها باختبار المصل المتعادل والتى تم حمايتها عند اجراء اختبار التحدى بعترة فيروس السعار ذو الضراوة العالية ولذا يوصي باستخدام مادة أوكسيد الكالسيوم المستخلص من قشر البيض كمادة طبيعيه مثبطة لفيروس السعاروالتى تؤدى الى توفير الوقت والتكلفة اللازمة لعملية التثبيط٠