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ROLE OF ULTRA-DILUTED BELLADONNA EXTRACT IN THE IMMUNE MEDIATED HEALING OF JE VIRUS INFECTION IN MICE

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ABSTRACT

Japanese encephalitis (JE) is highly prevalent in South East Asian countries, and is gradually spreading in adjoining countries, as there is a great obscurity in preventing the disease by vaccines, due to lack of adequate accessibility to remote places in the endemic region of developing countries. There are evidences that "Belladonna 200" –an ultra diluted homeopathic medicine, is capable of prevention, as well as remission of JE in mice. Such encouraging result prompted us to investigate, whether there is any role of this alternative medicine in immune alleviation of JE, in Webster strain of Swiss albino mice model, induced by virulent Nakayama strain of JE virus. RNA was extracted from brain tissue of JE infected mice (controls), and "Belladonna 200" treated JE infected mice (experimental group), followed by estimation of JE viral load, and relative mRNA expressions of CCR-5, IFN-β, TLR-3, TLR-7, IL-6, TNF α, ISG 15, MX1 and IFIT1 by real time PCR, and the results were compared and analyzed, between the experimental group and the control group. In the experimental group five out of six mice did not show any sign of sickness till 6th day post inoculation of JE virus; while in the control group the inoculated mice developed signs of sickness from the 3rd day, and death occurred in three of six

inoculated mice by 5th-6th post inoculation day. The results revealed that there was a considerable decrease of JE viral load, along with marked increase in relative mRNA expressions of IFN-β, MX1, and CCR-5, in "Belladonna 200" treated mice, compared to the control group; while there was no change in TLR-3 and TNF levels. Thus these findings indicate an explicit role of "Belladonna 200" in immune activated mitigation of JE.

KEYWORDS: Japanese encephalitis, Belladonna 200, CCR-5, IFN-β, TLR-3, TLR-7, IL-6,TNF, ISG 15, MX1 and IFIT1 .

INTRODUCTION

Approximately 2 billion people live in countries, where Japanese encephalitis (JE) presents a significant risk to humans and animals, particularly in China and India, with at least 700 million potentially susceptible children. JE is now regarded as a major health threat in Asian countries.[1,2] Mostly children and immune compromised persons are affected[3], with a case fatality rate of 30% and significant neurological sequels up to 50% of the affected persons.[4] JE cases appear to be increasing throughout Southeast Asia, probably as a result of increases in population density, deforestation, and the expanding irrigation of agricultural areas. No specific antiviral therapy is currently available, despite an emergence and resurgence of Flavivirus-mediated diseases.

JE virus is a single stranded positive sense RNA virus, under the family Flaviviridae. JE is a zoonotic disease, mainly prevalent in rainy season, when the mosquito vector Culex tritaeniorhynchus summorosus could breed easily. It has been noted that, antigenic changes occurs in JE virus through mutations in nucleotides, as well as in protein sequences. Again, another important problem is the vaccine induced sequence alterations, increasing the viral neurovirulence, as well as the host immune responses.[5] These recently observed changes indicate a possible major catastrophe due to JE in the coming years. Another problem which is associated with this dismal picture, is diagnostic problems of JE. Clinical features are quite nonspecific, and some conventional tests like IgM capture ELISA, immuno-fluorescence test, virus overlay protein binding assay (VOPBA), immuno-typing DNA microarray, are although highly sensitive and confirmatory, but indicates little regarding neuro-invasiveness and thus could not assess the risk of the infection, and molecular diagnostic tools are available in only a few centres. Thus there is a scope to find out other immunological tools, related to cytokines, so that a rapid diagnosis of JE virus invasion of neuronal cells could be diagnosed. In a study with CSF in JE virus infected patients, it was found that levels of interleukins,

complements, cytokines and intracellular viral proteins are helpful in predicting JE risks.[6] Recently it has been noted that sequential changes in serum cytokines/ chemokines are good biomarkers of JE.[7] Thus increased levels of pro-inflammatory and antiinflammatory cytokines and monocyte chemoattractant protein-1, which is a chemokine, are increased in serum of JE virus infected rats.[7]

