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Research Article

A COMPARATIVE STUDY OF 'BELLADONNA-200' AND JE VACCINE (SA-14-14-2) IN PREVENTION OF JAPANESE ENCEPHALITIS IN SWISS ALBINO MICE

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ABSTRACT

Objectives: Vaccination is the only means present with us to fight Japanese encephalitis which is widespread in South-East Asian countries as there is no effective treatment at present. However, vaccination of rural mass population in this specified region is extremely complex due to climatic, geographical, socio-economical barriers. In this study we explored possible use of "Belladonna-200" –an easily applicable homeopathic medicine to prevent this important disease.Methods: In this experiment initially adult mice JE virus adaptation was achieved by repeated passage of the virus and 50% lethal dose (LD_{50}) of the virulent Nakayama strain virus was calculated by successive experiments with several lots of mice (6 to 8 wks age). Following this 63 adult mice (control group) were challenged with the virus by a standardized intracerebral inoculation technique, similarly 33 mice which were fed with "Belladonna- 200" (experimental group) and 28 mice (vaccine control group) which were protected by the Japanese Encephalitis Live Vaccine (SA14-14-2), were also challenged with the same doses of the virus.Results: The results confirmed a definite protective action of "Belladonna 200" against JE which was almost equal to the protective action of Japanese Encephalitis Live Vaccine (SA14-14-2).Conclusion: Considering the insignificant expenditure of this medicine which can be administered orally without any side effects, "Belladonna-200" appears to be an important alternative to the vaccine..

Keywords: Japanese encephalitis, Belladonna- 200, J E Vaccine.

INTRODUCTION

No prequalified well recognized JE vaccine is available till date for the prevention of Japanese encephalitis (JE) - a recently globalized fatal disease.. This serious disease is a scourge of human race since epidemics which had been recorded in Japanese literature in 1870 and 1924 with a death rate of 62% [1]. This 'flavi' virus-mediated disease was first described in Japan and its causative agent Japanese B encephalitis virus first recognized in the year 1938, when the virus was isolated from the vector *Culex tritaeniorhynchus,* which breeds in stagnant water in paddy fields. Apart from this vector, *Culex vishnui* (in India), *C. gelidus* and *C. fuscocephala* (in India, Malaysia. and Thailand), as well as *C.pipiens* are now accepted as vectors of this life threatening virus from infected pig to humans.

In transmission cycle of this disease, the pig is the amplifying host and become extremely viremic without any evidence of symptoms of encephalitis except abortion in pregnant pigs. The reservoirs of this viral disease are migratory or domestic birds. Initially the mosquito acquires virus from birds (primary host), bites the pigs which gradually become highly viremic; and finally when virus loaded mosquito bites human beings causing the disease this becomes the dead end of the cycle which is completed mainly in pigs. So transmission is not possible from human to human.

According to a recent study, about 3 billion people now live in JE endemic area and more than 700 million children are now vulnerable to this disease throughout the world and the fact which sets us worrying is that, this geographical area where the disease in now endemic, is increasing day-by-day [2,3]. Annual rate of the disease in the endemic zones is about 50,000 with 10,000 to 15,000 human deaths per year. Children in between 3-15 yrs of age are more prone to infection (5 - 10 times) than adult age group [4, 5] ,which may be due to higher immunity in them. The mortality rate of Japanese encephalitis is about 25- 30% and 50% of the survivors suffer from neuropsychiatric problems [6]. Recently in an outbreak of Japanese encephalitis in India affecting 17 states lead to a death toll of 884 persons; however, it included 501deaths due to acute encephalitis syndrome where the etiology was not properly defined

in rural areas of Uttar Pradesh . The size of the affected population may be much higher as in only 1 in 300 infections is symptomatic in humans.

No specific therapy is available till date against Japanese Encephalitis. Since the Second World War, efforts had been made to prepare Japanese encephalitis vaccine [19], Which was first developed in Japan from infected mouse brain (inactivated) in the year 1965 [20] for immunization of Japanese kids against JE infection. However, substantiate data have been available regarding its efficiency from those vaccinated children except a few. Internationally three types of JE vaccine are available at present originating from inactivated JE infected mouse brain, inactivated cell culture derived and live attenuated JE vaccine (SA 14-14-2) also originated from cell culture but they are used in a restricted manner [6]. Some other new JE vaccines with higher effectiveness and minimizing the adverse events are under preparation which includes recombinant protein based vaccines, chimeric vaccine and DNA vaccines.

