Int J Biol Med Res.2019 ;10(1):6635-6640

Contents lists available at BioMedSciDirect Publications

International Journal of Biological & Medical Research

Journal homepage: www.biomedscidirect.com

Original Article

Suppression of viral load by Belladonna 200c through modulation of TLR and type-I IFN signalling pathways

Urmita Chakraborty, Moonmoon Sinha, Arghyadeep Bhattacharjee, Debdatta Nayak, Anil Khurana, Raj kumar Manchanda, Debabrata Sarkar, Satadal Das

ARTICLE INFO

Keywords:
Belladonna
embryonated egg
Japanese encephalitis
IFN
TLR

ABSTRACT

Toll like receptor (TLR) mediated intracellular signalling plays a pivotal role in regulating the pathogens in the initiation of the innate immune response via the induction of interferons (IFNs) controlling the viral replication and subsequent severity of the infection. In complementary medicine, ultradiluted extract of *Atropa belladonna* (Belladonna 200C) is widely used in Japanese encephalitis (JE) treatment. The antiviral property of Belladonna 200C has already been established by in-vitro and in-vivo experiments. However, the antiviral mechanism of Belladonna in connection with the TLRs has not been studied in detail for JE. Thus under this circumstances the present study was focussed to investigate the role of TLRs in experimentally infected chick eggs with JE virus. Pre-treatment with Belladonna 200C significantly reduced the overall viral load ($p<0.0001$) in chorioallantoic membrane (CAM) and brain which correlated with the morbid pathological changes of the organs. TLR4 expression was significantly upregulated among the direct virus infected group compared to the Belladonna 200C pretreated virus infected group. There were increased expressions of TLR3, TLR7 and TLR8 as well as IFN-α and IFN-β in CAM and brain among the Belladonna 200C pre-treated group. Taken together, the result indicates that Belladonna 200C exerts the antiviral effect by influencing the TLR signalling pathway which is one of the contributing factors in the immune-pathogenicity of JE virus infection. The present study may help in development of targeted immunotherapy by Belladonna 200C against JE virus in future by altering innate immune signalling.

1. Introduction

Japanese encephalitis virus (JEV) is a significant cause of neurological disease in human which is considered to be the major cause of viral encephalitis throughout South East Asia. It is now known that host innate immune response is vital in pathogenesis as well as the outcome of the disease. After entry into the host the virus first propagate inside the monocytes, thereafter enters the central nervous system (CNS) which leads to the death of the neurones [1, 2]. The generation of antiviral response through the production of pro-inflammatory mediators are essential to control the virus. However, the overproduction of pro-inflammatory response may lead to the neuronal death [3].

Considering the limited efficacy of the vaccine and unavailability of antiviral drugs against JE, various natural products especially the plant extracts are now getting importance compared to the synthetic chemicals for treatment. It is established that ultra-diluted Belladonna extract derived from *Atropa belladonna* has the anti-JEV property [4]. The main alkaloid of *A. belladonna*, atropine has the potent antiviral effects against various viruses [5, 6] and it affects viral attachment to the cell, influence both innate and adaptive immune cells [7].

The induction of strong innate immune response is essential in controlling early viral replication, dissemination and subsequent infection [8-10]. Type I interferons (IFN-α/β) act as the first line of defence against viral infection [11,12] and their induction is mediated by various molecules including toll like receptors (TLR) which belongs to the type I pathogen recognition receptors (PRRs) [13]. TLRs primarily recognise the virus during the initial interaction and viral particles during replication of the virus inside the cells leading to the activation of innate immune signalling. The viral genome is recognised by TLR3, TLR7, and TLR8. However, the viral proteins mainly the envelop glycoprotein binds TLR4 [14-16]. Activation of these TLRs during infection with JEV leads to the production of type I interferons (INFs). It has been found that TLR3 is indispensible while the TLR4 deficiency imparts resistance against JE in mice [17]. TLR7 is also very important against JEV infection as it play an important role in the immune response and regulation of myeloid cells. TLR7-deficiency leads to upregulation