A pilot study was conducted in the Virology unit of School of Tropical Medicine (STM), Kolkata, to find out whether homoeopathic medicines can diminish the infectivity of JE virus, on chorio-allantoic membrane, and in suckling mice.[8-10] It was found that different homeopathic ultra diluted preparations of Belladonna (3,6,30,200), could significantly inhibit JE virus infection, on chick chorio-allantoic membrane, and the mean survival rate of JE infected mice was 72.2% when treated with Belladonna 200, as opposed to 52.7% in the control group ($p<0.01$). Hence, this study was designed, to find out any under-lying protective immune- mediated mechanism of Belledonna-200 in the clearance of JE virus infection in experimental mice model.

MATERIALS AND METHODS

The ultra diluted homeopathic preparation: Belladonna 200 was procured from Homeopathic drug company, approved by Central Council of Research in Homoeopathy, Govt. of India where it is commercially prepared according to standard guidelines of Homeopathic Pharmacopoeia of India.

Virus stock: JE virus stock (Nakayama strain) which is maintained in School of Tropical Medicine, Kolkata was used in this study. After standardisation, the LD_{50} dose of the Japanese encephalitis virus in adult mice, was $10^{-2.36}$.

Ethical committee permission: The experiment was started after ethical clearance from the Institutional Ethics Committee.

The Experimental Mice: Randomly selected Swiss albino mice, Webster strain, were taken from the mice colony, of the Virology unit of School of Tropical Medicine, Kolkata, and they were arranged in two groups viz. control group and experimental group. Each group consisted of 6 mice.

The groups were treated as follows:

Group1: Control Group.

Randomly selected adult swiss albino mice, 6 in number, were challenged intra-cerebrally with 0.02mL of $10^{-2..36}$ JE viral dilution (LD_{50} dose), in each mouse. The mice were then observed daily, for signs of sickness or death.

Group 2: Experimental Group.

Randomly selected adult Swiss albino mice, 6 in number, were challenged intra-cerebrally with 0.02mL of $10^{-2..36}$ JE viral dilution (LD_{50} dose) in each mouse. The mice were observed for signs of sickness, and usually on the third day post inoculation, when the mice developed features of sickness viz ruffling of fur, and rounding of the body, experimental group mice were treated orally, with two doses of 0.06 mL of "Belladonna 200", mixed with a little distilled water (60 µL Belladonna 200 mixed with 10 µL distilled water- 60 µL of this mixture was fed orally, to the infected mice. A similar repeat dose was administered one hour later.[9]

The mice were then observed daily, for further progress of sickness, recovery from the disease or death. The mice that survived the JE virus infection, in both control and experimental groups, were sacrificed on the 10^{th} day post injection, and their brains were collected aseptically. The brains were then immediately preserved, in -80°C deep freezer. RNA extraction was done, from the mice brain, and real time PCR was done for JE viral loads along withrelative m-RNA expression of CCR-5, IFN-β, IL-6, TLR-3,TLR-7, TNFα, ISG 15, MX1and IFIT1 .

Procedure for RNA extraction from mice brain: The mice brains (50 – 100 mg) were homogenized, in 1mL trizol reagent (Invitrogen); using a power homogenizer. Following homogenization, insoluble materials were removed from the homogenate, by centrifugation at 12,000g for 10 min at 2-8 ^{o}C. The resulting pellet consisted extracellular membranes, polysaccharides, and high molecular DNA, while the supernatant contained RNA. The clear homogenate was transferred to a fresh tube, and proceeded for phase separation, with chloroform. The homogenate was then incubated for five minutes, at room temperature, to permit the complete dissociation of the nucleoprotein complexes. 0.2mL of chloroform was added, per 1mL of trizol reagent. Sample tubes were capped securely, and were vigorously shaken by hand for 15 sec, and incubated at 15^{o}C for two to three minutes. The samples were then centrifuged at 12000g for 15 min at 2 to 8^{o}C. Following centrifugation, the mixture then separated into a lower red, phenol-chloroform phase, an inter phase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous

