Global Veterinaria 13 (5): 774-778, 2014
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2014.13.05.86164

Comparative Studies on the Inactivation Effect of Ascorbic Acid and Binary Ethylemaine (BEI) on Rabies Virus

[1]Hemmat S. Elemam and [2]Abeer A. Boseila

[1]Department of Pet Animal Vaccine Research (DPAVR),
Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt
[2]Department of Viral Control and Research, National Organization for Drug Control and Research (NODCAR), 6 Abou Hazem St., Pyramids Ave., Giza 12553, Egypt

Abstract: The current used inactivating agent for rabies virus, Binary ethylemaine (BEI) is a very expensive carcinogenic agent, this thing directed the attention to evaluate an alternative natural agent which can inactivate the virus without affecting its antigenicity. Ascorbic acid was of choice in the present work where the inactivation effect of three concentrations (0.1; 0.5 and 1mg/ml) were tested in comparison with BEI. It was found that 3% of 0.03M-BEI induced complete virus inactivation of fixed rabies virus (ERA) strain propagated on BHK_{21} cell culture within 4 hours at 37°C while 0.1; 0.5 and 1mg/ml of ascorbic acid induced complete virus inactivation within 144; 72 and 63 hours at 37°C respectively. Two experimental rabies vaccine batches were prepared from the obtained BEI and 0.5mg ascorbic acid/ml inactivated virus portion adjuvant with 20% Alhdragel where all of these preparations were found to be free from foreign contaminants; safe and potent. The results of the potency test revealed that these vaccine batches had antigenic values 0.8 and 1.0 respectively while the reference vaccine had an antigenic value 1.1. These findings indicate non-significant differences between the determined antigenic values obtained by BEI and ascorbic acid inactivated vaccines suggesting that ascorbic acid can be used as an inactivating agent for fixed rabies virus grown in cell culture for preparation of safe potent inactivated rabies vaccine.

Key words: Rabies Virus · Inactivation · Ascorbic Acid · Vaccine Preparation

INTRODUCTION

Rabies is an acute highly fatal infectious disease affecting all worm blooded animals and man. Rabies is usually manifested by fatal encephalomyelitis; signs of mania; motor irritation; inability of swallowing; ascending paralysis which started from the hind limbs then moving forward (trunk and fore limbs) then recumbence and ends with death [1]. The disease is of worldwide distribution and of economic public health significance in many countries. It is caused by a filterable virus that belongs to family *Rhabdoviridae* [2].

Rabies experts estimate the disease kills 55,000 people each year in Africa and Asia alone and seems on the rise in China, the former Soviet Republics, southern Africa and Central and South America [3].

Regarding Egypt, rabies is enzootic in jackals and common in dogs as reported by Thomas and Rivers [3]. El-Kanawati *et al*. [5] concluded that dogs and wolves are the primary vector animal for transmission of rabies to cattle in the Middle East.

An essential step for controlling such disease is the prophylactic and emergency vaccination using specific potent vaccines. Inactivated cell culture rabies vaccines became of wide use where they were found to be completely safe in human and animal use [6-9].

Virus inactivation is a process where the virus loss its infectivity and its ability to replicate. Such process could be carried out by using chemical and natural agents. Various methods, which are still valid, have been used to render the viruses non- pathogenic or essentially inactivated (Killed) as vaccines. These include, but are

Corresponding Author: Abeer, A. Boseila, Department of Viral Control and Research ,
National Organization for Drug Control and Research (NODCAR),
6 Abou Hazem St., Pyramids Ave., Giza 12553, Egypt.
Tel: +2-02-35851278, Fax: +2-02-35855582.

not limited to beta-propiolactone (BPL), UV light and acetylethylamine as well as other amines. Phenol and formaldehyde are no longer recommended for virus inactivation [10]. ß-propiolactone, similarly to pepsin, can also reduce efficiently the complement activation by the tested sera nevertheless; the ß- propiolactone treatment didn't alter neutralization titers, while considerable reduction was observed after treatment with pepsin [11]. Attyat [12] and Naglaa [13] prepared inactivated vaccines from mice brain using Beta-probiolactone (BPL) and binary ethyleneimine (BEI) respectively.

It is well known that both of BPL and BEI are carcinogenic agents, so many vaccine producers directed their research to find a natural agent provides complete safe virus inactivation. One of these natural inactivators was found to be ascorbic acid as reported by [11, 14] indicating safe inactivation of human immunodeficiency and rabies viruses without any adverse effect on their antigenicity.

