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PREPARATION OF INACTIVATED RABIES SUCKLING MOUSE BRAIN VACCINE ADJUVANTED WITH NATURAL IMMUNE STIMULANTS

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SUMMARY

The challenge virus standard (CVS-11) strain of fixed rabies virus was propagated in suckling mouse brains for several passages. These passages were titrated in 3-4 weeks old white Swiss mice using mouse inoculation test (MIT). The harvested infected brains of suckling mice were inactivated using 1/4000 beta-propiolactone (BPL) and adjuvanted using Nigella sativa oil and combination of vitamin E & Selenium. The prepared plain and adjuvanted rabies suckling mouse brain vaccines were subjected to quality control tests including its safety, sterility and potency using either National Institute of Health (NIH) or immunogenicity test in mice. The obtained results were discussed and revealed that , the adjuvinated mouse brain rabies vaccine with either Nigella sativa or vitamin E and selenium were highly immunogenic than the plain vaccine and can be used in human or animals safely.

INTRODUCTION

Rabies virus was recognized in Egypt before 2300 B.C. and was described by Aristotle in ancient Greece. It was characterized as one of the oldest and most life threatening of both man and animals. It is the most lethal of all infectious diseases and has the widest host range of any virus [Fenner and White, 1994].

The inactivated rabies vaccines were firstly produced from the nervous tissues of rabbits [Pasteur, et al. 1881], sheep and goat [Fermi, 1908 and Semple, 1919] and suckling mouse brain [Fuenzalida, et al. 1964] or produced from chicken embryo origin (CEO) [Peck, et al.1955]. Such vaccines were antigenically potent and inexpen-

sive to produce. However, the nervous tissue (N.T.) and chicken embryo contains a great encephalitogenic components (mainly protein) which can cause neurological complications that were avoided by the development of cell culture vaccines after [Wiktor and Koprowski, 1965]adapted the fixed rabies virus to human diploid cell cultures (HDCS). But the high cost and small yield of the HDCS vaccine had been limited its use [Lin, et al. 1983]. Several new types of inactivated rabies vaccines are produced which are more safe and more immunogenic such as the fixed rabies virus grown in newborn mice brain [Fuenzalida, et al. 1964] or that prepared on cell culture [Abelseth, 1973].

Although the inactivated cell culture rabies vaccine cause no or fewer reactions than the nervous tissue or the chicken embryo vaccines due to they are relatively free from aggregates that could protect ineffective virus particles so the virus titer obtained require further concentration. However, suckling mouse brain vaccine (SMBV) often contains very high titers of virus and if probably inactivated is entirely safe for use in any species of animals [Sikes, 1975 and Bear, 1975].

In the present study , a trial to prepare a new naturally adjuvanted suckling mouse brain rabies vaccine (SMBV) using CVS-11 strain for propagation in suckling mice brains, inactivation using 1/4000 beta-propiolactone and adjuvantion using naturally immune stimulants such as Nigella sativa oil and or combination of vitamin E & Selenium to increase its efficacy and potency was done.

MATERIAL AND METHODS

Propagation& titration of CVS-11 rabies virus strain:

CVS-11 rabies virus strain(fixed rabies virus strain derived from the original Pasteur strain was obtained from Abahyrab Company for Rabies Vaccine Production, India had a titer of 105.46 $MICLD_{50}$ / 0.03 ml.), was propagated in suckling mice (from Helwan Animal House &VACSERA) for 5 passages by inoculating 100-1000 $MICLD_{50}$ / 0.03 ml in each mouse, not more than 4 days old, with a dose of 0.01 ml / mouse intracerebrally according to the method described by [Fuenzalida and Palacios 1955].

Approximately 96 hours, the mice were collected and the brains were harvested and subjected to virus titration . The propagated virus was titrated in weanling mice weighing (14-16 g) and expressed in terms of the $MICLD_{50}$ / 0.03 ml according to the method described by [Koprowski, 1973 b].

Virus Identity :

The test was done by mixing equal volumes of the harvested virus (master seed virus) 300 $MICLD_{50}$ / 0.03 ml with equal volume of 1/500 of equine antirabies hyper immune serum (Equirab, BHARAT Serums and Vaccines Limited, India) and or with normal horse serum (VACSERA) according

to the method described by [Johnson, 1973].

Virus Inactivation:

5% of the harvested virus was inactivated using 1/4000 b-propiolactone (BPL) according to (Diaz, 1996)

Vaccine preparation:

A) Plain vaccine: brain of the inoculated SM with CVS-11 rabies strain were collected, homogenized with normal horse serum 2% diluent as a stabilizer according to (Wilbur and Aubert, 1996). 5% of this harvested virus was inactivated using 1/4000 BPL [Diaz, 1996] according to and used as plain vaccine.

