Decreased intensity of Japanese encephalitis virus infection in chick chorioallantoic membrane under influence of ultradiluted belladonna extract

Bandyopadhyay, Bhaswati; Das, Satadal; Sengupta, Milan; Saha, Chandan; Das, Kartick Chandra; Sarkar, Debabrata; Nayak, Chaturbhuj

Introduction

Neglect of the basic requirements of health; poor political support for health; a weak public health capacity; centralized programs for control based on selective interventions and poorly-planned development projects have created conditions ideal for the outbreak of JE like diseases (Bhargava and Chatterjee, 2007). A good community awareness of encephalitis, a prompt referral system and a good supportive treatment for the patients and a good surveillance system was found to help in the reduction in cases, deaths and disabilities due to this disease (Gupta et al, 2008). Approximately 2 billion people live in countries where JE presents a significant risk to humans and animals, particularly in China and India, with at least 700 million potentially susceptible children (Gould et al., 2008). In Southeast Asia around 50.000 cases and 10.000 deaths occur per year affecting essentially children below 10 years of age. Further threats to humanity are there because the JE virus has shown a tendency to extend to other geographic areas. The combined effects of climate change, altered bird migratory patterns, increasing movement of humans, animals and goods, increasing deforestation and development of irrigation projects will also help this geographic dispersal of the virus producing an enhanced threat. The disease is also highly prevalent in animals. In Nepal, sero-prevalence of JE in pigs, ducks and horses was 48.11, 26.79 and 50.0%, respectively (Pant, 2006). At present in Nepal JE is seasonally endemic to the Terai region (Wierzba et al., 2008) and in Kathmandu valley (Partridge et al., 2007) affecting population of both lowland plains as well as the hilly regions. Phylogenetic analysis showed that JE isolates in India belonged to genogroup 3 (Parida et al., 2005).

Belladonna or Atropa belladonna (deadly nightshade)-the source of the drug atropine belong to genus Atropa along with two other species (Hunziker, 2001). Although the phylogenetic affinity of Atropa to Hyoscyameae has been controversial for over a century (Hunziker, 2001), however, in a recent study this affinity was conclusively proved (Yuan et al., 2006). The plant Atropa belladonna is surrounded by myth, fear and awe. That this plant contains poison is known from the ancient Greek and Roman civilization to medieval witches, professional poisoners, sorcerors and ultimately in 1830s atropine was isolated from the plant (Lee, 2007). Atropa belladonna is rich in tropane alkaloids, primarily atropine and scopolamine (Talaty et al., 2005).

Roots are the major organs of tropane alkaloid biosynthesis and after their formation in roots, tropane alkaloids are transported to the aerial parts of the plant. Putrescine N-Methyltransferase (PMT) is the pivotal enzyme for the biosynthesis of tropane alkaloids. The PMT cDNAs were cloned from A. belladonna and were found to encode a protein homologous to spermidine synthases indicating the evolutionary origin of PMT from spermidine synthase. In A. belladonna, PMT is located in pericycle and

xylem cells of the root. Root cultures of A. belladonna form the tropine derived alkaloids hyoscyamine, scopolamine and calystegines.

Corresponding Author: Bhaswati Bandyopadhyay, Department of Microbiology, Virology Unit, School of Tropical Medicine, Kolkata-700073, India

While hyoscyamine and scopolamine are also found in other related species to A. belladonna, thus calystegines appear to be the key agent which is present in high concentration to A. belladonna only and thus differentiates biological activities of belladonna from other related plants. The biosynthetic pathway of tropane alkaloids is very complex. Putrescine first converted into N-methyl putrescine by PMT. N-methyl putrescine yields tropinone. In tropinone I pathway tropinone is converted to tropine, tropine is converted to hyoscyamine and finally converted to scopolamine by the action of hyoscyamine-6-hydroxylase. In the relatively less investigated tropinone II pathway at first there is formation of pseudotropine, which is then converted to calystegine.

Although most human infections are mild or asymptomatic, about 50% of patients who develop Japanese encephalitis suffer permanent neurologic defects and 30% of them die due to the disease (Babu et al., 2006).

