

Suckling Mice of “Belladonna 200” Fed Mothers Evade Virulent Nakayama Strain Japanese Encephalitis Virus Infection

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Abstract: In many countries the prevalence of Japanese encephalitis is still increasing producing a significant number of deaths and disability-adjusted life years. Global warming with consequent increase in vector population may accentuate the occurrence of the disease in near future. There is no effective medicine against the disease and vaccination of rural poor population and pig population in a wide endemic zone appears not practicable. Thus we have to venture all possible sources to find out a remedy against this disease. In this experiment suckling mice of “Belladonna 200” fed mothers were challenged with virulent Nakayama strain of Japanese encephalitis virus and statistical analysis of the results indicated a protective role of this medicine when compared with controls. A hypothetic pathway of its action mediated by calyestegines involving envelope glycoproteins, synthesis of amino acid residues of E protein and Jak-Stat signaling cascades has been proposed.

Key words: Belladonna • Japanese encephalitis • Suckling mice • calyestegines

INTRODUCTION

Japanese encephalitis (JE) is essentially a zoonotic disease where wading water birds mainly herons and egrets are the reservoirs with frequent spill over even in epidemics to pigs, members of the family of equidae mainly horses and donkeys as well as humans. The most important vector of the disease is zoophilic *Culex tritaeniorrhynchus* related to *Cx. gelidus* complex. It primarily affects central nervous system and can produce severe neurological complications and even death [1].

This disease is presently prevalent in south Asia, south east Asia, east Asia and in the Pacific with a 3 billion population at risk, an annual incidence of 30,000 to 50,000 cases, with 10,000 to 15,000 annual deaths [2] and a global impact of about 7,00,000 disability-adjusted life years (DALYs) [3]. Local incidence rates usually range from 1-10 cases per 100,000 populations but can reach

more than 100 cases per 100,000 populations during outbreaks. Among countries particularly India, Nepal, Thailand, Malaysia, Myanmar, Japan, China, Indonesia, Bangladesh extending in a wide zone from Pakistan to Siberia and Japan the disease is now prevalent [1]. In seven countries (Cambodia, Laos, Bangladesh, Myanmar, India, Indonesia, Pakistan), JE virus infection is presently increasing with a significant percentage of total population living in rural JE-endemic area and among these countries DALYs are the greatest in India [12]. Recently more than 1300 children died in outbreaks of JE in the north eastern state of Uttar Pradesh, India although possibility of other enterovirus infection mixed with JE virus has not been ruled out [19].

According to Igarashi live attenuated vaccine against JE was developed about 40 years back [4] and newly developed vaccines like live inactivated yellow fever virus-chimeric vaccine [2] and adjuvanted SA 14-14-2

vaccine [5] grown on Vero cell lines, are not only safe but also effective in single doses. However, two important obstacles for an effective vaccination program are there which are very difficult to solve: difficulties in delivering the vaccine in rural poor population and failures of vaccination in newborn pigs having maternal antibodies and with a high turnover rate of their population [5].

Experiments on intermittent irrigation (alternate wetting and drying) of paddy fields was found effective in vector control but its implementation is also very complicated because all paddy fields could not be covered simultaneously in such a program due to the need to cover a vast area, sufficient water may not be available in rice-growing season and countrywide educational as well as supportive program may not be practicable [6]. The transmission of JE virus is multi-factorial with at least five variables directly or indirectly influences the rate at which the virus is transmitted - the viral strain, vector, wild vertebrate hosts, humans and environmental factors; thus incomplete targeting to one factor has got very little influence in this complex system of transmission.

Alterations in temperature and rainfall patterns induced by recent global climatic changes may lead to a significantly increased vector population in the endemic areas which will be very difficult to control [6].

Chemical control of the vectors with pyrethroids, organophosphates, carbamates etc. was also not found suitable due to their short term effects and rising levels of insecticide resistance [7].

The disease usually starts as a flu-like illness with fever, headache, nausea, vomiting and weakness. Altered mental status usually occurs from mild confusion to agitation to coma. Seizures develop in about 66% of sick children, while headache and meningismus are commonly found in adults. There is no specific medicine to treat the patients suffering from JE. According to the World Health Organization [3] the disease is fatal in up to 30 percent of cases and there is a possibility that those who survive may be disabled for life.

The homeopathic medicine "Belladonna200" is prepared with the root and the leaves of *Atropa belladonna*, which is also known as Deadly Nightshade, Dwale, Black Cherry, Strygium and Strychnon. In ancient times, the Venetians named this plant as Belladonna because at that time ladies used a distilled product of the plant as a cosmetic; hence the name "Bella-donna" or beautiful lady. One important indication of Belladonna is its use as a medicine in the treatment of patients with cerebral congestion as mentioned in homoeopathic

pharmacopoeia. In a previous study we also observed preventive role of Belladonna in JE virus infected chick chorio allantoic membrane [8].