Inactivated mouse brain vaccine was developed from Nakayama, Beijing-1(P1) strain initially manufactured in Japan (1954), and later in India, Korea, Taiwan, Thailand, and Vietnam [21]. In 2005, Japanese government had withdrawn this inactivated mouse brain JE vaccine from immunization schedule due to adverse events of acute disseminated encephalomyelitis which occurred in vaccinated population [7]. The production of inactivated mouse brain vaccine is now withheld in almost all countries due to these adverse events on human body [8, 9, 10, 13] although its higher efficacy rate (91%, confidence interval of 70-97%) [14].

A similar inactivated primary hamster kidney cell-derived Japanese Encephalitis vaccine (P3 inactivated vaccine) is also out of favor due to its local and systemic reactions. .In a recent study (1999-2009)

conducted by U.S Vaccine Adverse Event Reporting System has reviewed the factual status of unpleasant events following Japanese Encephalitis Vaccination in USA[15]. It is relevant to mention here that JE-VAX(Inactivated mouse brain vaccine) which was only licensed JE vaccine in USA is not manufactured since May 2011[16]

"SA-14-14-2" .live attenuated Japanese encephalitis vaccine though yet to be prequalified by WHO is now widely accepted by JE prone countries due to its low cost, higher efficacy, less and mild adverse local and systemic reactions. The vaccine has been prepared from serial passage of wild SA14 strain in primary hamster kidney cell cultures [22]. This vaccine was first launched in China in the year 1988 and more than 20 million Chinese children are vaccinated by this vaccine annually [17] One case control study was conducted in Terai of Nepal in 1999 to verify the long term protective effect of SA-14-14-2 live attenuated JE vaccine by a single dose. The observer claimed that SA-14-14-2 live attenuated JE vaccine in a single dose can confer 98.5% efficacy in Nepalese children one year after immunization[18].This vaccine has also been officially accepted by Korea, Sri Lanka, Nepal and India Governments and thus covered 50% of total production of currently available Japanese encephalitis vaccine worldwide. In India, it has been reported that some unpleasant events occurred during JE vaccine immunization program without any further confirmation. The outcome showed a significant number of hypersensitivity reactions, neurological problems and grave adverse effects in vaccinated population. Thus more precautions, keen experimental observations are required in vaccine productions and all adverse reactions should be investigated carefully.

No current therapy is in hand to protect against JE virus infection except vaccination and spread of this life threatening disease worldwide is very much alarming since last five decades in spite of existence of Japanese encephalitis vaccine. So a new avenue of therapy will be useful to combat that fatal infectious disease.

With this precarious position with no specific therapy in hand and preventive aspect is not fully dependable we started working with alternative medicines to find out a new avenue to challenge the disease and in this process our first interest went to the homeopathic medicine "Belladonna 200".

Records of activities of homeopathic preparation of Belladonna has a long past record as back as seventeenth and eighteenth centuries. Thus it showed a good prophylactic power in an epidemic of highly infectious scarlet fever in Konigslutter during summer of 1799 which was investigated by Dr. Samuel Hahnemann, a renowned German Physician and medical scientist[23] when he observed that all the children who took a very small dose of Belladonna in time , remained impervious by this highly infectious disease.

Later this finding was further strengthened by the works of Bloch, Cramer, Velson, Behr and others who administered this medicine on thousands of patients during epidemics and C.W. Hufeland, a famous pharmacologist, published a special article "On the prophylactic power of Belladonna in scarlet fever" in 1826, which are the collective evidence of prophylactic power of Belladonna in scarlet fever available at that time [24].

Many physicians at that time adopted the above-mentioned Hahnemann's protocol for preventing the scarlet fever. According to writings of Dr. Dudgeon (1820-1904), 10 conventional physicians used prophylactic Belladonna on 1646 children and only 123 (7.4%) were affected. These excellent results of Belladonna revealed its effectiveness in scarlet fever epidemic when the rate of infection was 90% at that time [25].

