

Formulation, Efficacy and Immunogenicity Studies of a Liquid State Rabies Vaccine with Magnesium Chloride as Stabilizer

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Abstract

Propagation of fixed rabies virus strain in vero cell line, ultra filtration of rabies viral harvest, betapropiolactone inactivation subsequent purification, total protein nitrogen quantification, Rapid Fluorescent focus inhibition test, MgCl₂ based liquid rabies vaccine formulation (TCALRV-B) and immune response analysis. The vero cell derived PV 11 rabies viral titers were found that 10^{-5.3}, ultra filtrated, betapropiolactone inactivated and viral protein qualities were within the range (WHO). The total protein concentration and PN₂ was 577.6, 0.26 mg/ml respectively. The host cellular protein and residual cellular DNA was 16 ng/single human doses and below 100 pg/ml respectively it reveals within the limit. The RFFIT titer of the TCALRV-B possessed higher immune response (8 IU/ml) of rabies neutralizing antibodies on 14th on 21st day the titer was four fold increasing even after 90th day it reveals its immunopotency. The TCALRV-B contained all the quality attributes to fulfill the regulatories although it contained potency, immunogenicity and safety as in vivo study shows lesser immunogenic during the booster doses. This may be due to the unadsorption of rabies viral proteins by MgCl₂ as adjuvant.

Key words:

Rabies vaccine; Tissue culture; Human albumin; Magnesium chloride; RFFIT; SRID

Introduction

Rabies is one of the neglected tropical diseases caused by the lyssavirus, causing severe encephalomyelitis throughout the world. It thought to be one of the oldest diseases of mankind. In Asia, rabies is one of the most important diseases because high human mortality rate and high costs spend for prevention and treatment, A survey has shown that Asia carries a larger part of the public health burden of with an estimated 32,000 deaths [1], and 20,000 of human deaths in India every year [2]. India has approximately 25 million dogs, with a dog: man ratio was 1:36. 1.1 to 1.5 million persons gets post-exposure rabies vaccine annually in India and hence the annual requirement for rabies vaccine is approximately 6-10 million doses. The post exposure prophylaxis of rabies disease requires 4-5 doses of highly expensive cell culture derived vaccines for the entire period of immunization, in which case the poor people are unable to bear the cost. The high cost of cell culture rabies vaccine is due to the increased cost of production. Most of the available cell culture vaccines are in freeze dried form whereas processing of the rabies vaccine the maximum cost is needed for downstream purification and lyophilization process [3].

The currently available cell culture rabies viral vaccines were formulated with human albumin and maltose as a freeze dried state, various stabilizers are added during the preparation, in case of freeze dried vaccines it is mandatory, the immunogen presence was weak amount. If sufficient amounts of various preservative materials were

not added while lyophilization, the vaccine would not be readily observable and undoubtedly adhere to the wall of the vaccine vial. But during the freeze drying process there is a losses of considerable antigenicity (immunogenic and potency) in the presence of human serum albumin the binding strength of rabies viral protein [4].

The Lyophilization process is a multistage operation in which, each step is utmost importance and critical, this process has to be adapted to individual vaccines according to the specific requirements, low-temperature behavior of the different products. The formulated and filled vaccine materials are hardened while freezing process due to low temperatures, during this period; all fluids present become solid bodies, either crystalline, amorphous, or glass. Keeping this view in this study to do the liquid formulation of vero cell derived rabies vaccine with MgCl₂ as a stabilizer as well as adjuvant.

Materials and Methods

Vero cell propagation of rabies virus

Vero cells (CCL-81) were obtained from the American Type Culture Collection (ATCC) at the passage number 125 as monolayer's frozen cells and propagated with appropriate medium [5]. Further it was revived, sub cultured and seeded into roller bottles (2.5 × 10⁻⁶ cells/ml) [6] and the cultures were incubated at 37°C at 0.6 rpm. Fixed strain of Pasteur virus (PV-11) which was obtained from Institute Pasteur France was propagated and infected with vero cell line and the virus infectivity was analyzed [5,6]. The rabies infected vero cell supernatant (viral harvest) was collected and replenished with maintenance medium at every intervals of 72 hrs for 4 harvests, it was

tested their infectivity in mice (LD50) [7] and the viral titer was also calculated [8]. All the pertained chemicals are prepared with exact molar strength with distilled water, 30.49 g of magnesium chloride 6-Hydrate HPLC grade (E Merck) was dissolved in 15ml of sterile distilled water before formulation all the reagents was checked its sterility test.

Concentration, inactivation and purification of rabies virus

Those viral harvests which have passed the in process quality control tests subsequently processed for concentration (CON), βPL inactivation (1:3000) [5,9], the βPL inactivated concentrate was tested for its quality [10]. Concentrated, inactivated rabies viral proteins were underwent purification for the removal of impurities like host cellular protein (HCP) and residual cellular DNA, the purified materials are analyzed the tests like total protein concentration, mouse infectivity titer, in vitro potency.

