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Preparation of Lyophilized Anti-Rabies Immunoglobulin

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Abstract:

This work aims to provide anti-rabies IgG in a purified concentrated dose smaller than that of the liquid anti-rabies whole serum. Such purpose was fulfilled through lyophilization of anti-rabies IgG precipitated from rabies hyper immune serum prepared in horse. Such serum was found to have an antibodies titer of 105IU/ml with immunoglobulin value of 4.3g/dl. Testing of the obtained final product revealed that it was free from foreign contaminants, safe in mice and able to stop rabies infection in experimentally infected mice. It was found that administration of IgG in mice experimentally infected with rabies virus at concentrations of 4.30, 2.20, 1.10, 0.54, 0.27 and 0.14g/dl showed protection percentage of 100; 90; 80; 80; 70 and 60% respectively when such treatment was applied from the zero time to the 3rd day post exposure. Latent treatment leads to progression of the infection where the protection rate decreased to be 40, 30, 30,20, and 10% and when administration of IgG was carried out on the 4th day post exposure to record 0% by the 6th day. Depending on these findings, it could be concluded that the lyophilized anti-rabies IgG is safe and able to withstand the virus infection when administrated on the correct time with the suitable dose.

Keyword: Immunoglobulin, anti-rabies, lyophilization

1. Introduction

Rabies provokes acute and fatal encephalitis in all warm blooded mammal hosts; it is caused by a neurotropic, RNA virus belonging to the order Mononegavirales, family Rhabdoviridae, genus Lyssa-virus (Fauquet et al., 2005). Rabies infection usually occurs through infected saliva reaching a bite wound or skin scratches and breached mucous membranes. The severity, location, multiplicity of bites inflicted on the victim, biotype of the virus and the susceptibility of the recipient influence the outcome of potential exposure to infection. Bites on head and neck are associated with the shortest incubation period (Green and Rupprecht, 2006)

The disease represents a serious public health problem in developing countries, where approximately 35000 to 50000 human deaths occur due to rabies each year (WHO, 1996).

Control of diseases causing significant mortality has made outstanding progress and immunization of one individual is to prevent diseases (Mohanty et al, 2007).

Post exposure vaccination against rabies along with anti-rabies immunoglobulin prevents the development of the disease. In this respect Al-Behwar (2009) concluded that the best immunization of farm animals post exposure to rabies infection could be achieved by administration of rabies vaccine and anti-rabies serum on the suitable time (1-3 days post exposure).

There is a great attention directed toward animal protection against fatal diseases as rabies infection. In this aspect many studies were carried out including prophylactic vaccination with potent vaccine and post exposure intervention using specific antiserum and immunoglobulin (Khodeir and Daoud, 2008).

Freeze-drying is a process used to improve the stability of pharmaceutical proteins, including snake anti-venoms. This additional step confers these with a higher stability in comparison to liquid formulations, especially in tropical regions where high temperatures could affect the activity of immunoglobulin.

Freeze-drying confers stability to proteins, particularly at high temperatures. However, during this procedure antibodies can experience physical and chemical modifications, which may cause irreversible changes and have a negative

impact on protein activity and aggregation, as well as on rehydration of the product (Sarciaux et al., 1999 and Maury et al., 2005). Aggregation is the main evidence of antibody degradation (Mahler et al., 2009), which could affect the efficacy and safety of these proteins. This effect could be prevented during freeze-drying by the addition of excipients like sugars and polyols

Concerning lyophilized monoclonal antibodies, the thermal characterization and the stability of different freeze dried anti-venoms formulated with sorbitol, sucrose or mannitol have been reported (Herrera et al., 2014).

Burton et al (1981) in a study with 15 neonatal foals (5 per treatment group), foals were fed within 4 hours of birth as follows: 250 ml of colostrum, 250 ml of lyophilized serum reconstituted at 5 times the original concentration, or 250 ml of a mixture (1:1) of colostrum and lyophilized serum. Foal serum samples were tested for immunoglobulin (IgG) concentration and titrated for anti-equine rhinovirus 1 and anti-equine influenza A1 and A2 antibodies at 0 and 24 hours after foals were born. There were no significant differences found in the serum IgG concentrations and antibody titers among the 3 treated groups. Seemingly, IgG was absorbed efficiently from both serum and colostrums, so that the use of reconstituted lyophilized serum as a prophylactic measure of conferring passive immunity to a new-born foal deserves serious consideration.

The freeze-dried formulation maintained anti-snake venom neutralizing potency and did not show marked signs of instability, thus indicating that freeze-drying could confer EchiTAB+ICP(A pan-African anti-venom used for the treatment of snakebite)with improved thermal stability required for distribution and storage at room temperature in sub-Saharan Africa (Herrera et al, 2017)

Aiming to provide a specific anti-rabies agent of high quality, this work was designed to prepare and investigate the efficacy of freeze-dried anti-rabies IgG to be used in emergency post exposure intervention.

