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Original Research Article

Preventive and Curative Role of "Belladonna 200" Against Japanese Encephalitis Virus Infection in Adult Mice

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K e y w o r d s

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A B S T R A C T

About 3 billion people are now at risk of Japanese encephalitis (JE) having its focus in South East Asian and Oceanian countries. There is no treatment of this disease and vaccination is still not adopted as a national program by most countries endemic to the disease. Thus this study was aimed to see possible preventive and curative effect of "Belladonna-200" against JE. This is an ultra-diluted homeopathic medicine without any known side effect and suitable for easy oral administration in inhabitants of remote villages and forest areas in the affected countries. The experiment was done in adult mice with adapted virulent Nakayama strain JE virus by a novel passage technique developed by us. In the control group 43.4% mice died after the adapted JE virus challenge. In the vaccinated group with SA14-14-2 JE vaccination all mice remained alive after the virus challenge and in the "Belladonna-200" treated group as preventive, curative and simultaneously treated options 13.6%, 22.8% and 50.0% mice died respectively after the challenge. The difference of outcome with SA14-14-2 JE vaccinated group and "Belladonna-200" preventive treatment was not statistically significant.

Introduction

The burden of Japanese encephalitis (JE) is still very high despite better vaccine coverage (Campbell *et al*., 2011). JE is now endemic in 24 Asian and Oceanian countries with an estimated 67,900 JE cases occurring annually (Campbell *et al*., 2011) with about 3 billion population is at risk. JE virus belong to the genus Flavivirus under the family Flaviviridae and is classified phylogenetically (Uchil, 2001; Li, 2011; Mohammed, 2011) into five genotypes (G1-G5) and the mode of JE virus dispersal study throughout the Asian countries using Bayesian Markov

Chain Monte Carlo (MCMC) simulations indicated its potential for introduction into non-endemic areas (Gao et al., 2013). JE is a mosquito borne disease with a 20-30% case fatality rate and neurologic sequel in 30-50% of survivors ((Campbell et al., 2011). The state of JE surveillance and immunization program in 2012, showed that there was no immunization program in 12 out of 24 countries and national program was present only in five countries among them. Again different types of vaccinations were used in different countries, thus in that study, live attenuated SA 14-14-2 JE vaccine (LAV) was used in five countries, inactivated mouse brain- derived JE vaccine (MB) was used in six countries and inactivated Vero cell culture derived JE vaccine (VC) was used in two countries. In China they used both LAV and VC. Thus it is a long way to improve awareness of JE disease and implementation of a national JE vaccine program in all the endemic countries. In our previous studies (Bandyopadhyay, 2010; Bandyopadhyay, 2011), we observed that simple homeopathic medicines like "Belladonna 200" could prevent JE virus infection in Swiss albino mice and in chorio-allantoic membrane of chick. Thus in this study we compared preventive efficacy of this medicine with LAV in experimental JE virus infection in adult Swiss albino mice along with a preliminary observation on this medicine's curative role also against JE.

Materials and Methods

The Medicine "Belladonna 200"

In this study we used **"**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 Homeopathic Pharmacopoeia of India (Ministry of Health, Government of India, 1971, 1:1, 7-16, 72).

Procurement and storage of Japanese Encephalitis Vaccine

The Japanese Encephalitis Live attenuated Vaccine; (SA14-14-2) was kindly gifted by the State District Hospital, Government. of West Bengal, India which was maintained as per manufacturer's instructions. The Government of West Bengal (a state in India) originally received the vaccine from Govt. of India (Lot no-200811CO57-4) manufactured by Chengdu Institute of Biological Products, Chengdu, China.

The adult mice

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 the virulent Nakayama strain (Source human, year 1935, location Japan, GenBank accession no. EF571853, Genotype III) was used.

Adult mice adaptation and inoculations

To study the effect of "Belladonna 200" on JE virus infection in adult mice, the experimental model was redesigned mainly for preventive aspect study as all previous experiments were mainly done on suckling mice which succumb within 72-96 hrs and immune-conversion is not possible in suckling mice within this short period of time. However, the real problem

was the virus from infected suckling mice brain does not affect the adult mice when inoculated intracerebrally probably due to absence of receptors, thickening of the skull bone of adult mice etc. Thus initially we established the JE virus infection by gradual passage inoculation of the virus obtained from infected suckling mice brain in the adult mice (6 -8 wks). The 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 10^{-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 (Bonamin and Endler, 2010; Berdai *et al.*, 2012).

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 observation were immersed in a closed vessel containing chloroform and later discarded according to statutory guidelines for Biomedical Waste management.

Experimental design

Control Group

In control experiment, litters were not orally fed with the medicine nor were they vaccinated. These mice were only challenged with intra-cerebral inoculation of LD50 dose (as determined initially) of JE virus.

Experimental Group

Group 1 (Preventive aspect)

Randomly selected adult mice (6 in number in each lot) were taken from litters in which every mouse was orally fed with 0.06 mL of "Belladonna 200" for 7 days. The mice were challenged with LD50 (as determined initially) JE virus suspension (intra-cranially) after 21 days of the first dose of administration of homoeopathic medicine.

Group II (Vaccinated group)

Randomly selected in-bred adult mice (6 in number) in each lot) were initially treated with two intra peritoneal doses of 0.03 ml of SA 14-14-2 JE vaccine on 0 and 7days. These mice were subsequently challenged intra-cerebrally with LD50 dose (as determined initially) of the JE virus suspension after 21 days of first dose of vaccine.