Quality attributes of rabies viral proteins

The purified, quality tested desalted rabies viral proteins were formulated with magnesium chloride and human serum albumin, Further it was subjected to their quality control tests like sterility test, pH, abnormal toxicity, and innocuity [5].

The single human dose was used for immune response analysis, the total protein was measured using with Bicinchoninic acid protein assay kit [11,12], protein nitrogen content was analyzed by the sulphuric digestion (microkjeldhel) method [5]. The immune sera's of guinea pig were confirmed through RFFIT [13]. Local reference vaccine (zonal centrifuge purified) which calibrated against WHO standard vaccine, was used as standard.

Liquid vaccine formulation

All the components were prepared with analytical grade chemicals and deionized water was used for formulation. Human albumin 20% was obtained from Reliance life sciences, the purified rabies virus protein (PRVP) (total protein- 577.6 mg/ml) and 1M magnesium chloride was taken. As per regulatory the purified rabies proteins, stabilizers, additives and adjuvants should be more than 2.5 IU, 5%, 1% and less than 2 mg/single human dose respectively. Human albumin was added as additives to a final concentration of 1%, the vaccine was formulated (Table 1), and it named as tissue culture anti liquid rabies vaccine-B (TCALRV-B). This formulation was carried

out in the +4°C to avoid protein loss as well as avoid protein clumps, after addition of all the formulators it was under mild shaking for proper mixing, and same time without froth formation the same was repeated up to 2 days.

Vaccine	Constituent present in Liquid rabies vaccine				
	Purified rabies viral proteins (%) Total (BCA)	Human Serum Albumin (%) protein	MgCl ₂ (ml)	Total protein µg/dose	pH
TCALRV-B	54.70%	1	20.3	65	7.2

Table 1: Traits of liquid rabies vaccine at formulation.

Analysis of seroconversion rate of rabies antibodies

Group of 2 guinea pigs were immunized intraperitoneally with two doses of 0.03 ml of TCALRV-B and references vaccines (HO23 and TCARV) with pertained adjuvants. The animals are challenged intracerebrally with 20 LD₅₀/0.003 ml of CVS, the immunogenicity of the vaccines was performed with pre exposure schemes (D₀, D₇, D₁₄, and D₂₁). Three milliliters of whole blood specimens were collected on day 0, 14 and 45 after the first dose injection respectively. The serum samples were separated from the collected blood specimens and stored at -20°C until using. Rabies virus neutralizing antibody levels were measured using the rapid fluorescent focus inhibition test (RFFIT), as recommended by the WHO [13].

Reaching adequate rabies virus neutralizing antibody (RVNA) concentrations of 0.5 IU/ml was defined as seroconversion based on the WHO criteria and considered to be positive [14].

Results and Discussion

Preparation and quality control of TCALRV-B

The PV 11 rabies viruses were harvested at 72 hours intervals until the 4th day, and viral titers were determined and it found that 10^{-5.3} furthermore it was concentrated about 20 times using with 0.45 µm microfiltration (clarification) and ultrafiltration (100 KDa), then inactivated with β-propiolactone (1:3000) at 4°C [9]. After completion of quality tests, chromatographic purification was conducted and the quality attributes of the samples are within the limit as IP 2014 (Table 2).

Sample	Sterility	pH	Protein purity		Total protein (BCA) (mg/ml)	PN ₂ (mg/ml)	Host cellular BSA (ng/ml)	Residual DNA (ng/ml)	SRID
			A ₂₆₀ /A ₂₈₀	% Protein					
PRVP	Passed	7.02	1.562	82	577.6	0.26	Below 16 ng	Below 100 pg	+
+: Positive									

Table 2: Quality attributes of purified rabies viral protein before formulation

The total protein concentration of sample was 577.6 mg/ml, and it reflects their viral protein concentrations. In case of PN₂ content (microkjeldhel method), their results were found to be 0.26 mg/ml, it showed higher amount protein as well the purity because most virulent part of the rabies virus as protein hence the protein contents play a main role in the part of potency determinant. The sample was

further tested for host cellular BSA by immunoenzymetric assay kit, and the values are below 16 ng, it was lower than the level of 50 ng/ml [5]. For the impurities analysis the residual cellular DNA by slot blot hybridization [15]. And the result values are below 100 pg/ml this test values are within the regulatory stipulated norms i.e 10 ng/ml [5].

The PRVP was formulated with 1M MgCl₂ (20.3%) human serum albumin (1%) the total protein concentration was kept as 65 µg/dose, during the liquid formulation the pH of the vaccine was 7.2. The final concentration of MgCl₂ was analyzed by titan yellow method [4,16] and the concentration was 20.3 ml (20%). Magnesium chloride present in vaccine sample was estimated using titan yellow as a coloring agent, the concentration was as 0.083 in single human dose (SHD) [4].

Human serum albumin (HSA), the most abundant protein in plasma, is a major antioxidant, transport, and depot protein. This globular 66 KDa protein contains 585 amino acids including 18 tyrosines, 6 methionines, 1 tryptophan, 17 disulfide bridges, and only 1 free cysteine (Cys34). Many commercial HSA preparations come as a sterile aqueous solution prepared by a cold alcohol fractionation method from pooled human plasma obtained from venous blood. The HSA products are available at 20% (20 g/100 ml). It is used predominantly in the preparation of viral vaccines [17] and it is also used as vaccine additives [18].