Botanically *Atropa belladonna* (Deadly nightshade), from which this homeopathic medicine "Belladonna 200" was prepared, is one of the most important species of Solanaceae family having various chemical ingredients like atropine, hyoscine (scopolamine) and hyoscyamine. The whole plant is used for the preparation of homeopathic drug "Belladonna-200" as per Homeopathic pharmacopeia of India [26]. The powder of the root of this plant was used as a prophylactic medicine against hydrophobia in the past although not as a homeopathic medicine.

According to Homeopathic pharmacopeia, Belladonna is a very useful remedy in arterial congestion of the brain from almost any cause [27].It is also indicated in homeopathic practice for inflammation and congestion with extreme burning heat, redness and throbbing in the affected regions.

With a feedback from all the above-mentioned facts, in the present study, we used the homeopathic medicine "Belladonna 200" and compared its possible preventive action to JE vaccine SA-14-14-2 in prevention of JE in adult Swiss albino mice, as our initial experiments on JE infected chick chorio allantoic membrane and suckling mice [28] .showed encouraging results.

MATERIALS AND METHODS

Place of Study

The study was carried out at the Virology unit, Department of Microbiology, School of Tropical Medicine, Kolkata.

The Medicine "Belladonna 200"

In this study we used aqueous preparation of "Belladonna 200" which was procured directly from reputed homeopathic drug company, Hahnemann Publishing Co. Pvt. Ltd (HAPCO), Kolkata, India. The medicine was prepared by the company according to standard procedures mentioned in HomeopathicPharmacopoeia of India (Ministry of Health, Government of India, 1971, 1:1, 7-16, 72). Initially we started the experiment with "Belladonna 6" and although average survival time of the mice treated with "Belladonna 6" after inoculation of the virus was increased from 36 h in controls to 50 h, but all the mice died. Later we found that C. V. Boenninghausen [20], a veterinary doctor as well as renowned homoeopathic practitioner described in 1843 that "Belladonna 200" is the ideal medicine in experiments with mice. Thus we used "Belladonna 200" in all lots of animals in this experiment.

Procurement and storage of Japanese Encephalitis Vaccine

The Japanese Encephalitis Live Vaccine, (SA14-14-2) was kindly given by the Medical Officer. State District Hospital, Govt. of West Bengal, India which was maintained as per manufacturer's instructions. The Vaccine, was originally supplied by Govt. of India (Lot no-200811CO57-4) manufactured by Chengdu Institute of Biological Products, Chengdu, China.

The Experiment

The most common choice of host for isolating arboviruses is still the mice [11]. In this experiment, adult Swiss albino mice (Webster strain) were used after getting permission from the Ethical Committee of the Institute and maintained in the mice colony of the School of Tropical Medicine, Kolkata. In this experiment thevirulent Nakayama strain (Source human, year 1935, location Japan, GenBank accession no. EF571853, Genotype III) was used.

Adult adaptation and inoculations

To study the preventive effect of " Belladonna 200" on JE, the experimental model needed to be established in adult mice as suckling mice succumb within 72-96 hrs. To set up this experiment on adult adaptation of the virus a careful protocol was established. This is due to the fact that virus from suckling mice brain does not affect the adult mice when inoculated intracerebrally due to several reasons like absence of receptors, thickening of the skull bone of adult mice etc. So JE virus infection was gradually adapted in the adult mice (6 -8 wks) from infected suckling mice brain. The virulent Nakayama strain virus was consecutively passaged three times in mice and the virus suspension was prepared from the third stage which was designated as the 10^{-1} stock suspension. For determination of a 50% lethal dose (LD_{50}), several lots of mice (6 to 8 wks age) were injected intracerebrally with dilutions of the stock suspension from 10^{-1} to 1^{-9}. All the inoculated mice were observed daily, their survival capacity was noted and following this LD_{50} value was calculated by the standard method [10,11]. After completing inoculations each mouse was returned to the cage and was properly labeled and kept in the rack. Mice showing severe disease signs or those that died within 2 h of observations were immersed in a closed

vessel containing chloroform and later discarded according to statutory guidelines for Biomedical Waste management.