B)-Preparation of the adjuvated vaccines :

1) Vitamin E & Selenium adjuvanted suckling mouse brain vaccine :

E-SELEN (composed from Vitamin E acetate 150 mg/ml & sodium selenite 1.67 mg/ml, produced by MAM Egypt) was diluted depending upon the LD_{50} of sodium selenite in mice (0.9 mg / kg body weight) according to [Toxic Rep. Ser. 1994] and for vitamin E (100 mg / kg body weight) according to [Toutain, et al. 1992] was mixed with the inactivated virus in a ratio of 1:1 to make a homogenous mixture, kept at 4C and subjected to quality control tests.

2) Nigella sativa oil (natural oil) adjuvanted suckling mouse brain vaccine:

One part of the water phase(The inactivated virus was thoroughly mixed with 1% tween 20) was thoroughly mixed with one part of the oil phase (one part of span 80 was thoroughly mixed with 9 part of the Nigella), kept at 4°C and subjected to quality control tests.

Quality control of the prepared three types of vaccines

1)-Safety Test:

Group of mice, each weighing 18-20 g, were inoculated intracerebrally with 0.03 ml of the inactivated vaccine. All mice were observed for 30 days and any one of them showing signs of rabies after 5 days p.i. was considered positive for rabies. Another group of suckling mice (≤ 4 days of age) were inoculated with 0.01 ml of the prepared inactivated vaccine observed for at least days p.i. The safety test was done for inactivated vaccine before addition of adjuvants to ensure adequate inactivation of the vaccine.

Sterility Test:

According to [Sikes and Larghi 1967] , the prepared vaccines were inoculated on thioglycolate broth (specific for aerobic and anaerobic bacteria) and Sabouraud's agar (specific for fungi) , then incubated at 37°C for 7 days and 22°C for 15 days, respectively.

Abnormal Toxicity Test:

Three groups of Swiss mice 5 per each group, weighing 13-16 g, were inoculated intraperitoneally with 0.5 ml of each of the prepared vac-

cine per mice . The inoculated mice were observed for 7 days for matching clinical signs, deaths, and decrease in body weight.

Potency Test by NIH:

Potency test was done for the prepared plain (not adjuvanted) inactivated suckling mouse brain vaccine by the NIH test according to the method described by [Seligmann 1973].

Potency Test by immunogenicity:

This test was done instead of NIH method to determine the potency of the three types of the prepared suckling mouse brain vaccines according to the method described by [European Pharmacopoeia, 1998].sera of vaccinated mice were collected and tested by ELISA using indirect ELISA according to [Hubschle, et al. 1981] , negative mouse serum(from negative control group of mice) and positive mouse anti rabies serum (prepared by using combination of vitamin E & Selenium added to the Verorab vaccine and inoculated I/P in ten mice, weighing 18-20 grams, for 4 injections ,sera were collected after 10 days from the last injection) were involved in the assay as controls, mouse anti rabies peroxidase conjugate was used as enzymatic reaction

RESULTS

Table (1): The titer of the serial passages of the master seed virus in brains of weaned and suckling mice :

Virus Code No.	Passage No.	Host	Log_{10} $MICLD_{50}$/ 0.03 ml
Original	Original	Weaned mice	5.46
SMB	3rd	Suckling mice	6.84
SMB	5th	Suckling mice	7.25
SMB	6th	Suckling mice	8.20
SMB	7th	Suckling mice	7.80

Data presented in table (1) shows clearly that the master seed virus has 6.84 log_{10} $MICLD_{50}$ / 0.03 ml after three passages in suckling mice from the original strain. The identified 2nd passage that has 8.2 log_{10} $MICLD_{50}$ / 0.03 ml was used as virus inoculum for vaccine preparation.

Table (2): Identity test of propagated rabies virus using normal horse serum and equine antirabies hyper immune serum.

Tested sample	Clinical symptoms of rabies on mice
1. Virus with equine antrabies hyper immune serum	-ve
2- Virus with normal horse serum	+ve (typical signs of rabies with 100% deaths 5-7days post inoculation

Data presented in table (2) shows that the propagated virus was clearly identified as rabies virus strain by mice neutralization test using equine antirabies hyper immune serum.