Vaccines for JE have been available for many years and their use has been effective in reducing the incidence of JE disease in several countries (Diagana et al., 2007) but, as disease incidence has decreased, concerns regarding adverse events following immunisation have increased (Beasley et al., 2008). Childhood mass immunization programs with first generation, mouse brain-derived vaccines showed occurrence of severe side effects in Japan.

No specific antiviral therapy is currently available (Gould et al., 2008) despite an emergence and resurgence of flavivirus-mediated diseases (Ray and Shi, 2006). There are few recent studies, which were aimed to treat JE with new drugs. A plant lignan arctigenin was found to reduce viral load and viral replication within the brain, neuronal death and secondary inflammation and oxidative stress resulting from microglial activation, suggesting its potential for treating JE, however, unless it is tested in human beings it can not be used in treatment of JE (Swarup et al., 2008). Similarly, Interferon alpha-2a was tested in children with JE, but with negative results. There is thus a real need for antivirals that can reduce the toll of death and neurological sequelae resulting from infection with JE virus (Gould et al., 2008). Therefore, this study was aimed to see whether ultradiluted belladonna has a role in this infection.

Materials And Methods

Ultradiluted belladonna: For the study we selected Ultradiluted Belladonna 3,6,30,200. These medicines were included in this study because they are claimed by practitioners and researchers of alternative medicine to have a positive role in the treatment and prevention in Japanese Encephalitis (JE). All these medicines were available commercially and were prepared according to standard procedures advocated by homeopathic pharmacopoeia of India (Ministry of Health, Government of India, 1971, 1:1, 7-16, 72).

Procurement of ultradiluted belladonna for this study: Medicines were reconstituted in sterile pyrogen free water immediately before their applications after complete elimination of alcohol.

The aqueous dilution of Belladonna (3, 6, 30, 200) was prepared and procured from reputed Homeopathic drug company, Hahnemann Publishing Co. Pvt. Ltd (HAPCO), Kolkata.

Virus stock: JE virus stock (Nakayama strain), which is maintained in School of Tropical Medicine, Kolkata, was used in this study.

Embryonated chick egg inoculations: For preventive studies one dose (50 [micro]L) of aqueous dilution of each selected medicine was inoculated in the chorioallantoic membrane followed by the administration of 50 [micro]L of the JE viral suspension, 5-10 min later. The chorio allantoic membrane consists of an outer layer of stratified epithelium, which constitutes the respiratory surface of the embryonated egg and an inner layer of endoderm (the lining of the allantoic cavity).

Dermotropic viruses (poxviruses and some herpes viruses) and JE viruses grow on this membrane and at low concentrations, they produce discrete foci of cell proliferation and necrosis (pocks). The membrane was therefore used to assay JE viruses in this study. Different viruses cause pocks of different color and morphology and this is also of diagnostic value for distinguishing between different viruses. One-day-old fertile hen's (White leghorn) eggs were obtained from State Poultry Farm of Govt. of West Bengal, Tollygunge, Kolkata. They were collected from healthy flocks, which were maintained on a well balanced and antibiotic free diet. The eggs were incubated at 37[degrees]C within a special egg incubator with 65% humidity.

On the 12th day eggs were candled with the help of an illuminator in a dark room to check viability, movements of embryos and the area of the blood vessels was defined. The air space was marked on the eggshell with a pencil and a point was selected on CAM avoiding injury of large blood vessels. The air space was punctured with a pointed end of hand punch. The shell was also punctured after clearing with a sterile cotton swab on the marked spot over the CAM, using the hand punch with slight rotatory motion, avoiding injury to the shell membrane. The shell dust was blown away with capillary pipette. A drop of sterile normal saline was placed over the inoculation site. The tip of the blunt instrument was inserted through the drop of saline. This tore the cell membrane and the drop of fluid was sucked inside as the CAM fell away creating a new air space. Slight suction with a rubber teat over the hole at the blunt end of the egg was applied to have a complete dropping of the membrane confirmed by candling.

