

IN VITRO EFFECT OF DIFFERENT INACTIVATORS ON RABIES VIRUS

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Abstract

Ascorbic acid, Beta propilacton and binary ethylenamine (BEA) were evaluated as inhibitors of rabies' virus (Evely-Retkitincki-Abeleseths skauis) in optimized BHK21 cell cutrures. Ascorbic acid inactivated rabies virus at concentrations of 0.1, 0.5 and 1mg/ ml after 22, 18 and 14 hours at 37° C respectively, while BPL inactivated the virus after 4, 6, 8 hours with concentrations of 1/500, 1/10000, and 1/20000 respectively at 37°C. BEI inactivated the virus after 6 hours when used with 0.3 M. These Inactivators did not affect the antigincity of rabies virus in three prepared experimental vaccine batches. Ascorbic acid appears to be the most preferable one as safe natural non-carcinogenic agent when compared with BPL and BEI.

INRODCTION

Rabies is a highly fatal infectious disease affecting most worm blooded animals including man. It is an infection of the central nervous system which is usually fatal (OIE 2012). El-Gaballi (2008) reported that there is an increasing in the number of bitten persons by street dogs representing a huge problem facing the ministry of health. Number of affected persons was 121509 in 2003. This number reached 207694 during 2007 with a medication cost of 25 million Egyptian pounds. Since rabies is known to be an incurable disease, so vaccination is a powerful tool in combating the disease. WHO (1996) recommended the prevention of usage of all live vaccine to be administrated for vaccination either in humans or in animals to avoid the recurrence vicinal strains. In order to elicit the production of a safe inactivated vaccine for immunization of pet and farm animals has been recommended.

Since rabies virus is highly neurotropic and can cause fatal encephalitis, necessary precautions should be taken while handling. Inactivation of rabies virus is essential for preparation of vaccines, diagnostic reagents and research purposes (Madhusudana *et. al.*, 2004).

The present study aims to comparatively evaluate the inactivation effect of β P; BEI and ascorbic acid on rabies virus to select the best inactivator that provides the safe and potent vaccine.

MATERIALS AND METHODS

1-Rabies virus strains

1.1-Vaccinal strain

Evely-Retkitincki-Abeleseths (ERA) strain of rabies virus adapted on BHK₂₁ cell culture (Wunner, 1985) was supplied by the Department of Pet Animal Vaccine Research (DPAVR); Veterinary Serum and Vaccine Research Institute (VSVRI).

1.2-Challenge strain

Challenge rabies virus strain (C.V.S) was adapted on mice brain. It has a titer of $10^{3.5}$ MLD₅₀ /mice (Minimum lethal dose). This virus was used in the test of National Institute of Health (NIH test) to detect the antigenic value of the experimentally prepared vaccine batches. This virus was supplied by DPAVR.

2- Reference Vaccine

Defensor-R vaccine supplied by Mycoform, U.K. was used as a reference vaccine in NIH test.

3-Baby Hamster kidney cell culture (BHK-21)

BHK-21 cell culture was supplied by DPAVR for propagation of rabies virus and virus titration. These cells were passaged and maintained using minimum essential medium (MEM) supplemented with 10% new born calf serum.

4-Virus titration

Virus titration was carried out using the micro-titer technique according to WHO (1996) and the virus titer was calculated according to Reed and Muench (1938).

5- The influence of cell culture density on the titer of the rabies virus (Multiblicity of infection "MOI")

To determine the optimal concentration of BHK₂₁ cells, which could be infected with ERA strain, different cell densities have been investigated for virus productivity after being infected with the virus. Cells were counted by using modified Fuchs Rosental.

6- Effect of sequential cell growth on rabies virus productivity

Different times of cell culturing have been followed to investigate the optimal time of virus infection that yield the highest virus titer. The cell line has been infected at zero time, 18 hours, 24 hours and 48 hours post culturing.

*Cell virus infection was carried out using a multiplicity of infection (MOI) of 1.04: 1.

7-Chemicals

7.1- Ascorbic acid and copper sulphate

These salts were supplied by Sigma Chemical Company (USA). Stock solutions containing 0.01 M of copper sulphate and 0.5 M of ascorbic acid were prepared and sterilized by membrane filtration. The stock solutions were added to the virus suspension to obtain a final concentration of 5 µg /ml of copper sulphate with 3 concentrations of ascorbic acid (0.1 mg/ml, 0.5mg/ml and 1 mg /ml).