Thus considering all these facts this study was done to find out any protective role of Belladonna against JE when the infection was challenged experimentally in suckling mice.

MATERIALS AND METHODS

Place of Study: The study was conducted at the Virology unit, Department of Microbiology, School of Tropical Medicine, Kolkata, during the period May 2008 -Sept 2010.

Medicine: 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 Homeopathic Pharmacopoeia 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 suckling mice of treated mothers 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. Boeninghausen [20], a veterinary doctor as well as a renowned homoeopathic practitioner described in 1843[20] that "Belladonna 200" is the ideal medicine in experiments with mice. Thus we used "Belladonna 200" in all successive lots in this experiment.

Experiment: The most common choice of host for isolating arboviruses is still the suckling mice [11]. In this experiment, 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.

Standardization of the Viral Inoculums: The virulent Nakayama strain virus was consecutively passed 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 suckling mice were injected intracerebrally (i.c.) with dilutions of the stock suspension from 10^{-1} to 10^{-9} . All the inoculated suckling mice were observed

daily to note their survival and following this LD₅₀ value was calculated by a standard method [10,11].

Methods of Inoculation of Suckling Mice: This was done following the method described by Gould and Clegg [11]. 0.02 mL of the virus suspension was inoculated into one of the cerebral hemisphere penetrating no more than 1.5 mm. The needle is kept in position for 2 Sec and then removed slowly. The procedure was repeated until the complete litter had been inoculated. For determination of LD₅₀ dose, inoculations were commenced with the highest dilution, so that the same needle and syringe could be used with each viral dilution. 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. 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 ethanol IP (Indian Pharmacopoeia) 95% v/v and the scalp was removed using one pair of sterile scissors and forceps. The skull cap was then lifted up and the brain was removed by the closed ends of the scissors.

Experimental Design: Suckling mice (2-3 days old) were taken from litters in which mothers were orally fed with 0.06 mL of Belladonna preparations for 7/14 days. In control experiment, suckling mice were similarly taken from mothers not orally fed with the medicine. After this suckling mice of both the groups were challenged with 0.02 mL of the supernatant of clarified JEV infected mice brain emulsion (10%) diluted to a LD₅₀ dose i.c. 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 particularly in the last 24 h were refusal to feeds, became disarranged in the nest, tremors and muscular spasms, ataxia and hind-limb paralysis followed by death within a few hours. Those died within the first 24 h were considered as non-specific deaths. For preparation of infected brain emulsion required for LD₅₀ determination and standardization of the inoculums, brains were collected close to the time of death when the animal showed acute signs of uniform sickness (usually on third post inoculation day), their brains were harvested aseptically. The brains after weighing, were ground in a

homogenizer (Lourde's homogenizer) placed in ice bath to give a 10% w/v suspension in BAPS (Bovine Albumin Phosphate saline, pH 7.3 ± 0.1) with antibiotics. The suspension was initially centrifuged at 2000 rpm at 4°C for 15 minutes, following which the supernatant was recentrifuged at 10,000 rpm at 4°C for one hour (in a refrigerated centrifuge). The supernatant was carefully collected and was kept in small aliquots of 0.5 ml screw capped vials, labeled and sealed and stored at - 70°C.

RESULTS

LD₅₀ of the virulent Nakayama strain JE virus was 7.0 log₁₀ /0.02mL. The average survival rates of the control group (n=96) (where mothers were not treated with the medicine), experimental group (n=67) (suckling mice from mothers treated with "Belladonna 200" for 7 days) and the last experimental group (n=53) (suckling mice where mothers were treated with the medicine for 14 days) were 47.92, 80.60 and 79.24%, respectively. Details of the outcome of this study and statistical analysis (χ^2 test) are given in Table 1 and Table 2 respectively. The statistical analysis (χ^2 test) indicated a highly significant difference with p value significant at 0.01 levels.

DISCUSSION

The major findings of this study are that administration of "Belladonna 200" for 7/14 days, significantly improved the survival rate (p=0.001) of JE infected suckling mice (80.60%) as compared with the controls (47.92%). Previously, we reported the effect of administration of ultradiluted Belladonna on chick chorioallantoic membrane of embryonated eggs which could significantly decrease the pock count when challenged with JE virus [8], indicating the beneficial role of ultradiluted Belladonna in the prevention of JE. Unexpectedly, in the present study, we did not observe any significant advantage of administration of "Belladonna 200" for 14 days over 7 days so far improvement in the survival rate of suckling mice were concerned by comparing with the controls.

Mosquitoes in the genus *Culex* have a strong tendency to ornithophagy probably due to relatively inactive thrombocytes present in birds because anti-thrombin peptides are probably less in the saliva of these mosquitoes, only recently they are gradually adapted to mammals[7]. It appears that recent changes due to global warming will accentuate their behavior in adaptation and a great havoc in human population may occur in near future by this virus unless we are prepared.