The present work aims to evaluate the use of ascorbic acid as a natural inactivator to prepare an inactivated cell culture rabies vaccine in a comparison with that vaccine prepared by BEI inactivated virus.

MATERIALS AND METHODS

Cell Culture: Baby hamster kidney cell culture (BHK_{21}) established by Mackpherson and Stocker [15] used for propagation and titration of rabies virus.

Virus Strains

Evely-Retkitincki-Abeleseths (ERA): ERA strain of rabies virus adopted on BHK_{21} cell culture [16] was supplied by the Department of Pet Animal Vaccine Research (DPAVR); Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt.

Challenge Virus Strain: Challenge rabies virus strain (C.V.S) was adopted on mouse brain. It has a titer of $10^{3.5}$ MLD_{50} / 0.03 ml (Minimum lethal dose fifty). This virus used in the test of National Institute of Health (NIH test) to detect the antigenic value of the experimental prepared vaccine batches. This virus was supplied by DPAVR, Abbasia, Cairo.

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

Preparation of Rabies Virus Suspension: In order to prepare the virus suspension, ERA virus was replicated at multiplicity of infection (MOI) rate of 2:1 of virus/BHK_{21} cells.

Virus Titration: Titration of the obtained virus was performed using the micro-titer technique and the virus titer was calculated according to Reed and Meunch [17].

Chemicals

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 1mg/ml) according to Madhusudana *et al*. [11].

Binary Ethylenamine (BEI): Bromo ethylamine hydro bromide 95% f.w. 204.90 lot 08517 HV was supplied by Aldrich chemical Co. Ltd Grillingham Dorest. England. It was prepared according to Girard *et al*. [18] with molarity of 0.03 in 0.5 M of sodium hydroxide.

Sodium Thiosulphate Solution: It was used at a final concentration of 2% to stop the reaction of the inactivator (BEI).

Aluminum Hydroxide Gel ($ALOH_3$): Aluminum hydroxide gel 2% was supplied by Superfos Biosector, Denmark. It was used as adjuvant to the prepared inactivated rabies vaccine experimental batches.

Virus Inactivation:

Virus Inactivation by Binary Ethylene Amine (BEI): BEI was added at 37°C to the viral suspension with a final concentration of 3% of 0.03M. The mixture was stirred continuously at 37°C according to W.H.O. [19]. Inactivation process was stopped by addition of cold sodium thiosulphate with a final concentration of 2%.

Virus Inactivation by Ascorbic Acid: Three concentrations of ascorbic acid (0.1 mg/ml, 0.5mg/ml and 1 mg /ml) were added to three portions of rabies virus suspension with stirring continuously at 37°C according to Madhusudana *et al.* [11].

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

Preparation of Experimentally Inactivated Rabies Vaccine Batches: Two portions of inactivated rabies virus infected fluid was prepared (one was inactivated with BEI and one portion was inactivated with 0.5mg ascorbic acid/ ml) 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 $AlOH_3$ was added to the inactivated virus as adjuvant and subjected to quality control tests as recommended by W.H.O. [19] and Edries [20].

Quality Control Tests: The prepared experimental inactivated rabies vaccine batches were subjected to quality control tests including the freedom of foreign contaminants; safety and potency tests according to W.H.O. [19].

Test of the National Institute of Health (NIH): NIH was carried out to evaluate the antigenic value of the prepared vaccine batches according to W.H.O. [19] where this value should be not less than 0.3.

RESULTS AND DISCUSSION

Vaccine producers usually search to find safe inactivating agents to avoid the possible carcinogenic hazard of chemical substances directing their effort to natural materials. Earlier studies have indicated that ascorbic acid can be used as inactivating agent for both RNA and DNA viruses [21-23].