Table (3): Time of inactivation :

Vaccine Type	Log_{10} $MICLD_{50}$/0.03ml		
	Before treatment	treatment 3 hours	4hours
SMBV	8.2	<1.0	0.0

Data presented in table (3) shows clearly that complete inactivation of the suckling mouse brain virus is at approximately 4 hours of inactivation using 1/4000 beta-propiolactone (BPL) at 37C in water bath.

Table (4): The quality control of the prepared three types of SMB vaccines.

Virus Code No.	Types of vaccines	Sterility Tests		Safety Tests	
		Thioglycolate broth	Sabaroud's agar	I/P Mice	I/C Mice
SMB6	Plain SMBV	No turbidity	No colonies	No detah	No detah
Vit E + Sel. Adj.	Vit.E + selenium adjuvant	No turbidity	No colonies	No death	ND*
SMB6 N.Sativa Adj.	SMBVN igella sativa adjuvant	No turbidity	No colonies	No death	ND*
SMB6	SMBV				

Data depicted in table (4) shows that the prepared vaccines are sterile (when inoculated on specific media for cultivating aerobic, anaerobic and fungi) and safe when injected in mice I/P & I/C.

Table (5): The Potency test of the prepared plain inactivated S.M.B. rabies vaccine against another inactivated and adjuvanted T.C. rabies vaccines that was commercially available for human use.

Dil	Plain suckling brain vaccine			Commercial vaccine*		
	mice survived	Total no. of mice	Log_{10} of ED_{50} end point dilution	mice survived	Total no. of mice	Log_{10} of ED_{50} end point dilution
$10^{-0.7}$	9	9		9	9	
$10^{-1.4}$	7	8	2.25	8	9	2.142
$10^{-2.1}$	5	9		4	7	
$10^{-2.8}$	2	7		1	9	

Data presented in table (5) shows that the prepared plain inactivated suckling mouse brain vaccine has a R.P. (2.25) higher than that of the commercial one.

Table (6) : The optical densities (O.Ds) of the collected sera from vaccinated mice with different prepared SMBV.

Dil	Plain SMBV*		ES-SMBV**		NS-SMBV***	
	14 d.p.i	21 d.p.i	14 d.p.i	21 d.p.i	14 d.p.i	21 d.p.i
1/10	1/711	1/713	1/601	1/727	1.621	1.569
1/20	1.364	1.484	1.574	1.566	1.388	1.328
1/40	1.242	1.343	1.445	1.523	0.929	1.072
1/80	0.745	0.762	0.839	0.808	0.459	0.416
1/160	0.493	0.485	0.505	0.432	0.426	0.225
1/320	0.275	0.268	0.394	0.268	0.164	0.120
1/640	0.120	0.135	0.198	0.124	0.081	0.058
1/1280	0.164	0.107	0.116	0.118	0.084	0.053

* The prepared SMBV without any adjuvants.
**The prepared SMBV adjuvanted using combination of vitamin E & selenium.
The prepared SMBV adjuvanted using Nigella sativa oil.***
Cut off value = 0.015

Data presented in table (6) shows clearly that the ODs of the prepared vaccines increased with lower dilutions of collected sera and decreased with higher dilutions. The ODs of collected sera from weaned mice vaccinated with combination of vitamin E & selenium at any used dilutions are higher than these collected from mice vaccinated with either plain vaccine or Nigella sativa adjuvanted vaccine.

DISCUSSION

The scientist exerted great efforts for preparing good immunogenic, safe, efficient, cheap and potent rabies vaccines However, most of these prepared vaccines are of limited use because most of them are not ideal with refer to their quality. keeping control, potency, cost, safety and availability. Although the inactivated cell culture rabies vaccines cause no or fewer reactions than the nervous tissue or the chicken embryo vaccines due

to their relatively free from aggregates that could protect ineffective virus particles, the obtained virus titer require further concentration (Sokol, 1973).

However, suckling mouse brain rabies vaccine (SMBV) often contains very high titers of virus and if properly inactivated is entirely safe for use in any species of animals (Sikes, 1975 and Bear, 1975). Also Diaz, A.M. (1982) encouraging the use of prophylactic course of suckling mouse brain vaccine for many benefits : the sizeable percentage of reactors with a high neutralizing antibody response in a short period of time, the small number of persons requiring a booster dose 30 days after the first vaccine dose, the rapid and intense secondary immune response and the absence of serious post vaccinal reactions. Moreover, Harry, et al. 1984 said that suckling mouse brain and fetal bovine kidney cell rabies vaccine were both equally efficacious and well tolerated. In view of the simple technology required and the resultant lower cost, the SMBV was being recommended for production and use in post exposure treatment in Nigeria and also, (Favi, et al. 2004) compared the immunogenic capacity of antirabic vaccines (suckling mouse brain vaccine) and Verorab (tissue culture vaccine) that was used in pre exposure prophylaxis in human using RFFIT, it was found that at day 42 days post inoculation no difference were observed in the two groups vaccinated with either vaccine