Table 1: Outcome of the experiment in which suckling mice of treated or untreated mothers with Belladonna 200 were challenged with a LD₅₀ dose of virulent Nakayama strain of JE virus.

Suckling mice	Control (Mice' Mothers without any treatment)			Mice' Mothers treated with Bell-200 for 7days			Mice' Mothers treated with Bell-200 for 14 days		
	No.	Death	Alive	No.	Death	Alive	No.	Death	Alive
Lot-1	8	4	4	6	1	5	8	1	7
Lot-2	7	3	4	6	1	5	7	2	5
Lot-3	8	4	4	5	1	4	--	---	--
Lot-4	9	4	5	8	1	7	7	1	6
Lot-5	8	4	4	--	---	---	7	1	6
Lot-6	6	4	2	6	1	5	--	--	--
Lot-7	6	4	2	6	1	5	--	--	--
Lot-8	8	3	5	8	1	7	7	1	6
Lot-9	6	3	3	6	2	4	--	--	--
Lot-10	4	2	2	4	1	3	--	--	--
Lot-11	6	3	3	6	2	4	--	--	--
Lot-12	6	4	2	6	1	5	--	---	--
Lot-13	6	3	3	--	--	--	9	2	7
Lot-14	8	5	3	---	---	---	8	3	5
Totaland%	96	50(52)	46(48)	67	13(19.4)	54(80.6)	53	11(20.8)	42(79.2)

Table 2: Statistical analysis (χ^2 test) showing the significant protective action of "Belladonna 200" in suckling mice challenged with LD₅₀ dose of virulent Nakayama strain of JE virus.

Suckling Mice	Control (Mice' Mothers without medicine)	Experimental group 1 (Mice' Mothers treated with Belladonna 200 for 7 d)	Experimental group 2 (Mice' Mothers treated with Belladonna 200 for 14 d)	Total
Attacked with JE	50	13	11	74
Not attacked with JE	46	54	42	140
Total	96	67	53	214
Expected attacks (50%)	48	33.5	26.5	
(Observed-Expected) ²	4	420.25	240.25	
(Observed-Expected) ² /(Expected) (χ^2 test)	0.08	12.54	9.07	21.69
				(p value significant at 0.01 level)

Medicinal properties of Belladonna are known from time immemorial. Many homoeopathic practitioners used it in prevention of JE, although there is no experimental proof in favor of it [8, 20, 21]. This experiment clearly indicated preventive role of this medicine against JE which is statistically highly significant.

Although such action of Belladonna is difficult to explain we here propose a hypothetic action pathway of this medicine in preventing JE virus infection. If we look into the occurrence of calystegines and related compounds in *A. belladonna* and related plants then it is obvious that these are present in significantly higher amounts in *A. belladonna* [12]. Calystegines are well known selective glycosidase inhibitors in comparison to common tropane alkaloids atropine and scopolamine of *A. belladonna*, which are parasympatholytic. Like most

glycosidase inhibitors, calystegines compete with the substrate for binding to the active site as observed in kinetic interaction measurements. Most glycosidases perform enzymatic hydrolysis reaction with the aid of a glutamic acid residue in the active cleft and calystegines mimic the transition state of this reaction [12].

Most enveloped viruses like human immunodeficiency virus, hepatitis B virus etc. showed altered life cycle during invasion of cells in which glycosidase-mediated N-linked oligosaccharide trimming is inhibited and N-linked oligosaccharide processing events in the endoplasmic reticulum are important for the secretion of some enveloped viruses [13] characterized by sequential trimming of the glucose residues on oligosaccharide precursor. It has also been demonstrated that Dengue virus envelope glycoprotein processing in

cells was strongly affected by this important mechanism [14]. Thus Belladonna appears to act through this pathway in preventing JE virus infection in suckling mice [8]. This also may lead to inhibition of the synthesis of key amino acid residues of the E protein of Nakayama strain J E virus (E107,Leu; E138,Glu; E176,Ile; E177,Thr; E264,Gln; E279,Lys; E315,Ile; E439,Lys) which are important for neurovirulence. It also blocks JE virus in evading action of interferon on them. Although interferon has been identified as the most promising antiviral agent against JE virus [15], but inside the body JE virus usually target the Jak-Stat (Janus Kinase-signal transducer and activation of transcription) signaling cascade to evade the interferon response leading to failure of interferon treatment in JE infected children [16]. In this connection, it is important to note that cluster of genes encoding the interferon - induced 2'-5'-OAS (2',5'-Oligoadenylate synthetases) is present in chromosome 5 in mice- known as Flv gene is also responsible for susceptibility to JE virus infection in mice [17]. It has been observed that some newly synthesized proteins are required to block Jak-Stat signaling by JE virus [18]. Thus protein synthesis inhibitors may play a role in decreasing pathogenicity of JE virus where this medicine may play a role through the above mentioned pathways.

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