2. Materials and Methods

2.1. Animals

2.1.1. Horses

Two apparently healthy native breed horses, free from external and internal parasites of about 4 years old were housed under hygienic measures receiving balanced ration and adequate clean water were used for preparation of the anti-rabies hyper immune serum.

2.1.2. Mice

Four hundred and fifty weaned Swiss Albino mice were used to challenge the experimental rabies infection using the prepared lyophilized immunoglobulin.

2.2. Rabies Vaccine

Locally prepared inactivated cell culture rabies vaccine (Batch No. 1704) prepared according to Edries (1994) was supplied by the Department of Pet Animal Vaccine Research (DPAVR), Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt. It was used for immunization of horses in order to prepare anti-rabies hyper immune serum.

2.3. Viruses

2.3.1. Live Attenuated Virus

BHK-21 cell culture adapted Evelyn Rokintniki Abelseth(ERA) strain of rabies virus with a titer of 10^7 TCID₅₀ /ml (Edries, 1994) was supplied by (DPAVR), (VSVRI) and used in serological test (SNT).

2.3.2. Virulent Strain

Mice brain adapted rabies virus; challenge virus strain (CVS) with titer of 10^6 MLD₅₀/ml was supplied by (DPAVR), (VSVRI) and used for induction of experimental infection in mice.

2.4. Cell Culture

Baby hamster kidney cell line (BHK-21) established by Mackpherson and Stocker (1962) was used in serum neutralization test. This cell culture and its solutions were supplied by DPAVR, (VSVRI).

2.5. Preparation of Anti-Rabies Hyper Immune Serum

Preparation of anti-rabies hyper immune serum was carried out in horses using 5 successive doses (4 ml/ dose)with a week interval injected subcutaneously according to the method described by Lepine and Atanasiu (1973).Serum samples were obtained from immunized horses on week intervals post immunization then serum was collected on the 14th day after the last injection and subjected for titration of induced rabies antibodies through application of serum neutralization test. The prepared hyper immune serum was subjected to sterility tests and proved to be free from aerobic and anaerobic bacteria, fungi, and mycoplasma contaminations following the directions of WHO (1992)

2.6. Preparation of Rabies Immunoglobulin

Precipitation of the immunoglobulin (IgG) of the prepared hyperimmune serum was carried out using ammonium sulphate according to Narin and Marrack (1964) and the globulin content was estimated according to Weichselbaum (1946) and found to be 4.3 g/dl.

2.7. Lyophilization of the Prepared Rabies IgG

The prepared immunoglobulin solution was stabilized with sucrose in a ratio of stabilizer to protein was 1:1 (w/w); sodium concentration and pH were adjusted to 120 ± 10 m Eq/l and 7.0 ± 0.1 , by adding 1.0_M NaCl and 10% NaHCO_3 , respectively, and finally the protein concentration was adjusted up to $10.0 \pm 0.2\%$ with 0.3_M glycine. The protein solution was filtered and dispersed in neutral glass vials under sterile conditions and lyophilized following the method adopted by Sisti et al. (2001) where the vialled materials were frozen and held at -40°C for 6 hours at atmospheric pressure and subsequently the temperature was decreased to -50°C for another 10 hours (super freezing) at a vacuum of 10m and maintained during the rest of the process. The primary drying time was 48 hours, after which the temperature was gradually increased to 35°C and maintained for a further 8–10 hours, depending on the doses. The lyophilized immunoglobulin was stored at room temperature (25°C).

2.8. Serum Neutralization Test (SNT)

It was carried out using micro titer technique according to Bass et al. (1982) to determine the antibody titer of prepared hyper immune serum of immunized horses. The antibody titer was calculated according to Lepine and Atanasiu (1973) as IU/ml.

2.9. Experimental Design

Four hundred and fifty weaned Swiss Albino mice were divided into 9 groups each of the first 6 groups consisted of 70 mice while each of the other 3 groups consisted of 10 mice. The first 6 groups were experimentally infected with the CVS through the intra cerebral route using a dose of 0.03 ml/ mouse according to Sellgmann (1973). Each of these groups was sub-divided into 7 sub-groups (10 mice/ sub-group) where very sub-group (of each group) received the lyophilized, reconstituted immunoglobulin daily started from the time of infection (Zero time) up to 6 days post-experimental infection. Five two-fold dilutions were prepared from the reconstituted rabies immunoglobulin starting from 4.30 reaching 0.14 g/dl where each dilution was inoculated in mice sub groups as mentioned above. The 7th group was experimentally infected by the CVS only while the 8th group was inoculated with undiluted immunoglobulin only and group 9 was kept without any inoculation as normal test control.