The inoculum was deposited into the CAM with the help of tuberculin syringe and the inoculated egg was rotated to facilitate the dispersion of the inoculum. The hole in the air sac was sealed and the inoculated eggs were incubated at 37[degrees]C for 48 h in a horizontal position. After 48 hours the shell over the false air sac was painted with tincture Iodine and the shell membrane was broken with a blunt forceps for maximum exposure of the CAM. The membrane was cut out with a sterile pair of scissors and placed in a Petri dish for further examination.

Control study: Japanese encephalitis virus (50 [micro]L) in the same concentration ([10.sup.-3]) in bovine albumin phosphate saline pH 7.20 mixed with equal volume (50 [micro]L) of sterile pyrogen free distilled water was also inoculated on chorioallantoic membrane. The pock count of this control virus study was considered as baseline data and any deviation from this baseline was noted after application of different medicines in the test series. An initial experiment was also done with different concentrations of viruses to find out the dilution which gave the maximum number of pocks on CAM (optimum dilution). If during the study, there was death of the inoculated eggs or membranes were not found properly the data of that lot were excluded.

Apart from this virus control experiment, similar control studies were also done with all the medicines without the virus in equal dilutions with water. Control studies were also performed with Bovine albumin in phosphate saline pH 7.2 and potentised distilled water and studied similarly as viral dilutions.

Observation of growths on CAM: Inoculated CAMs were observed after 48 h of inoculation particularly to see the formation of pocks and other associated changes on CAM.

Results

Optimum dilution of the JE virus was studied first which can produce significant number of pocks on CAM with different concentrations of the virus-Neat (10% infected brain suspension), [10.sup.-1], [10.sup.-2], [10.sup.-3], [10.sup.-4], [10.sup.-5], along with control (buffer solution without the virus). The results showed that [10.sup.-3] dilutions showed maximum pock count and thus this optimum concentration was used throughout the experiment.

Results of different experiments with Belladonna 3, 6, 30, 200 are given in Table 1, Fig. 1 and 2. Control studies with different medicines without virus and bovine albumin phosphate saline showed no significant findings on CAM. The results showed significantly decreased pock count when JE virus infection on CAM was challenged with belladonna.

Discussion

In this study JE virus infections on CAM and in mice were challenged with different ultradiluted Belladonna preparations-Belladonna 3, 6, 30, 200. Encouraging results were obtained with all ultradiluted Belladonna 3, 6, 30, 200 preparations used in this study showing inhibition of viral growth on CAM. Therefore, this initial study conclusively showed the beneficial role of these medicines in JE infection. However, it is not possible to explain this action with our current poor knowledge about these ultradiluted preparations. Various mechanisms of these ultradiluted preparations were postulated by many workers but there is no confirmation of all these hypothetic research (Andersson et al., 1997).

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 only. Thus amounts (microgram per gram fresh mass) of calystegine A3 in young leaf, flower, mature leaf and in root of A. belladonna are 280, 146, 62 and 14 respectively. Similarly amounts (microgram per gram fresh mass) of calystegine B2 in young leaf, flower, mature leaf and in root of A. belladonna are 380, 263, 70 and 13 respectively (Draeger et al., 1995).

Calystegines are selective glycosidase inhibitors in contrast to common tropane alkaloids atropine and scopolamine of A. belladonna, which are mainly parasympatholytic.

Like most glycosidase inhibitors, calystegines compete with the substrate for binding to the active site. There are evidences that N-linked oligosaccharide processing events in the endoplasmic reticulum are important for the secretion of some enveloped viruses (Mehta et al., 1998) characterized by sequential trimming of the glucose residues on oligosaccharide precursor. It was found that Dengue virus envelope glycoprotein processing in cells was strongly affected by this unique mechanism. Thus it is probable that these ultradiluted preparations may also act in a similar way by calystegines.

Conclusion

In conclusion, we may claim that ultradiluted Belladonna 3,6,30,200 have potential role in diminishing JE virus infection on CAM. The probable mechanism of action of these ultradilued preparations appeared to be due to glycosidase inhibitor action of calystegines present in Belladonna.