7.2- Beta propiolactone (BPL)

B. propiolactone reinst (2. oxetanone research grade) C₃O₂ of M.W. 72.06 was supplied by SERVA electrophoresis/D. 69115 Heidel barge Carl. Benz str.7. It was prepared according to kopowski (1996) where 3 different molarities (1/5000; 1/10000 & 1/20000) were prepared in double distilled water.

7.3- Binary Ethylenamine (BEI)

Bromo ethylamine hydro bromide 95% f.w. 204.90 HV was supplied by Aldrich chemical Co. Ltd Grillingham Dorest. England. It was prepared according to Girard *et. al.*, (1977) where different molarities (0.01; 0.02 and 0.03) were prepared in 0.5 M of sodium hydroxide.

7.4- Sodium thiosulphate solution

It was used at a final concentration of 2% to stop the reaction of the inactivators (BPL and BEI).

7.5- Aluminum hydroxide gel (AlOH₃)

Aluminum hydroxide gel 2% was supplied by Superfos Biosector, Denmark. It was used as adjuvant to the prepared inactivated rabies vaccine experimental batches.

7.6- Trypan blue stain

5% trypan blue stain was used to determine cell viability and counts during preparation BHK₂₁ cell culture.

8- Preparation of experimental inactivated rabies vaccine batches

Different portions of rabies virus infected fluid (obtained from infected BHK-21 cell culture) were subjected to inactivation processes using the above mentioned chemicals at 37° C with determination of the less time allowing complete virus inactivation. Complete virus inactivation was confirmed by virus titration in cell culture and mice inoculation that revealed zero virus titer and safety for mice. 20% of AlOH₃ was added to the inactivated virus as adjuvant and subjected to quality control tests as recommended by WHO (1996).

9- Mice

Albino Swiss mice, 3-4 weeks old, were bred in the mice colony in the Department of Pet Animal Vaccine Research. These mice were used for titration of CVS virus, test of National Institute of Health (NIH) and safety of the prepared vaccines.

10-Test of the National Institute of Health (NIH)

NIH was carried out to evaluate the antigenic value of the prepared vaccine batches according to WHO (1996) where this value should be not less than 0.3.

RESULTS AND DISCUSSION

Table 1. Effect of initial seeding density of BHK₂₁ cells on the infectivity titre of rabies virus

BHK ₂₁ cell concentration (X10 ⁴ /ml)	Virus infectivity titer (log ₁₀ TCID ₅₀ /ml)	Multiplicity of infection (MOI)
1.5	7.8	5.2:1
1.75	5.2	2.98:1
3.0	6.2	2.06:1
3.75	6.7	1.78:1
7.5	7.8	1.04:1
8.75	4.1	0.48:1

Table 2. Effect of sequential cell growth on ERA rabies virus productivity

Age of cell culture (HPS*)	Virus infectivity titer (log ₁₀ TCID ₅₀ /ml)
Zero	7.8
18 HPS= 60-70 % confluence	7.8
24 HPS= 80-90% confluence	7.8
48 HPS =100% confluence	6.5

*HPS= hours post seeding (Time of incubation)

Table 3. Inactivation of ERA rabies virus by BpL at 37°C

Conc. Of BpL	Virus infectivity titer ($\log_{10}TCID_{50}/ml$) on HPI*												
	0 HPI	2 HPI	4 HPI	6 HPI	8 HPI	10 HPI	12 HPI	14 HPI	16 HPI	18 HPI	20 HPI	22 HPI	24 HPI
1/5000	7.8	2.4	0	0	0	0	0	0	0	0	0	0	0
1/10000	7.8	4.5	2.3	0	0	0	0	0	0	0	0	0	0
1/20000	7.8	5.7	3.5	1.2	0	0	0	0	0	0	0	0	0

*HPI= hours post inactivation

Table 4. Inactivation of ERA rabies virus by BEI at 37°C

Conc. Of BEI	Virus infectivity titer ($\log_{10}TCID_{50}/ml$) on HPI*												
	0 HPI	2 HPI	4 HPI	6 HPI	8 HPI	10 HPI	12 HPI	14 HPI	16 HPI	18 HPI	20 HPI	22 HPI	24 HPI
0.01 M	7.8	6.8	5.4	4.3	3.2	2.6	2.1	1.6	1.2	0	0	0	0
0.02 M	7.8	6.3	4.8	3.1	1.7	1.3	0	0	0	0	0	0	0
0.03 M	7.8	4.6	2.4	0	0	0	0	0	0	0	0	0	0