3. Results and Discussion

Tested Antiserum	Total Protein (G/DI)	Albumin (G/DI)	Globulin (G/DI)
Normal horse serum	5.7	2.6	3.1
Prepared rabies antiserum	7.0	2.7	4.3

Table 1: Serum Proteins in Normal the Prepared Horse Rabies Antiserum

Rabies Serum Neutralizing Antibody Titer (IU/MI) /WPI*							
Tested IgG	0 time	1WPI	2WPI	3WPI	4WPI	5WPI	7WPI
Pre-lyophilization	0	15	30	60	90	100	105
Post-lyophilization	0	15	30	60	90	100	105

Table 2: Rabies IgG Titer in Horse Serum

*=Weeks Post Inoculation

Mice Groups	Inoculated Dose Of Igg (G/Dl)	Number of Survived Mice/Total Infected Treated Mice						
		Zero day	1 DPI**	2DPI	3DPI	4DPI	5DPI	6DPI
1	4.30	10/10	10/10	10/10	10/10	4/10	2/10	0/10
2	2.20	10/10	10/10	10/10	9/10	3/10	2/10	0/10
3	1.10	10/10	10/10	10/10	8/10	3/10	1/10	0/10
4	0.54	10/10	10/10	10/10	8/10	2/10	1/10	0/10
5	0.27	10/10	10/10	8/10	7/10	1/10	0/10	0/10
6	0.14	10/10	10/10	7/10	6/10	0/10	0/10	0/10
7	Infected untreated control	Typical rabies symptoms began by 4 th day and death of all mice by the 6 th day post experimental infection.						
8	Immunoglobulin control							
9	Normal control							

Table 3: Efficacy of the Lyophilized Immunoglobulin

**= Days Post Infection

The total protein content in the prepared anti-rabies hyper immune serum was higher than in normal horse serum, this rise could be attributed to the increased globulin content from which antibodies are formed (Table-1). These results came in agreement with those of Kataria and Sharma (1993) and Hala et al. (2003).

Estimation of rabies IgG pre and post lyophilization using serum neutralization test showed that it had a titer of 105 IU/ml without any adverse effect of the lyophilization on such titer (Table 2). These results were supported by those of Lepine and Atanasiu (1973), WHO (1992); Edries et al. (1999) and Khodeir and Daoud (2008) who concluded that antiserum with such titer could be considered hyper immune serum and they recommended that protective serum rabies antibody titer should not be less than 0.5 IU/ml.

Considering the experimental infection of mice using CVS, it was found that mice received the prepared (lyophilized) immunoglobulin were able to withstand the experimental infection when it was administrated on the 1st, 2nd and 3rd days post inoculation of CVS while those mice received such treatment on the following days did not survive and showed paralysis of the hind limbs by the 4th day and died by the 6th day after experimental infection. Administration of IgG in mice experimentally infected with rabies virus at concentrations of 4.3, 2.20, 1.10, 0.54, 0.27 and 0.14g/dl were able to withstand the virus infection in percentage of 100, 90, 80, 80, 70 and 60% respectively when applied from the zero time to the 3rd day post exposure. Latent treatment resulted in progressed infection where the protection rate decreased to be 40, 30, 30, 20, 10% when the IgG was administrated on the 4th day post exposure to record 0% by the 6th day in corresponding manner to the used doses. It was clarified that the preferable dose of rabies IgG is 4.30g/dl followed by 2.20g/dl inducing the highest protection percentage (100 and 90% respectively) concerning that the lowest dose that could induce acceptable protection is 0.54g/dl giving 80% protection; also, it was clear that the best time for administration of anti-rabies immunoglobulin was within the first three days post exposure to rabies infection (Table-3). These results indicated that post exposure treatment of rabies should be as soon as possible as any delay in intervention leads to rapid disease arise. There were no available data about emergency immunization of animals against rabies using anti-rabies immunoglobulin but Barnas (2001) stated that anti-rabies immunoglobulin could be applied for treatment of animals exposed to natural infection. In addition, Khodeir and Daoud (2008) found that passive immunization of experimentally infected mice could protect them when they received rabies antiserum on 1st, 2nd and 3rd days post experimental infection but can't survive after that. Also, Al-Behwar (2009) mentioned that post exposure intervention should be carried out as soon as possible after exposure to infection and recommended the same periods. He added that either homologous or heterogenous species rabies anti-sera could be safely used in cases of emergency.

So, this work indicated that lyophilized anti-rabies immunoglobulin conserved its viability, biological activity and safety and could be reliably used in emergency intervention for different animals as post exposure treatment of rabies infection representing passive immunization. In addition, the prepared immunoglobulin was more purified, concentrated and of less chance to cause hyper sensitivity than the whole anti-rabies serum can do when used for treatment of different animal species. This treatment is recommended besides rabies vaccination which is considered fundamental treatment as a source of active immunity.

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