Acknowledgement

This study was done as a part of the project study (No.17-88/2006-2007/CCRH/Tech/Coll/STM) financed by CCRH, AYUSH, Govt. of India. We thank Prof. D.K. Neogi, Ex Professor, Department of Virology, School of Tropical Medicine, Kolkata; Dr. P.C. Mal, Dr. N.R. Dey of CCRH, Govt. of India for fruitful suggestions. We are also thankful to our technical personnel Surja Kumar Halder, Suniti Bikash Ghosh, Kajori Mukherjee and Kartick Chandra Roy, for their dedicated study in the project. Lastly we sincerely acknowledge the helpful suggestions given by Dr. Rathin Chakraborty, Member Scientific Advisory Committee, CCRH, Govt. of India.

References

Andersson, P.U., A. Tomsic, M.B. Andersson and J.B.C. Pettersson, 1997. Emission of small fragments during water cluster collisions with graphite surface. Chem. Phys. Lett., 279: 100-106. DOI: 10.1016/S0009-2614(97)00990-1.

Babu, G.N., J. Kalita and U.K. Misra, 2006. Inflammatory markers in the patients of Japanese encephalitis. Neurol. Res., 28: 190-192. DOI: 10.1179/016164106X98062

Beasley, D.W., P. Lewthwaite and T. Solomon, 2008. Current use and development of vaccines for Japanese encephalitis. Expert Opin. Biol. Ther., 8: 95-106. DOI: 10.1517/14712598.8.1.95

Bhargava, A. and B. Chatterjee, 2007. Chikungunya fever, falciparum malaria, dengue fever, Japanese encephalitis ... are we listening to the warning signs for public health in India? Indian J. Med. Ethics, 4:18-23. PMID: 18630215

Diagana, M., P.M. Preux and M. Dumas, 2007. Japanese encephalitis revisited. J. Neurol. Sci., 15: 165-70. DOI: 10.1016/j.jns.2007.06.041

Draeger, B., A. van Almssick and G. Mrachatz, 1995. Distribution of calystegines in several Solanaceae. Planta Medica, 61: 577-579.

Gould, E.A., T. Solomon and J.S. Mackenzie, 2008. Does antiviral therapy have a role in the control of Japanese encephalitis? Antiviral Res., 78: 140-914. DOI: 10.1016/j.antiviral.2007.10.005

Gupta, N.C., K. Karmakar, S. Jain, S.K. Venkatesh, S. Lal and S. Bellary, 2008. India achieves negligible case fatality due to Japanese encephalitis despite no vaccination: An outbreak investigation in 2004. Ind. J. Pediatr., 75: 31-7. http://medind.nic.in/icb/t08/i1/icbt08i1p31.pdf

Hunziker, A.T., 2001. Genera Solanacearum: The Genera of Solanaceae Illustrated, Arranged According to a New System. Koeltz Scientific Books, Konigstein, Germany, ISBN 3-904144-774, pp: 500.

Lee, M.R., 2007. Solanaceae IV: Atropa belladonna, deadly nightshade. J. R. Coll. Phys. Edind, 37: 77-84. DOI: 10.1.1.106.7273

Mehta, A., N. Zitzmann, P.M. Rudd, T.M. Block and R.A. Dwek, 1998. [alpha]-Glucosidase inhibitors as potential broad based anti-viral agents. FEBS Lett., 430: 17-22. DOI: 10.1016/S0014-5793(98)00525-0

Pant, G.R., 2006. A serological survey of pigs, horses and ducks in Nepal for evidence of infection with Japanese encephalitis virus. Ann. N. Y. Acad. Sci., 1081: 124-129. DOI:10.1196/annals.1373.013

Parida, M., P.K. Dash, N.K. Tripathi, A.S. Sannarangajah and P. Saxena et al., 2005. Japanese Encephalitis Outbreak, India. Emerg. Infect Dis., 12: 1427-1430. http://www.cdc.gov/ncidod/EID/vol12no09/06 0200.htm

Partridge, J., P. Ghimire, T. Sedai, M.B. Bista and M. Banerjee et al., 2007. Endemic Japanese encephalitis in the Kathmandu Valley, Nepal. Am. J. Trop Med. Hyg., 77: 1146-1149. http://www.ajtmh.org/cgi/reprint/77/6/1146