phase was about 60% of the trizol reagent, used for homogenization. The aqueous phase was then transferred to a fresh tube, and the organic phase was discarded. The RNA was precipitated from the aqueous phase, by mixing with isopropyl alcohol(0.5mL of isopropyl alcohol was used per 1mL of trizol reagent, used for the initial homogenization). The samples were incubated at 15^{o} C for 10 min, and centrifuged at 12000 g for 10 min at 2 to 8 ^{o}C. The RNA precipitate, often invisible before centrifugation, formed a gel like pellet on the side and bottom of the tube. The supernatant was then removed. The RNA pellet was washed twice with 75% ethanol. The sample was mixed on vortex, and centrifuged at 7,500g for 5 min at 2 to 8^{o}C. The RNA pellet was air dried, and dissolved in nuclease free water (Ambion) and stored at -80 ^{o} C.

Preparation of cDNA of the extracted RNA, from mice brain samples, and procedure for RT PCR

Total RNA was extracted from the mice brain tissue, using trizol reagent, as described above. cDNA was synthesized using random 9-mers using RNA LA PCR kit (AMV) Ver.1.1 (Takara). Approximately 1µg RNA was used for cDNA preparation, using the following thermal cycler program: 10min at 30^{o}C, followed by 42^{o}C for 30min, after that heat treatment at 95℃ for 5 minutes was performed to inactivate the reverse transcriptase. Quantitative PCR (qPCR) was performed in triplicate, by the real-time fluorescence detection method, with the fluorescent DNA binding dye SYBR green (Power SYBR Green PCR master kit; Applied Biosystems), using an ABI PRISM 7000 sequence detection system (Applied Biosystems), according to the Manufacturer's protocol. Oligonucleotide primer pairs against mice CCR-5, IFN-β, IL-6, TLR-3,TLR-7, TNF, ISG 15, MX1, IFIT1 and JE virus are listed in Table 1.

The PCR amplification parameters were as follows: 2 min at 50^{o}C, 10 min at 95^{o}C followed by 40 cycles of denaturation at 95^{o}C for 15 s, and annealing and extension at 60^{o}C for 1 min. Amplification was performed in a final volume of 20 µL, and each sample contained 2 µL of 5times diluted cDNA, 5µM of forward and reverse primers and 10 µL of Fast Start Universal SYBR Green Master Mix. Each quantitative PCR reaction was performed in triplicate, and the mean Ct value for each sample was used for data analysis. Samples were normalized to Actin; results are expressed as fold changes of Ct values relative to controls by using the 2-ΔΔCt formula.

RESULTS

Control Group

The mice developed signs of sickness from the 3rd day. Death occurred in three mice out of six, by 5th-6th day of JE virus inoculation.

Experimental Group

Five out of six mice did not show any sign of sickness till 6th day of JE virus inoculation. One mouse developed signs of sickness and died on 6th day.

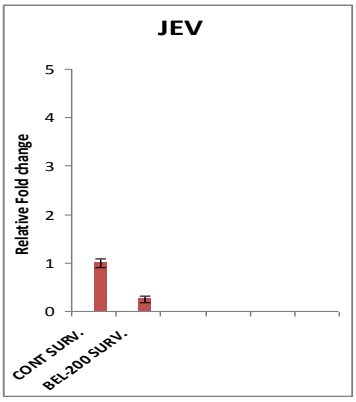
Results of The Molecular Tests

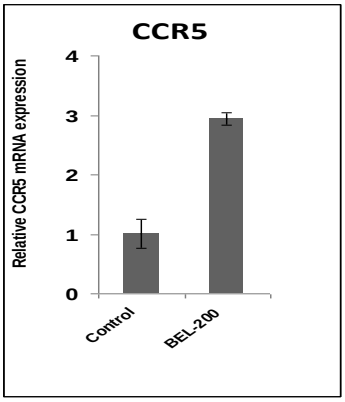
The results revealed that there was marked decrease of JE viral RNA levels (Fig1) and marked increase in relative m-RNA expressions of IL-6, CCR-5, TLR-7, IFN-β, ISG 15, MX1 and IFIT1 levels in "Belladonna 200" treated mice, compared to the control group(Figs. 2,3 5- 8 and 10); while there was no change in TLR-3 (Fig 4) levels between the control and the experimental group. Relative mRNA expression of TNF (Fig 9) was slightly lowered in the Belladonna treated mice, than the control group.