*HPI= hours post inactivation

Table 5. Inactivation of ERA rabies virus by ascorbic acid at 37°C

Conc. Of ascorbic acid (mg/ml)	Virus infectivity titer ($\log_{10}TCID_{50}/ml$) on HPI*												
	0 HPI	2 HPI	4 HPI	6 HPI	8 HPI	10 HPI	12 HPI	14 HPI	16 HPI	18 HPI	20 HPI	22 HPI	24 HPI
0.1	7.8	7.3	6.8	6.1	5.5	4.2	3.3	2.5	2.0	1.6	1.1	0	0
0.5	7.8	7.1	6.5	5.8	4.6	3.7	2.8	2.1	1.5	0	0	0	0
1.0	7.8	6.5	5.9	5.1	3.5	2.3	1.1	0	0	0	0	0	0

*HPI= hours post inactivation

Table 6. Final evaluation of the prepared inactivated vaccine

Type of vaccine	Safety in mice	Antigenic value by NIH
BpL inactivated vaccine	Safe	2.3
BEI inactivated vaccine	Safe	2.2
Ascorbic acid inactivated vaccine	Safe	2.2
Defensor reference vaccine	Safe	2.5

Concerning the preparation of an inactivated rabies virus for vaccine production, the present work optimized some conditions for yielding a high virus titer to be suitable for preparation of a potent inactivated vaccine.

Table (1) showed the influence of cell density on the replication of ERA rabies virus in BHK₂₁ cells with different concentration (cell seeding densities) reflecting that the virus titer was significantly decreased in culture with the highest cells density (8.75×10^4). Low cell density (1.75×10^4) was not as successful as anticipated, the thing which could be attributed to the high alkalinity required for virus replication affecting cellular division where the used medium for virus replication should be maintained at pH (7.4-7.6) (Wiktor, 1973).

The best cell concentration was found to be from 1.5×10^4 to 7.5×10^4 cells/ml. This result is similar to that obtained by Chapman *et. al.*(1973) and with Wiktor (1973) who found that the range of cell counting of 0.5×10^6 to 2.5×10^6 cells/ ml resulted in increased infective antigen titre and immunogenicity but not in direct proportion.

The yielding of infective rabies virus in BHK₂₁ cells supported these finding as shown in table (2) where the highest titer was obtained when the virus infection of the cells was being carried out at 0; 18 and 24 hours post seeding of the cells where the cells did not reach a complete confluence. The lower infectivity titre was obtained when the virus infection of the cells was done at 48 hours post seeding with complete confluence of cell sheet. These results came to be confirmed by Wiktor and Clark (1973) and OIE (2012) who mentioned that the total virus yield are often higher when cells are infected in suspension and then plated and allowed to grow to confluence, than in case of infection of already confluent cell sheet.

The importance of inactivated infectious viruses in preparation of inactivated vaccines or in diagnostic reagents cannot be minimized, since various degree of laboratory safety guidelines are practiced around the world (White *et. al.*, 1986).

Through the present study two types of alkylating agents were used (BPL and BEI) in comparison to Ascorbic acid. Most human commercial inactivated vaccines are inactivated by BPL (OIE, 2012). BPL acts as nucleic acid denaturant, as a result of alkylation of imidazol functional groups in the viral nucleic acid as described by Fishbein *et. al.*, (1970).

As shown in table (3); 3 concentrations of BPL were used providing complete virus inactivation achieved after 4, 6 and 8 hours with BPL final concentrations of 1: 5000, 1:10000 and 1:20000 respectively. The complete virus inactivation was confirmed by cell culture infection (showing no CPE) and inoculation of weaned mice through intracerebral route (showing no rabies signs). In this respect; Nicolas *et.*

al.(1978) used BPL for preparation of inactivated rabies vaccine for human use at 1:4000 for 24 hours at 4°C and 2 hours at 37°C, while Sazonova *et. al.*(1980) inactivated Moscow strain in cell culture at a dilution of 1/8000 for 3 hours at 4° C and 2 hours at 20° C. Attyat (1988) used 1:1000 BPL to inactivate mice brain adapted rabies virus for 6 hours at 4°C. These results confirmed that BPL inactivation rate differs according to the temperature of inactivation and type of the used tissue for virus propagation.

