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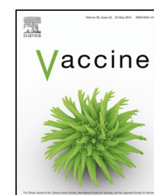

Inactivation of rabies virus by hydrogen peroxide

Q1 Asmaa A. Abd-Elghaffar[a], Amal E. Ali[b,*], Abeer A. Boseila[c], Magdy A. Amin[b]

[a] Viral Control Unit, National Organization for Research and Control of Biologicals (NORCB), 51 Wezaret El-Zeraa Street, Agouza, Giza, Egypt
[b] Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Kasr Al-Aini Street, Cairo 11562, Egypt
[c] Viral Research and Control Lab., National Organization for Drug Control and Research (NODCAR), Kafr El-Gabal, Giza, Egypt

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ABSTRACT

Development of safe and protective vaccines against infectious pathogens remains a challenge. Inactivation of rabies virus is a critical step in the production of vaccines and other research reagents. Beta-propiolactone (βPL); the currently used inactivating agent for rabies virus is expensive and proved to be carcinogenic in animals. This study aimed to investigate the ability of hydrogen peroxide (H_2O_2) to irreversibly inactivate rabies virus without affecting its antigenicity and immunogenicity in pursuit of finding safe, effective and inexpensive alternative inactivating agents. H_2O_2 3% rapidly inactivated a Vero cell adapted fixed rabies virus strain designated as FRV/K within 2 h of exposure without affecting its antigenicity or immunogenicity. No residual infectious virus was detected and the H_2O_2-inactivated vaccine proved to be safe and effective when compared with the same virus harvest inactivated with the classical inactivating agent βPL. Mice immunized with H_2O_2-inactivated rabies virus produced sufficient level of antibodies and were protected when challenged with lethal CVS virus. These findings reinforce the idea that H_2O_2 can replace βPL as inactivating agent for rabies virus to reduce time and cost of inactivation process.

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1. Introduction

Q3 Rabies is considered one of the ancient diseases known to human race. Rabies is acute, progressive viral encephalitis caused by rabies virus which is classified as single stranded negative sense RNA genome enclosed into lipoprotein envelope. It belongs to *Mononegavirales* order, *Rhabdoviridae* family and the *Lyssavirus* genus [1].

Rabies transmission from animal to animal or to human occurs by exposure to saliva mainly through animal bites. Other sources of viral infection may include exposure to scratches, immunization with incompletely inactivated vaccine, transplantation of virus infected organs and virus inhalation. Rabies is a disease infecting mammals involving dogs, wolves, foxes, raccoons, cats and bats which serve as reservoirs or vectors [2].

Rabies is considered as a worldwide serious public health problem, it is a lethal disease as once the manifestations appear; death is inevitable. Rabies disease cannot be under estimated as according to WHO; the annual number of human deaths by rabies globally was estimated in 2010 to be from 26,400 to 61,000 with estimated annual cost of rabies about 6 billion US$. Approximately 15 million people receive post-exposure vaccine treatment every year after being exposed to rabies-suspect animals. Asia and Africa recorded the highest number of cases (95%) and the majority of death incidences (84%) occur in rural areas especially in children [3,4]. Therefore, the development of vaccines, diagnostic tools and research is continuous in this field.

Rabies virus inactivation is a critical step in production of vaccines and diagnostic reagents. The recommended inactivating agent used in rabies vaccine production is beta-propiolactone (βPL), but it is a very expensive chemical and proved to be potential carcinogen. *In vitro* studies on nucleic acids extracted from *Escherichia coli* showed that βPL produces complexes between DNA and proteins [5]. Many studies in different experimental animals showed that βPL caused tumors at various tissue sites, and by different routes of exposure. A study showed that oral exposure to βPL caused squamous cell carcinomas in rats. When topically administered in mice and guinea pigs, skin tumors have been reported. Upon subcutaneous injection; local tumors have been observed at injection site. Also lymphomas and hepatomas have been observed in mice after intraperitoneal injection with βPL [6]. Inhalation of βPL developed nasal cancer in experimental rats [6,7]. Other

Q2 * Corresponding author. Present address: Department of Microbiology and Immunology, Faculty of Pharmaceutical Sciences & Pharmaceutical Industries, Future University in Egypt, Egypt. Tel.: +20 223639307.
E-mail addresses: Ph.asmaa.ahmed@gmail.com (A.A. Abd-Elghaffar), amal.ali@pharma.cu.edu.eg, amal.emad@fue.edu.eg (A.E. Ali), abeerboseila@gmail.com (A.A. Boseila), magdy.amin@pharma.cu.edu.eg, magdy_biotek3@yahoo.com (M.A. Amin).

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chemicals like formaldehyde and phenol are proved to adversely affect virus antigenicity. Thus, it is necessary to find replacement inactivating agents which are not expensive, safe and available [1].

Hydrogen peroxide (H_2O_2) is a strong oxidizing agent. Many studies investigated its ability to preserve antigenic epitopes and other studies used it as inactivating agent for development of viral vaccines against both DNA and RNA viruses including lymphocytic choriomeningitis virus (LCMV), yellow fever virus (YFV), West Nile virus (WNV), Vaccinia virus (VV) and monkeypox virus (MPV) [8–10].

Keeping this in mind, our study aimed to investigate the capability of H_2O_2 in causing a complete inactivation of fixed rabies virus without adversely affecting its antigenicity and immunogenicity.