Most of the cell culture vaccines commercially produced for animal and human were adjuvanted (principally with aluminium salts) that held the antigen at its site of deposition, delaying its adsorption and subsequently released antigen in a deduced secondary response (Glenny, et al. 1931 and Nakashima, et al. 1981. Therefore, the present study is conducted to prepare a modified Suckling Mouse Brain Vaccine for rabies virus inactivated using 1/4000 beta-propiolactone, and adjuvinated with Nigella sativa oil and or combination of vitamin E and selenium. Then the different prepared types of inactivated rabies vaccines were subjected to quality control tests and compared with the inactivated tissue culture rabies vaccine that adjuvanted with aluminium hydroxide and available commercially in Egypt for human use, on the basis of its efficacy and potency.

The choose of these natural immunostimulants depends upon :

1) Its effective stimulation of the immune cells like macrophage (Basil and Erwa 1990) and T-lymphocyte (El-Kadi, et al. 1990) as shown in Nigella sativa adjuvanted vaccines.

2) The powerful role of the combination of vitamin E & selenium as good immunopotentiators protecting the sensitive, rapidly proliferating cells of the immune system from oxidation damage and increase cell-cell interaction by membrane alteration (Tengerdy and Lacetera

1991). Also the immune responses investigated by the lymphoproliferative assays was found to be enhanced significantly when vitamin E was injected intraperitoneally in mice (Yasunaga, et al. 1982) and its significant enhancement for the formation of IgM & IgG in contrast to alum. (Inagaki, et al. 1984).

3) To overcome the disadvantages of the aluminium hydroxide salts that was shown by Redhead, et al. 1992 to cause a transient rise in the level of brain tissue aluminium that peaks around the second and third day after intraperitoneal injection of alum adsorbed vaccines into mice and this rise has not been seen in saline control group and with vaccine not containing aluminium. Also Jefferson, et al. (2004) noticed that alum adsorbed vaccine associated with local pain lasting up to 14 days in older children administered such vaccines. More over, Verdier, et al. (2005) observed histopathological lesions, similar to the Macrophagic Myofasciitis (MMF) described in humans, and was still present 3 months after aluminium phosphate and 12 months after aluminium hydroxide adjuvanted vaccine administration.

For achieving the aim , CVS rabies virus strain was propagated for 6 passages in suckling mice brains and titration was done in weaned mice to determine the highest titer of the virus which could be obtained after the 5th passage (8.2 $\log_{10}$ $MICLD_{50}$ / 0.03 ml) and this result is in agreement with that recorded by (Sikes and Larghi, 1967) who showed that suckling mice 3-4 days old when inoculated with 0.01 ml of fixed rabies virus had infectivity titer of $10^{7.1}$ - $10^{7.7}$ LD_{50} / 0.03 ml.

Data presented in table (1) shows clearly that the master seed virus has 6.84 $\log_{10}$ $MICLD_{50}$ / 0.03 ml after three passages in suckling mice from the original strain. The identified 2nd passage that has 8.2 $\log_{10}$ $MICLD_{50}$ / 0.03 ml was used as virus inoculum for vaccine preparation.

Concerning the identity of the seeded virus from contamination with other neurotropic viruses , the CVS rabies virus (100 $MICLD_{50}$ / 0.03 ml) was mixed with equal amount of equine antirabies hyperimmune serum and also with another normal equine serum , incubated for 1.5 hours at 37°C then 5 mice for each mixture were inoculated intracerebrally with a dose of 0.03 ml. The inoculated mice were observed for 21 days post inoculation. The obtained results (table 2) revealed that , no signs were developed on any of the inoculated mice using mixture of the CVS strain with the antirabies hyper immune serum while all mice received the virus with the normal horse serum showed signs of rabies and died after 5 days post inoculation. These results indicated that the seeded virus is a rabies virus and these results agree with that obtained with Johnson, 1973 The prepared suckling mouse brain vaccine (SMBV) could be inactivated efficiently in water bath at