Ray, D. and P.Y. Shi, 2006. Recent advances in flavivirus antiviral drug discovery and vaccine development. Recent Patents Anti-Infect Drug Disc., 1: 45-55. http://www.bentham.org/pri/samples/pri1-1/Shi.pdf

Swarup, V., J. Ghosh, M.K. Mishra and A. Basu, 2008. Novel strategy for treatment of Japanese encephalitis using arctigenin, a plant lignan. J. Antimicrob. Chemother., 61: 679-688. DOI: 10.1093/jac/dkm503

Talaty, N., Z. Takats and R.G. Cooks, 2005. Rapid in situ detection of alkaloids in plant tissue under ambient conditions using desorption electrospray ionization. Analyst, 130: 1624-1633. DOI: 10.1039/b511161g

Wierzba, T.F., P. Ghimire, S. Malla, M.K. Banerjee and S. Shrestha et al., 2008. Laboratory-based Japanese encephalitis surveillance in Nepal and the implications for a national immunization strategy. Am. J. Trop Med. Hyg., 78: 1002-1006. http://www.ajtmh.org/cgi/reprint/78/6/1002

Yuan, Y., Z. Zhang, Z. Chen and R.G. Olmstead, 2006. Tracking ancient polyploids: A retroposon insertion reveals an extinct diploid ancestor in the polyploid origin of belladonna. Mol. Biol. Evol., 23: 2263-2267. http://lseb.ibcas.ac.cn/cn/yjcg/pdf/lseb2006.pdf

1. Bhaswati Bandyopadhyay, (2) Satadal Das, (1) Milan Sengupta, (3) Chandan Saha, (4) Kartick Chandra Das, (4) Debabrata Sarkar and (5) Chaturbhuj Nayak

1. Department of Microbiology, Virology Unit, School of Tropical Medicine, Kolkata-700073, India
2. Department of Pathology and Microbiology, D.N. De H. Medical College, West Bengal University of Health Sciences, Kolkata-700046, India
3. Department of Clinical and Experimental Pharmacology, School of Tropical Medicine, Kolkata-700073, India
4. Drug Proving Research Centre, CCRH, Government of India, Kolkata-700 046, India
5. Department of AYUSH, Ministry of Health, CCRH, Government of India, JLN Anudandhan Bhawan, 61-65 Intitutional Area, Janakpuri, New Delhi 110058

Table 1: Changes in pock count on CAM with ultradiluted belladonna

Experiment	Pock count on CAM in number (Average [+ or -] SD [+ or -] SEM)
Belladonna 3 (N-500)	
Virus control	80.73[+ or -]31.91[+ or -]5.32

Virus + Medicine 25.32[+ or -]11.18[+ or -]1.86
Belladonna 6 (N-500)
Virus control90.97[+ or -]12.87[+ or -]2.14
Virus + Medicine 20.74[+ or -]7.43[+ or -]1.24
Belladonna 30 (N-200)
Virus control55.92[+ or -]15.56[+ or -]2.59
Virus + Medicine 13.31[+ or -]5.20[+ or -]0.87
Belladonna 200 (N-300)
Virus control53.97[+ or -]28.21[+ or -]4.70
Virus + Medicine 18.17[+ or -]12.66[+ or -]2.11

Experiment	t-value of the difference and its significance
Belladonna 3 (N-500)	
Virus control	9.84, P value highly significant
Virus + Medicine	at 0.01 level
Belladonna 6 (N-500)	
Virus control	28.43, P value highly significant
Virus + Medicine	at 0.01 level
Belladonna 30 (N-200)	
Virus control	15.61, P value highly significant
Virus + Medicine	at 0.01 level
Belladonna 200 (N-300)	
Virus control	6.95, P value highly significant
Virus + Medicine	at 0.01 level

CAM: Chorioallantoic Membrane; SD: Standard Deviation; SEM: Standard Error of Mean; N: Number of inoculated eggs; Eggs that were dead or yielded deformed or absent CAM, were not considered for calculation of the results