Table-1: Oligonucleotide primer pairs

MCCR5-F1	5'-TTTTCAAGGGTCAGTTCCGAC-3'
MCCR5-R1	5'-GGAAGACCATCATGTTACCCAC-3'
M- IFN-b1	5'-ATGAACAACAGGTGGATCCTCC-3'
M-IFN-b2	5'-AGGAGCTCCTGACATTTCCGAA-3'
M-Actin F1	5'-AGAGGGAAATCGTGCGTGAC-3'
M-Actin R1	5'-CAATAGTGATGACCTGGCCGT-3'
M-TLR3-F	5'-GTGAGATACAACGTAGCTGACTG-3'
M-TLR3-R	5'-TCCTGCATCCAAGATAGCAAGT-3'
M-TLR7-F	5'-ATGTGGACACGGAAGAGACAA-3'
M-TLR7-R	5'-GGTAAGGGTAAGATTGGTGGTG-3'
M- Mx1 - F	GACCATAGGGGTCTTGACCAA
M-Mx1 - R	AGACTTGCTCTTTCTGAAAAGCC
M-ISG15 - F	GGTGTCCGTGACTAACTCCAT
M-ISG15 - R	TGGAAAGGGTAAGACCGTCCT
M-IL6 - F	5′-GAC AAA GCC AGA GTC CTT CAG AGA G-3′;
M-IL6 - R	, 5′-CTA GGT TTG CCG AGT AGA TCT C-3′;
M-TNF - F	5′-ATG AGC ACA GAA AGC ATG ATC-3′;
M-TNF - R	5′-TAC AGG CTT GTC ACT CGA ATT-3′;
JEV - F	AGAGCACCAAGGGAATGAAATAGT
JEV - R	AATAAGTTGTAGTTGGGCACTCTG
JEV - Probe 6-FAM TAMRA	CCACGCCACTCGACCCATAGACTG

Relative JE viral load and relative m- RNA expression levels of CCR5, IFN-β,TLR3, TLR7, ISG15, MX1, IFIT1, TNF, IL6, genes in Belledonna treated JE virus infected mice as compared to untreated controls(conducted by real time PCR assay).





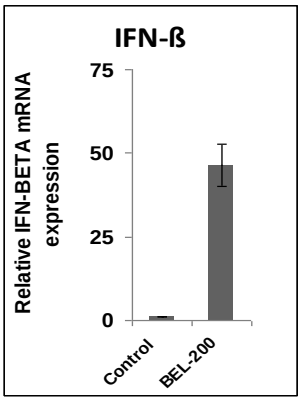
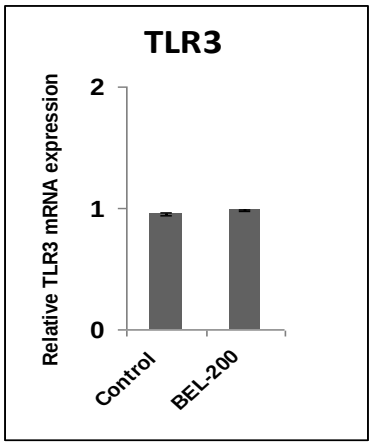
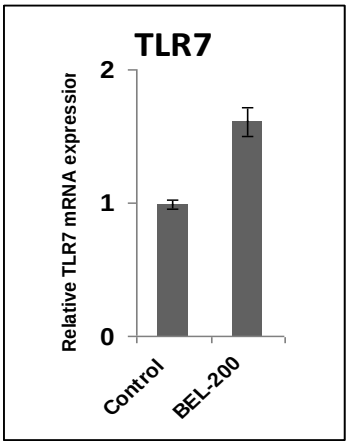


Fig1 Modest down regulation (p -value< 0.005)

Fig 2. Significant up-regulation (p -value= 0.006)