2. Materials and methods

2.1. Rabies virus

Fixed Pasteur strain designated as FRV/K adapted on Vero cells and concentrated 20-fold by ultra filtration by Prof. Dr. Rifky El-Karamany (ex-GM of R&D sector, VACSERA) [11] was a generous gift from rabies vaccine research unit, VACSERA.

2.2. Reference rabies vaccine standard

Lyophilized rabies vaccine produced by National Institute for the Control of Pharmaceutical and Biological Products, China was used as a reference standard. Its labeled potency was of 6.6 IU/ml.

2.3. Challenge virus strain (CVS)

Fixed rabies virus strain derived from the original Pasteur strain, was propagated and fixed in mice brain. The virus strain was obtained from Pasteur Institute, France in freeze-dried form.

2.4. Mice

Swiss albino mice were bred and housed in laboratory animal facility, VACSERA. Animals handling was according to guidelines [12,13]. Permission to conduct the study was obtained from Ethics Committee in Faculty of Pharmacy, Cairo University.

2.5. Determination of the infectivity titer of FRV/K rabies virus suspension

The virus suspension infectivity titer was determined by mouse inoculation test [14] where serial tenfold dilutions of the virus suspension were prepared in virus diluent (0.9% saline (Otsoka, Egypt) + 2% FBS (Biowest, France) and inoculated intracerebrally in groups of 10 Swiss albino mice strain with each dilution, each mouse receiving 0.03 ml. The mice were observed for 14 days and number of mice died from rabies or showing signs of rabies virus infection (shaky movement, convulsions or paralysis) in each dilution was recorded after the first five days. The 50% mouse lethal dose ($MICLD_{50}$) of the virus strain was calculated using the method of Spearman and Kärber [15].

The titrated virus suspension was aliquoted and stored in −80 °C freezer (NuAire, USA).

2.6. Inactivation kinetics of FRV/K rabies virus using H_2O_2

A 30% stock solution of H_2O_2 (Carl Roth, Germany) was sterilized by membrane filtration and kept in a dark sealed container. The virus suspension was treated with H_2O_2 stock solution to a final concentration of 3% and was kept at 2–8 °C refrigerators (SANYO, Japan). An aliquot was withdrawn from the bulk suspension every 30 min for 2 h and treated with catalase at 12.5 U/ml final concentration (MP Biomedical, USA) for 10 min at room temperature to remove residual H_2O_2 and this process was performed twice on each aliquot to ensure complete removal of H_2O_2 [16].

Each aliquot was evaluated for viral infectivity by mouse inoculation test [14] and the titer of each aliquot was calculated by the Spearman and Kärber method [15].

2.7. Inactivation of FRV/K rabies virus using βPL

Inactivation of the same viral harvest by βPL which is a standard inactivating agent for rabies vaccine production was used as a control versus the H_2O_2-inactivated virus. This procedure was done by treating virus suspension with βPL (SERVA, Germany) at a dilution of 1:4000, and virus suspension was kept for 24 h at 2–8 °C to complete inactivation and then transferred to 37 °C incubator (ALC, USA) for 2 h to remove residual βPL [1,17].

2.8. Evaluation of the prepared inactivated virus suspensions

The following tests aimed to compare between the two inactivated virus preparations concerning safety, antigenicity and immunogenicity.

2.8.1. Safety

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Both inactivated virus suspensions were evaluated using abnormal toxicity test, detection of residual infectious life virus, fluorescent antibody technique and detection of residual H_2O_2 content.

2.8.2. Abnormal toxicity test

The test was performed in accordance with European pharmacopoeia monograph which describes the test for immune sera and vaccines for human use [18]. Two groups of adult mice weighing 17–22 g, ten mice/group and two groups of healthy guinea-pigs weighing 250–350 g, two guinea-pigs/group were inoculated intra-peritoneally (I/P) with either H_2O_2 or βPL-inactivated virus suspensions. Each animal received 0.5 ml dose. All the inoculated animals must remain free of any signs of ill-health during a 7-days observation period. The test was performed in triplicate.

2.8.3. Detection of residual infectious live virus

Groups of twenty mice each weighing 11–14 g were inoculated intracerebrally with either H_2O_2 or βPL-inactivated virus suspensions, each mouse receiving 0.03 ml. The mice were observed for 28 days, any deaths occurring during the first 4 days are disregarded, but after this date there must be no deaths or signs of rabies infection. The test was performed in triplicate [19].

2.8.4. Direct fluorescent antibody test (FAT)

Test was performed as a confirmatory test to assure the effectiveness of inactivation process [17] using mice from residual live virus test. Test was controlled by positive and negative control slides. Positive control slides were prepared by grinding the entire brains of mice inoculated intracerebrally with rabies virus and killed after showing clear signs of rabies infection while negative control slides were prepared by grinding the entire brains of healthy mice. This procedure was carried out according to WHO protocol for the test [20,21] with modifications of using anti-rabies immunoglobulin of human origin (WHO second international standard. NIBSC code: RAI) diluted 1/10 followed by fluorescein-labeled antibody to human IgG (KPL, USA) diluted 1:10 instead of using fluorescein-labeled anti-rabies immunoglobulin. When properly stained, the positive control film and test films containing rabies antigen will contain brilliantly fluorescing apple-green or

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