Group III (Curative aspect)

Randomly selected in-bred adult mice (6 in number in each lot) were initially challenged intra-cerebrally with LD 50 dose (as determined initially) of the JE virus suspension. These mice were observed daily for signs of sickness. On day 3 p.i. when mice showed signs of sickness, they were treated with two doses of 70 microl of Belledonna 200 at an interval of one hour. The dose and dilution of Belladonna 200, administered for curative purposes was derived from the references of Boenninghausen C.V (1843-1863), Boerick G (1960) and Farrington H (1921). 60 microl of the medicine (commercially obtained Bell 200) was diluted with 10 microl of distilled water

and was shaken for 1 minute (Bradford 1921). 70 microl of the diluted medicine, thus prepared, was administered orally as a single dose to the JE infected mice. This was followed by a second similar dose, 1 hr later. Thus only 2 doses of diluted Belladonna 200 were administered to the sick mice (at one hour interval) and they were observed for a further period of 30 days.

Group IV (Simultaneously treated)

Randomly selected in-bred adult mice (6 in numbers in each lot) were initially challenged intra-cerebrally with LD 50 dose (as determined initially) of the JE virus suspension. These mice were then immediately treated with two doses of 70 microl of Belledonna 200 at an interval of one hour. The dose and preparation of the medicine is as mentioned in Group III.

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. In curative treatment "Belladonna-200" was given in 3 days post JE virus inoculation and in simultaneous treatment method "Belladonna-200" was administered simultaneously with virus inoculation.

Results and Discussion

In the control group in which the mice were neither vaccinated nor treated with "Belladonna-200" 43.4% mice died after the JE virus challenge. An overwhelming result was obtained in the vaccinated group in which no mice died in the experiment. In the "Belladonna-200" group in which the experimental mice were treated in a preventive aspect (Table 1), curative aspect (Table2) or simultaneously treated (Table 3) with the virus inoculation the percentages of dead mice were 13.6%, 22.8% and 50.0% respectively. In statistical analysis $\chi 2$ test value in between the control group results and the vaccinated group results was 22.19 and the similar value for the "Belladonna-200" group was 16.79. Both the $\chi 2$ test values were significant at 0.0005 level, while the differences of the $\chi 2$ test value in between the vaccinated group and the "Belladonna-200" preventive group was not statistically significant. Similarly if we look into the $\chi 2$ test value in the curative group (Table 2), the difference is not so significant between the control and experimental group (the $\chi 2$ test value only 3.60) and when we compare such values between belladonna treated group with vaccine treated group the $\chi 2$ test value is alsos not statistically significant. There is no statistically significant difference between the control group and simultaneously treated group and the same is true for the simultaneously "Belladonna-200" treated group.

Atropa Belladonna extract from which ultradiluted "Belladonna-200" is prepared contains many bioactive chemicals like hyoscyamine, atropine, hyoscine, apo-atropine(atro-pamine), belladonnine, bellaradine and cuscohygrine. There are also flavonoids; minerals like iron, copper, cobalt, manganese which have also different biological activities. As "Belladonna-200" is prepared from crude extract it is very difficult to predict which

Table.1 Shows the survival and death rate of JE infected adult mice (6 wks old) in preventive treatment with "Belladonna-200" for 7 days and compared with the control group and the vaccinated group.

Group	Total no. of inoculated mice	Number of survived mice (%)	Number of died mice (%)
Control	71	42 (59.15%)	29 (40.84%)
Vaccinated	42	42(100%)	00(00.0%)
"Belladonna-200"	59	51(86.4%)	08(13.6%)

Table.2 Shows the survival and death rate of JE infected adult mice (6 wks old) in curative treatment (3 days post JE virus inoculation) with "Belladonna-200" and compared with control group and vaccinated group.

Group	Total no. of inoculated mice	Number of survived mice (%)	Number of died mice (%)
Control	36	19(52.77 %)	17(47.22 %)
"Belladonna-200"	36	26(72.2%)	10(22.8%)

Table.3 Shows the survival and death rate of JE infected adult mice (6 wks old) in simultaneous treatment with "Belladonna-200" and compared with control group and vaccinated group.

Group	Total no. of inoculated mice	Number of survived mice (%)	Number of died mice (%)
Control	12	07(58.33 %)	05(41.66%)
Vaccinated	06	01(16.66%)	05(83.33%)
"Belladonna-200"	06	03(50.0%)	03(50.0%)

is the key anti JE component of this preparation. The whole plant is used for the preparation of homeopathic drug "Belladonna-200" as per Homeopathic pharmacopeia of India (HPI). 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 when investigated by Dr.Samuel Hahnemann, a renowned German Physician and medical scientist (Hahnemann, 1801). 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.

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 (Dudgeon 1853).

According to Homeopathic pharmacopeia, Belladonna is a very useful remedy in arterial congestion of the brain from almost any cause (Hughes 1886).It is also indicated in homeopathic practice for inflammation and congestion with extreme burning heat, redness and throbbing in the affected regions.

The results of this study indicated a good preventive role of "Belladonna-200" against JE comparable to the preventive action of SA 14-14-2 JE vaccine. This study results strengthen the possibility of effective use of this medicine to prevent JE in all endemic areas.

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