The obtained results through the present work revealed that BEI induced complete inactivation of rabies virus within 4 hours of inactivation at 37°C as shown in table (1). Similar results were obtained by Naglaa [13], Edries [20] and Amany [24]. Complete loss of rabies virus infectivity as demonstrated by cell culture and mice infections revealed that 0.1mg ascorbic acid/ml had no appreciable reduction in the virus infectivity titer which remained at high titer (4.5 log10$TCID_{50}$/ml) up to 144 hours (table-2) while complete loss of virus infectivity by 0.5 mg ascorbic acid/ml was evident at the end of 72-84 hours (table-3). With a concentration of 1mg ascorbic acid/ ml the virus infectivity titer dropped to half value by 24 hours to reach complete inactivation by 30-36 hours (table-4).Murata *et al*. [25] showed that the presence of oxygen is essential and ascorbic acid undergoing auto-oxidation results in the formation of OH groups that could bring about the inactivation of the cell free viruses. These findings come in complete agreement with those of Madhusudana [11] who showed that the minimum effective concentration of ascorbic acid was found to be 0.5mg/ ml without significant fall in pH while increasing the concentration fall in pH causing sloughing of Vero cells during titration. Also Bhupat *et al.* [14] mentioned that the presence of ascorbic acid in the medium has been reported to inhibit reverse transcriptase (RT) activity in vitro with consequent suppression of CAHIV replication in chronically infected cells. A concentration of 0.5mg/ml of ascorbic acid was therefore used for preparation of the experimental inactivated rabies vaccine which subjected to quality control tests in comparison with BEI inactivated vaccine and the reference vaccine. These tests revealed that such vaccine are free from foreign contaminants and safe inducing no clinical signs in inoculated mice in a parallel manner to the recommendations of W.H.O. [19]. Regarding the potency of the prepared experimental batches of inactivated rabies vaccine, as tested by NIH (table-5), it was found that the BEI and ascorbic acid vaccines had antigenic values 0.8 and 1.0 respectively while that of the reference vaccine was 1.1. These values appear to be higher than the recommended one which must be not less than 0.3 as reported by W.H.O. [19] and Larghi and Nebel [26].

Table 1: Inactivation of rabies virus by BIE

Period of the inactivation process (HPI*)	Virus titer (log_{10}/ml)	Loss in virus titer (log_{10}/ml)
0	7.5	0
1	6	1.5
2	3	3
3	1.5	1.5
4	0	0

*HPI= hours post inactivation

Table 2: Inactivation of rabies virus by 0.1mg ascorbic acid / ml

Period of the inactivation process (HPI*)	Virus titer (log_{10}/ml)	Loss in virus titer (log_{10}/ml)
0	7.5	0
24	7.0	0.5
48	6.5	0.5
72	6.0	0.5
96	5.5	0.5
120	5.0	0.5
144	4.5	0.5

*HPI= hours post inactivation

Table 3: Inactivation of rabies virus by 0.5mg ascorbic acid / ml

Period of the inactivation process (HPI*)	Virus titer (log_{10}/ml)	Loss in virus titer (log_{10}/ml)
0	7.5	0
12	6.25	1.25
24	5.75	1.25
36	4.5	1.25
48	3.25	1.25
60	2.0	1.25
72	0.75	1.25
84	0.0	0.75

*HPI= hours post inactivation

Table 4: Inactivation of rabies virus by 1mg ascorbic acid / ml

Period of the inactivation process (HPI*)	Virus titer log_{10}/ml)	Loss in virus titer log_{10}/ml)
0	7.5	0
12	6.0	1.5
18	4.5	1.5
24	3.0	1.5
30	1.5	1.5
36	0.0	0.0

*HPI= hours post inactivation

Table 5: Antigenic value of the prepared experimental batches of inactivated rabies vaccine batches

Tested vaccine inactivated with	NIH antigenic value
3% of 0.03 M-BEI	0.8
0.5 mg ascorbic acid/ml	1.0
Reference vaccine	1.1

NB. The antigenic value of inactivated rabies vaccine should not be less than 0.3

In addition, chemicals like formaldehyde and phenol not only inactivate the virus but also adversely affect its antigenicity [11]; beta propiolacton (BPL) and Binary Ethylenamine (BEI) are very expensive chemicals and potentially carcinogenic [27].

CONCLUSION

Depending on the present obtained results, it could be concluded that a cheaper natural material, safe and easily available is ascorbic acid which is suitable for preparation of safe potent inactivated rabies vaccine.

REFERENCES

1. Bear, G.M., 1975. The natural history of rabies. Academic press, New York.
2. Hummeler, K., N. Tommassini, K. Sokol, F. Kumert and H. Koprowski, 1968. Morphology of nucleoprotein component of rabies J. virol., 2: 1191-1194. W.B. Saunders Company USA, pp: 751.
3. Dolittle, E., 2012. New rabies vaccine. www.arkive.org
4. Thomas, M. and A. Rivers, 1952. Viral and Rickettsial infections. J.B. LIPPI, N.Cott Comp. 2nd ed. USA, pp: 267-296.