BEI was used for inactivation of rabies virus after cyclization of 2-bromoethylamine hydrobromide in alkaline solution (sodium hydroxide) with different concentrations as shown in table (4) including 0.01M ; 0.02M ; 0.03M. Complete inactivated rabies ERA virus was obtained after 18, 12 and 6 hours post treatment with such molarities respectively. These results were confirmed by cell culture infection (showing no CPE) and inoculation of weaned mice through intracerebral route (showing no rabies signs).

Depending on the obtained results, it could be deduced that inactivation rate of rabies virus differs according to the nature of infected tissue where Larghi and Nebel (1980) used BEI with a final concentration of 1% after 3 hours.

BEI and BPL have been used satisfactorily but their use in mass production lods put the producer at a risk because they are carcinogenic materials as concluded by Nitert *et. al.*(1974) and White *et. al.*(1986).

So, there is a need to find an alternative inactivating agent which is not expensive and always available as ascorbic acid (vitamin C) which is undergoing autooxidation results in the formation of OH groups that could induce the virus inactivation (Murata *et. al.*, 1986). Table (5) showed that 3 different concentrations of ascorbic acid (0.1; 0.5; and 1mg/ml) resulted in complete virus inactivation after 22, 18 and 14 hours respectively at 37°C. Similar findings were reported by White *et. al.*(1986) who used ascorbic acid to inactivate para influenza type 2 within 24 hours at 37°C with 1 mg ascorbic acid/ml. The loss of infectivity did not alter either the haem-agglutination or complement fixation qualities of the antigen.

Madhusudana *et. al.*, (2004) inactivated rabies virus (CVS) strain on VERO cell culture with ascorbic acid at a concentration of 0.5 mg/ml after 72 hours at 4°C and 1 mg/ml after 48 hours.

The adsorbance of rabies virus by Alhydra gel was done at a final concentration

20 % resulted in agreement with Larghi and Nebel (1980). The test of National Institute of Health (NIH) was carried out for testing the potency of the three prepared inactivated rabies vaccine experimental batches. As shown in table (6), the 3

preparations induced high potency with antigenic value ranged from 2.2 to 2.3 and that of the reference vaccine was 2.5. These values appear to be higher than the minimum requirement antigenic value (0.3) recommended by WHO (1996).

It could be noticed that there is no difference between the effects of the different methods used for inactivation of rabies virus on the potency of the prepared vaccines except the safe use of ascorbic acid in inactivation process. These results are similar to those obtained by Madhusuana *et. al.*, (2004).

From the present obtained results it could be concluded that ascorbic acid has a potential for being used as safe inactivating agent without affecting the antigenicity of the rabies virus.

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تأثير بعض المثبطات علي فيروس السعار معمليا

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تمت خلال هذه الدراسة المقارنة بين ثلاث مثبطات لعنزة لقاح السعار (ERA) المؤقلم على المزارع النسيجية لكلى اليربوع السورى الذهبى (BHK₂₁) بعد ضبط ظروف التزريع من حيث كثافة وعمر المزارع النسيجية حيث أوضحت الدراسة أن أنسب تركيز للخلايا يتراوح بين ١٥-٧ و ١٠×^٤ خلية/مل وقت التزريع أو بعد ٢٤ ساعة من التزريع . كما أوضحت الدراسة أن حامض الأسكوربيك (فيتامين سى) يحدث تثبيطا كاملا للفيروس بعد ٢٢، ١٨ و ١٤ ساعة عند استخدام تركيزات ٠،٠٥ و ١مل جرام/مل على التوالي بينما احدثت تركيزات ١:١٠٠٠٠، ١:٥٠٠٠، ١:٢٠٠٠٠٠ من البيتا بروبيولاكتون تثبيطا كاملا للفيروس بعد ٤، ٦ و ٨ ساعات فى حين أعطى ٠٣ مولاريتى من البيينارى إيثيلين أمين تثبيطا كاملا للفيروس بعد ٦ ساعات وقد أجريت جميع عمليات التثبيط عند درجة حرارة ٣٧ °م . وبتحضير ثلاث لقاحات تجريبية تبين أن كل منها آمن وفعال معطيا قيما أنتيجينية عالية طبقا لاختبار معهد الصحة القومى . وعلى ذلك يمكن القول بأن المثبطات الثلاثة صالحة لإنتاج لقاح سعار مثبط إلا أن أكثرها آمانا حامض الأسكوربيك كمادة طبيعية خلاف المثبتين الآخرين ذوى التأثير المسرطن .