Fig 3. Significant up-regulation





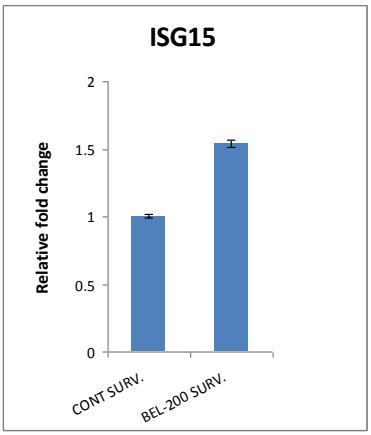
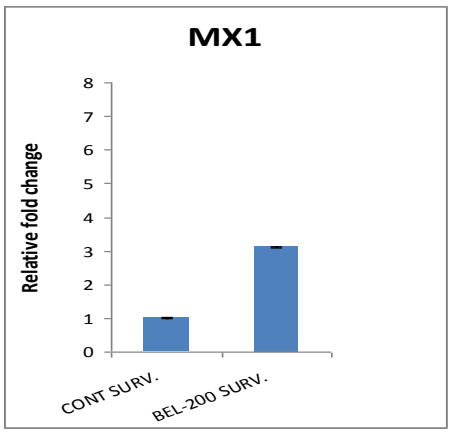


Fig 4. No change

Fig 5. Modest Up-regulation

Fig 6. Modest Up –regulation



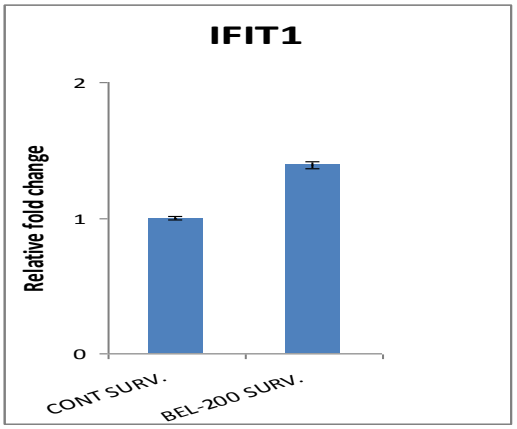
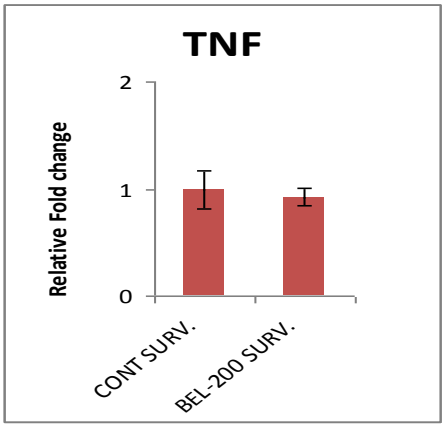


Fig 7.Significant Up-regulation

Fig 8 Modest Up-regulation



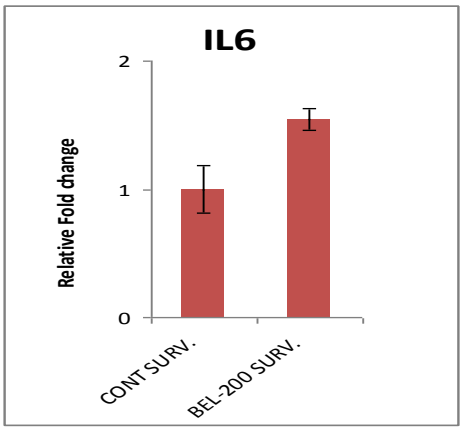


Fig 9 No Change **Fig10.Modest Up regulation**

(p value > 0.005)

DISCUSSION

A new finding was observed, when Japanese encephalitis (JE) virus infection, on chorio-allantoic membrane (CAM) and in mice were challenged with some homeopathic medicines. It was found that Belladonna 3,6,30,200, could significantly inhibit JE virus infection on chorio-allantoic membrane of embryonated chick eggs, and the mean survival rate of JE infected mice was 72.2%, when treated with Belladona 200, as opposed to 52.7% in the control group.[8-10] Belladonna-200 possibly clears JE-virus infection by immune mediated mechanisms was our hypothesis.