5. El-Kanawati, Z.R., Ikram, A. Karim, Afaf, Amin and A.A. El-Ebeedy, 2000. Occurrence of rabies in Egypt during 1997-1999. J. Egyp. Vet. Med. Ass., 60: 47-54.
6. Barnas, G.P., 2001. Rabies vaccination. WHO position, weekly epidemiological record, 77: 109-120.
7. Green, C.E., 2006. Rabies and other lyssa virus infections, In Infectious Diseases of the dog and cat. 3rd Ed. Chapter 22, pp: 168-172, Philadelphia, W.B. Saunders, Co.
8. Khodeir, M.H. and A.M. Daoud, 2008. Preparation of antirabies hyperimmune serum for emergency immunization of farm animals. 4th Int. Sci. Conf. NRC, pp: 1-9.
9. Albehwar, A.M.A., 2009. Studies on prophylactic and emergency vaccination of farm animals against rabies. Ph.D.Thesis (Infectious Diseases) Fac. Vet. Med. Cairo Univ.
10. Reculard, P., 1996. Cell culture vaccines for veterinary use In: laboratory techniques in rabies 4th ed (F-x Meslin; M.M. Kaplan and Koprowski, eds) pp: 314-323. Geneva: world health organization.
11. Madhusudana, S.N., R. Shamsundar and S. Seetharaman, 2004. In vitro inactivation of the rabies virus by ascorbic acid. Int. J. Inf. Dis. 8: 21-25.
12. Attyat, A.K., 1988. Studies on preparation of mice encephalon inactivated anti-rabies vaccine. M.V.Sc. Thesis, Microbiology, Fac. Vet. Med., Cairo Univ.
13. Naglaa, I.A., 1996. Comparative studies on some adjuvants used in production of inactivated anti-rabies vaccines. M. Vet. Sci. Thesis (Virology) Fac. Vet. Med. Cairo Univ.
14. Bhupat D. Rawatt, Francesco Bartoiini and Girish N. Vyas, 1995. In vitro inactivation of human immunodeficiency virus by ascorbic acid. J. of International Association of Biological Standardization, 23: 75-81.
15. Mackpherson, I. and M. Stocker, 1962. Polyma transformation of hamster cell clones. An investigation of genetic factors affecting cell competence. Nature, London 201: 1251-1256.
16. Wunner, H., 1985. Growth purification and titration of Rhabdo viruses in Mahy Bud, editor, virology : a practical approach. Oxford: IRL press, pp: 79-83.
17. Reed, L.J. and J. Muench, 1938. A simple method of estimating fifty percent points. Am. J. Hyg., 27: 493- 497.
18. Girard, H.C., O.I. Bayramoglu, ErLN and Burghut, 1977. Inactivation of viruses by binary ethylenimine. Bull. Int. Ipid. 87: 201-207.
19. WHO, 1996. WHO monograph "Laboratory techniques in rabies" 3rd Ed Geneva, world Health organization series, 23 : 101-123.
20. Edries, S.M., 1994. Production of inactivated tissue culture rabies vaccine. Ph.D. Thesis, Fac. Vet. Med., Virology, Cairo Univ.
21. Turner, G.S., 1964. Inactivation of vaccina virus by ascorbic acid. J. Gen microbial, 35: 75-80.
22. White, L.A., C.Y. Freeman, B.D. Forester and W.A. Chapel, 1986. In vivo effect of ascorbic acid on infectivity of herpes viruses and paramyxoviruses. J. Clin. Microbiol., 4: 527-531.
23. Rawal, B.D., F.Bartolini and G.N. Vyas, 1995. In vitro inactivation of human immunodeficiency virus by ascorbic acid. Biologicals. Mar, 23: 75-81.
24. Amany M. Abbas, 2008. Effect of some inactivators on the immunogenicity of rabies virus vaccine. M. Vet. Sci. Thesis (Virology) Fac. Vet. Med. Benha Univ.
25. Murata, A., M. Kawaaki, H. Motomatsu and F. Kato, 1986. Virus inactivating effect of D-isoascorbic acid. J. Nutr. Sci. Vitaminol., 32: 559-667.
26. Larghi, O.P and A.E. Nebel, 1980. Rabies virus inactivation by binary ethylenimine. New method for inactivated vaccine production. J. Clin Microbiol, 2: 120-122.
27. Nietert, W., L. Kellicut and H. Kubinski, 1974. DNA protein complexes produced by a carcinogenic B-propiolacton. Cancer Res., 34: 859-861.