Method of inspection and virus collection

During inspection the condition of each mouse was recorded as D (dead), S (sick), N (normal), or M (Missing). However, in this experiment there was no M category. When the infected brain was collected for preparation of the inoculums, the dead mouse was pinned on to the cork board placed over two paper towels, one pin was placed through the nose and the other through the base of the tail. The mouse was then soaked with sufficient amount of rectified spirit and the scalp was removed using one pair of sterile scissors and forceps. This was done by cutting across the back of the scalp using scissors that have a pointed and a blunt blade. The pointed blade of the scissors was inserted into the soft rear centre point of the skull and the

outside was cut down towards the nose. The skull cap was then lifted up and the brain was removed by the closed ends of the scissors.

Experimental design

Randomly selected adult mice were taken from litters in which every mouse was orally fed with 0.06 mL of "Belladonna 200" for 7 days and similarly other litters were treated with 0.03 ml of SA 14-14-2 JE vaccine on 0 and 7days (two doses) intraperitoneally. In control experiment, litters were not orally fed with the medicine nor were they vaccinated. After this, mice of all the groups were challenged with 0.03 mL of the supernatant of clarified JE virus (adult adapted) infected mice brain suspension (10%) intracerebrally diluted to LD_{50} dose as determined initially and observed for 30 days as post inoculation period. All the mice were observed daily after inoculation and every four hours after the onset of clinical signs. Clinical signs of the disease in mice were refusal to feeds, became disarranged in the nest, showed tremors and muscular spasms, ataxia, and hind-limb paralysis followed by death within a few hours. Those mice that died within the first 24 h were considered as non-specific deaths. For preparation of infected brain (adult adapted) suspension required for LD_{50} determination and standardization, the infected brains were collected close to the time of death.

RESULTS

The results are given in Table 1 along with statistical analysis. The results clearly indicated an excellent preventive role of "Belladonna 200".

Table 1: Results showing preventive action of "Belladonna 200" against JE in adult Swiss albino mice compared with control and vaccinated group.

	Control group (without vaccine or medicine)	**Vaccine group**	**"Belladonna 200" group**
Attacked with JE	26	0	5
Not attacked with JE	37	28	28
Total	63	28	33
Expected attacks (50%)	31.5	14	16.5
$(Observed-Expected)^2$	30.25	196	132.25
$(Observed-Expected)^2$/ Expected ($\chi 2$ Test)	0.96	14	8.01
		14.96 (P value significant at 0.005 level)	**8.97 (P value significant at 0.005 level)**

DISCUSSION

The results showed a definite protective action of "Belladonna 200" against JE. However, when we compared its action to vaccination then it was found that vaccination was slightly better than "Belladonna 200" in preventing JE. Thus out of 28 cases in vaccination group no animal was attacked with JE while five out of 33 animals were attacked with JE in "Belladonna 200" group. This difference was possibly due to the fact that "Belladonna 200" was administered orally in mice which may not be perfect in each time, but the JE vaccine was administered intraperitonially .by injection where there was practically no chance of a missed dose. Nevertheless, we may consider that vaccine is a bit better than "Belladonna 200". Now the important point is that the cost of this medicine is negligible, it can be administered orally thus will be widely accepted, it can be used even in remote places without any seasonal, storage and transport problems. Thus one may use it widely for general protection from JE in endemic areas and focal accentuations may be done by vaccinations in some areas of very high prevalence. This protocol may entirely change the global picture of JE and the disease can be mitigated to a great degree benefiting millions of people living in JE endemic areas.

Regarding probable mechanism of action of "Belladonna 200", we have practically no knowledge at the present moment. calystegines and related compounds present in *A. belladonna* are well known selective glycosidase inhibitors in comparison to common tropane alkaloids - atropine and scopolamine of *A. belladonna*, which are parasympatholytic and compete with the substrate for binding to the active site as observed in kinetic interaction measurements. Most enveloped viruses like human immunodeficiency virus, hepatitis B virus etc. showed glucosidase-mediated N-linked oligosaccharide trimming, which may be a possible mechanism by which calystegines of Belladonna may act on JE virus.

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