Japanese encephalitis virus (JEV) causes acute central nervous system (CNS) disease in humans, in whom the clinical symptoms vary from febrile illness to meningitis, and encephalitis. Tumour necrosis factor (TNF) is an important cytokine, in the innate immune response against various infections, including JEV, HSV-1 infection. It has recently become a molecular target of anti-cytokine treatment, in certain inflammatory diseases. Recent reports suggested that TNF-α has an immune-regulatory effect on pro-inflammatory cytokines in the CNS during JEV infection, and consequently protects the animals from fatal disease.[11]

In JE, acute inflammatory changes in brain lead to both local and systemic responses. Cytokines like IL-1, IL-6, TNF-α, IL-12 and chemokine receptors CCR1, CCR2 and CCR4 are related to acute flavivirus infection.[12,13] In vaccinated persons levels of IL-6, IL-8, monocyte chemo-attractant protein (MCP), macrophage inflammatory protein (MIP 1a and 1b) are increased[14] Levels of inflammatory cytokines mainly TNF-α can indicate any up-regulation of inflammatory cytokines.[15] TLR signaling also plays an important step in

immune responses against JE virus infection.[16,17,] Interferon antagonists of JE virus protein play a major role in JE pathogenesis.[18] Maximum levels of cytokines and chemokines particularly TNF-α, IFN-γ, IL-6 and IL-10 were found in the cortex of the brain mainly in 6 days post-inoculation of JE virus after which they gradually decline.[19] IL-6 plays an important role in promoting effective cell-mediated immune responses, thereby facilitating successful virus clearance.[20]

IFN-stimulated gene 15 (ISG15), an ubiquitin-like protein, is rapidly induced by IFN-alpha/beta, and ISG15 conjugation is associated with the antiviral immune response. Previous report suggested that over-expression of ISG15 inhibits replication of the Japanese encephalitis virus in human medulloblastoma cells.[21] Moreover, Tumpey et al.,[22] showed that the interferon-induced resistance factor Mx1 represents a key component of the murine innate immune system that mediates protection against epidemic and pandemic influenza viruses.

In order to understand the protective effect of Belladona-200 against lethal JEV challenge, we checked JE viral RNA level, IL-6, CCR-5, TLR-7, IFN-β, TNF, ISG 15, MX1 and IFIT1genes and compared their expression level with the untreated survived mice. We observed Belladona-200 treated mice showed enhanced expression of IL-6, CCR-5, TLR-7, IFN-β ,ISG 15, MX1 and IFIT1expression as compared to untreated survived mice.

CCR5, is expressed on natural killer (NK) cells, macrophages, and CD4+ and CD8+ T cells. In these cell types, it regulates chemotaxis and cell activation through interaction with the chemokine ligands CCL3, CCL4 and CCL5, which are up-regulated at the site of infection(23).NK cells form an important part of the cellular arm of the host's innate immunity, and function by killing virally infected cells and by release of the cytokines, IFN-ϒ and TNF-α. CD8 + T cells form the dominant cytotoxic arm of the adaptive immune system. CCR5 expression drives the migration of leukocytes into the CNS after infection, and thereby contributes to viral clearance and recovery from infection. In addition, recent findings suggest that CCR5, which is expressed in neurons, and is up-regulated in the brain in pathological conditions, could have a direct neuro-protective function by increasing neuronal survival.[23] CCR5 serves as a host antiviral factor against JE. This result is consistent with a mechanism by which CCR5 expression enhances lymphocyte activation, and thereby promotes host survival in JE. In many diseases, pathogenesis and disease outcome have been contained by the CCR 5 (24-32). CCR5 has got a regulatory role in controlling leucocyte

migration at the site of inflammation along with additional role like antigen recognition (33-35), inducing lymphocyte proliferation.[36-38] There are evidences of increased mortality in absence of CCR 5 expression.[39]

The significantly increased expression of CCR-5, IFN-β, and MX1 genes in Belladonna 200 treated mice corroborate the above mentioned findings and indicate that ultra-diluted Belladonna treatment might have some effect on innate immune response.

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