37°C for 4 hours using BPL at a concentration of 1/4000(table 3). These result is in agreement with (Diaz . 1996) who said that SMBV could be inactivated using BPL at concentration of 1/4000 . but disagree with him in time as he stated that complete inactivation was done in 3 hours at 37°C but here it needs 4 hours for complete inactivation and this result could be due to the higher titer of the virus used in this study ($10^{8.2}$ $MICLD_{50}$ / 0.03 ml) compared to that recommended by (Diaz , 1996) which was$10^{7.1}$-$10^{7.3}MICLD_{50}$/0.03ml. Also these results agree with the results obtained by (Sikes and Larghi. 1967) in using BPL in the inactivation of SMB vaccine but it is disagree with them in the concentration of the BPL (they used 1/10000) but in this work was 1/4000 and also disagreed with them in the time of the inactivation in that they used 48 hours at 4°C then make dialyzing in 0.01 M sodium phosphate buffer but here 4 hours at 37°C in water bath was sufficient to preserve the antigenicity of the virus The seeded virus was subjected to quality control tests like Inocuity. safety. sterility and potency as shown in tables (4.5). The relative potency of the prepared inactivated suckling mouse brain vaccine was due to its higher titer of the virus used ($10^{8.2}$ $MICLD_{50}$ / 0.03 ml). and this result is in agreement with (Habel . 1973) who stated that rabies virus is a relatively poor antigen in inactivated vaccines, so the presence in the final vaccine of a large amount of the antigen must be ensured by using a virus source of high titer for inactivated vaccine production. Also, Montano ñ Hirose, et al. 1995 found that SMB vaccines contained considerably higher amounts of N protein than most of the tissue culture vaccines. And this effect was confirmed by Herzog, et al. 1992 who said that suckling mouse brain vaccine could be able to stimulate a secondary antibody response composed of nucleocapsid- specific antibodies and glycoprotein-specific neutralizing antibodies instead of glycoprotein-specific neutralizing antibodies only in case of tissue culture vaccines.

For evaluating the efficacy of the prepared SMB rabies vaccines that was adjuvanted using combination of vitamin E and selenium and /or the Nigella sativa fixed oil , the immunogenicity test was done by inoculating mice and their sera were collected and tested by ELISA for determining the elevated antibody titers because there is no available reference SMB vaccine in hand and used in the NIH test to compare with it.

The immunogenicity test was done according to regulations of the (European Pharmacopoeia 1998) in controlling inactivated veterinary rabies vaccines and this test was done by vaccinating groups of 5 mice weighing 18g-20g with one fifth the dose recommended for the vaccine and the sera should be collected 14 days post inoculation and examined using the Rapid Fluorescent Focus Inhibition Test (RFFIT). Also (Perrin . et al. 1990) said that the replacement of the in vivo potency test [NIH test] for rabies vaccine evalua-

tion by in vitro methods was discussed by WHO expert working groups. Moreover, Lazarowicz, et al. (1982) stated that the antibody assay in immunized mice used for the NIH test seems to be the best possible manner to determine the potency of inactivated rabies vaccines. The antigenic value of the NIH test does not correlate with the antibody status in immunized cats and dogs but a correlation between antibody titers in mice and dogs appeared to exist. According to the (European Pharmacopoeia 1998) collected sera should be tested using the Rapid Fluorescent Focus Inhibition Test (RFFIT) and because of the absence of the possibility to do this test , we use instead an immuno-capture assay (ELISA) which was available , easy to perform and correlated well with the RFFIT and this was done according to Atanasiu , et al.(1977 , 1986) who reported the early detection of rabies antibody post vaccination by ELISA and suggested that the test may be a good way of testing the efficacy of a given vaccine. Also Mebatsion, et al. (1992) employed the enzyme linked immunosorbent assay (ELISA) and the Rapid Fluorescent Focus Inhibition Test (RFFIT) to detect levels of rabies antibodies in the sera from unvaccinated dogs and Simien Jackal in the Bale Mountains National Park [BMNP] of Southern Ethiopia.

Therefore , ELISA was used in this work to detect the antibodies produced by each type of prepared vaccines. The obtained results (table 6) revealed that the ODs of the prepared SMBV increased with lower dilutions of collected sera and decreased with higher dilutions. The ODs of collected sera from mice vaccinated with SMBV-adjuvanted vitamin E & selenium at any used dilutions are higher than those collected from mice vaccinated with either plain SMBV or Nigella sativa-adjuvanted SMBV. These data agree with Yasunaga, et al. 1982 who said that the immune responses investigated by the lymphoproliferative assays was found to be enhanced significantly when vitamin E was injected intraperitoneally in mice and also Inagaki, et al. 1984 found that vitamin E had a significant enhancement for the formation of IgM & IgG in contrast to alum.

In conclusion , the prepared inactivated SMB vaccine adjuvanted with vitamin E and selenium is considered safe , effective ,potent vaccine and can be used either in human or animals

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