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## Decreased Intensity of Japanese Encephalitis Virus Infection in Chick Chorioallantoic Membrane Under Influence of Ultradiluted Belladonna Extract

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**Abstract: Problem statement:** No specific antiviral therapy is currently available despite an emergence and resurgence of Japanese encephalitis in South-East Asian Countries. There are only few recent studies, which were aimed to treat Japanese encephalitis with newer drugs. There is thus a real need for study on antiviral agents that can reduce the toll of death and neurological sequelae resulting from infection with this virus. **Approach:** Optimum dilution of the JE virus was determined which could produce significant number of pocks on Chorioallantoic Membrane (CAM). Then ultradiluted belladonna preparations were used to see their inhibitory action on JE virus infection in CAM. **Results:** Ultradiluted belladonna showed significantly decreased pock count in CAM in comparison to JE virus control. **Conclusion:** Ultradiluted belladonna could inhibit JE virus infection in CAM, which may be mediated through glycosidase inhibitory role of calystegines present in belladonna.

**Key words:** Japanese Encephalitis (JE), Chorioallantoic Membrane (CAM), pock, belladonna

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### 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, seroprevalence 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

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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, sorcerers 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. 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  $\mu$ L) of aqueous dilution of each selected medicine was inoculated in the chorioallantoic membrane followed by the administration of 50  $\mu$ 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°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°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 µL) in the same concentration ( $10^{-3}$ ) in bovine albumin phosphate saline pH 7.20 mixed with equal volume (50 µL) 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),  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ , along with control (buffer solution without the virus). The results showed that  $10^{-3}$  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.

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

Experiment	Pock count on CAM in number (Average $\pm$ SD $\pm$ SEM)	t-value of the difference and its significance
<b>Belladonna 3 (N-500)</b>		
Virus control	80.73 $\pm$ 31.91 $\pm$ 5.32	9.84, P value highly significant at 0.01 level
Virus + Medicine	25.32 $\pm$ 11.18 $\pm$ 1.86	
<b>Belladonna 6 (N-500)</b>		
Virus control	90.97 $\pm$ 12.87 $\pm$ 2.14	28.43, P value highly significant at 0.01 level
Virus + Medicine	20.74 $\pm$ 7.43 $\pm$ 1.24	
<b>Belladonna 30 (N-200)</b>		
Virus control	55.92 $\pm$ 15.56 $\pm$ 2.59	15.61, P value highly significant at 0.01 level
Virus + Medicine	13.31 $\pm$ 5.20 $\pm$ 0.87	
<b>Belladonna 200 (N-300)</b>		
Virus control	53.97 $\pm$ 28.21 $\pm$ 4.70	6.95, P value highly significant at 0.01 level
Virus + Medicine	18.17 $\pm$ 12.66 $\pm$ 2.11	

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

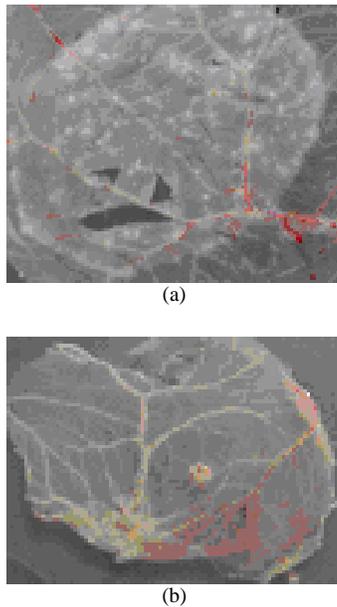


Fig. 1: Pocks on CAM-(a) with JE virus,  $10^{-3}$  dilution; (b) inhibition of pocks with JE virus,  $10^{-3}$  dilution and Belladonna 3

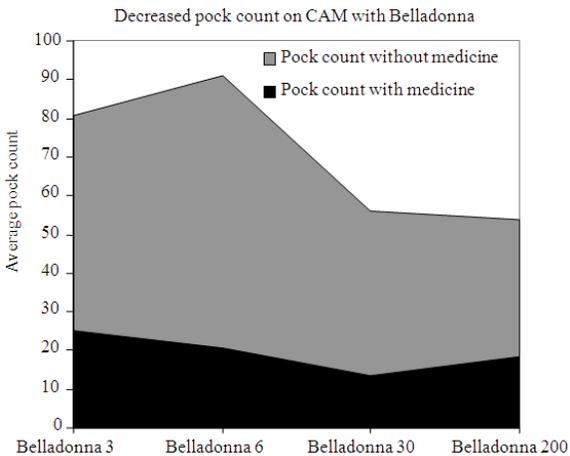


Fig. 2: Remarkable decreased pock count on JE virus infected CAM with Belladonna

### 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 A<sub>3</sub> 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 B<sub>2</sub> 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 parasymphatholytic.

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 ultradiluted preparations appeared to be due to glycosidase inhibitor action of calystegines present in Belladonna.

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