Storing proteins in liquid state has numerous advantages, the process of thawing and rehydration decrease its stability of protein. Less manipulation is required for the formulation than for freeze dried form. Protein stability is directly correlated with the ability of water in the hydration layer to fluctuate among different equilibrium structure [19]. Viral vaccines are highly sensitive, respond differently to physical stresses, commonly it can be affected differently by solvents, pH, and ionic strength of extreme temperatures (heat/cold) [20].

As per the regulatory requirements the TCALRV-B was passed its safety, efficacy and potency tests (Table 3), the abnormal toxicity test is the general requirement for all vaccines, the mice and guinea pigs were inoculated with TCALRV-B, reference vaccines through intraperitoneally, all the animals are observed for 7 days, during that period there was no death, necrosis and weight loss it revealed absence of abnormal toxicity.

Quality Tests	IP 2014 of rabies vaccine	TCALRV-B
Sterility test	Negative (-)	Negative (-)
Residual Albumin Serum	≤ 50 ng/dose	16 ng/dose
Residual BPL	Not more than 1:3500	Below the level
Potency	≥ 2.5 IU/dose	≥ 3.1 IU/dose
Residual Vero cell DNA	≤ 100 pg/dose	10 pg/dose
Bacterial Endotoxin	≤ 50 EU/dose	≤ 50 EU/dose
Protein content	≤ 80 µg/dose	65 µg/dose
pH	7.2-8.0	7.8
Abnormal Toxicity	Passed	Passed
Avirulence Test	Negative	Negative
Virus Amplification Test	Negative	Negative

Table 3: Quality attributes of TCALRV-B

Seroconversion of TCALRV-B

The *in vitro* potency of the TCALRV-B was analyzed through RFFIT method, due to avoiding the usage of more animals, the potency of inactivated rabies vaccines is conventionally determined by a mouse challenge test, and this method causes severe distress to the test animals and is known to be imprecise and time-consuming. So WHO and many countries drug agency suggest the method of rapid and reproducible *in vitro* the availability of alternatives, this method remains the standard method for measuring rabies-specific antibodies [21]. The RFFIT method spend only 2 days, and can detect a large number of samples once time, and do not require the use of animals (3R principles of experimental animals), WHO strongly recommended the method, RFFIT method has been the approved by the agency of drug regulatory of China for the potency detection of anti-rabies serum/immunoglobulin now [22].

The RFFIT results of the TCALRV-B was in the Table 4, and found to be the rabies neutralizing antibodies value of TCALRV-B, HO23 and reference freeze dried vaccine (control vaccines) on the 7th day it was <05 and it shows all the animals are not in the required values [18]. In case of 14th, 21st and 90th day the values are increased and it reached above 2.5 IU/ml it reveals higher immunogenicity, in case of the TCALRV-B it also possessed higher immune response. The TCALRV-B immunized guinea pigs blood sera's was showed 8 IU/ml (RFFIT) of rabies neutralizing antibodies on 14th day. The antibodies level rate was increased four folds after 21st day and continued the same increase rate even after 90th day it reveals its immunopotency.

Vaccine	No. of animals used	RFFIT				
		Rabies Neutralizing antibodies in IU/ml				
		0 th day	7 th day	14 th day	21 st day	90 th day
TCALRV - B	8	<05	<05	8	>32	32-64
Local reference (HO23)	8	<05	<05	16-32	64	64
Reference Freeze dried vaccine	8	<05	<05	16	32-64	64
Dose 1 ml, I/m on days 0, 7 th (booster) for both						

Table 4: Immunogenicity of TCALRV-B

Magnesium chloride is used as the stabilizer as well as adjuvants in oral polio vaccine for human use [23]. The magnesium chloride is boosting the immune response when combined with polio vaccine [24]. Additives are used to stabilize the vaccines from adverse conditions such as freeze drying and heat. In addition, additives are added to vaccines to avoid immunogens from adhering to the side of the vial. There is no published data available with respect to use of different stabilizers in the preparation of rabies vaccine as liquid state.

Conclusion

Wenqiang Jiao et al. [25] stated in that new kind of adjuvants which could be used to enhance the potency of the vaccine, it needs to be developed. Also immunization strategies could also be developed. Immunologic adjuvants are agents incorporated into vaccine formulation to enhance the immunogenicity of vaccine antigens. The immunogenicity of a vaccine is defined as its ability to evoke an immune response in the vaccinated individual [17]. Although the formulated vaccine contained its potency, immunogenicity and safety in *in vitro* studies as $MgCl_2$ as stabilizer, the *in vivo* study shows lesser immunogenic during the booster doses. This may be due to the unadsorption of rabies viral proteins by $MgCl_2$ as adjuvant. This research study reveals to need a further study to focus on the adjuvancy of magnesium chloride.

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