* Corresponding Author : **URMITA CHAKRABORTY**
urmitachak@gmail.com

of TLR8 and regulates the innate immune response, thereby increasing the survival rate in JEV infected mice. TLR7 is considered to have a pivotal role over TLR8 in JEV infection [18]. Interestingly, JEV can alter innate immune responses and subsequent adaptive responses in MyD88-dependent and independent pathways, which indicates that JEV may be recognized by certain TLR signal pathways, thereby affecting the outcome of JEV induced neurological diseases [19]. Thus, it is essential to evaluate the molecular mechanisms contributed by TLR signalling under the influence of Belladonna 200C in preventing JEV infection.

Among the various in-vivo model of JEV infection, embryonated egg is the cheapest and easily available model. The chorio allantoic membrane (CAM) of the embryo which is an extra embryonic membrane mimics the blood brain barrier and the underdeveloped immune systems make it an ideal model of host pathogen interaction in JEV infection [20]. Considering importance of TLRs in JEV infection and the antiviral role of Belladonna 200C the present study was aimed to explore the changes in the TLR signalling pathway in JE infection and its alteration by Belladonna 200C in embryonated eggs. Thus in this study we evaluated the expressions of TLRs, type-I IFNs and their regulatory factors (IRFs) both in CAM and brain in this experimental model by monitoring the changes of viral load.

MATERIALS AND METHODS

Embryonated chick eggs

Embryonated chicken eggs of Black Australorp (*Gallus gallus*) were procured from State poultry farm, Tollygunj, Kolkata, India. The eggs were kept in the incubator maintaining 37^{0}C with 60% relative humidity after disinfecting the shells. Day 3 onwards the eggs were candled and rotated 3-4 times per day to observe vascularisation and viability till 11th day.

Virus strain

The prototype strain (NIV no P-20778, M/K no M-52134) and the reference Nakayama strain (NIV no 753101, M/K no M-8117) of JEV was procured from National Institute of Virology, Pune, India. The copy no of the virus inoculums was determined by Path JEV realtime PCR kit (Primer design) according to the manufacturer's instruction.

Belladonna 200C

Belladonna 200C is a standard homoeopathic medicine (under alternative medicine group) prepared according to the Govt. approved Pharmacopoeia (India) from *Atropa belladonna plant.*

Experiment design

Live eggs of 12th day were selected and divided into 4 groups. The infection group (Group I) were challenged with JEV, the medicine treated group (Group II) were pretreated with Belladonna 200C followed by infection with JEV, the alcohol control group (Group III) were pre treated with potentized alcohol 200C followed by infection with JEV and among the matched control group (Group IV) Bovine albumin phosphate saline (BAPS) was applied. As the vehicle of the medicine was alcohol, the alcohol procured from the same source and termed alcohol 200C after following all the procedures of Belladonna 200C and was used as same. The virus was applied at LD_{50} dose and 50µl medicine, alcohol and BAPS was applied in the respective groups through CAM route following the method stated elsewhere [4]. After application of materials the holes over the eggs were sealed with molten wax and kept horizontally at 37ºC till 48 hours. In between the viability of the eggs were checked by candling every 12 hrs. After incubation the eggs were sacrificed followed by harvesting of CAM and brain.

RNA isolation and viral load determination, cDNA synthesis and semi-qRT-PCR

The RNA from CAM and brain were extracted using RNAiso plus (Takara, Japan) reagent following manufacturer's guidelines by taking 100mg tissue from each sample [21]. Viral load was determined directly from the extracted total RNA using real time PCR kit (Primer Design).

cDNA synthesis and semi qRT PCR of TLRs mRNA

For semi-qRT-PCR cDNA was prepared using the i-script cDNA synthesis kit (Bio-Rad, USA). Semi-qRT-PCR was performed using iTaq Universal SYBR Green Supermix (BioRad) with standard cycling conditions. The primer sequences for avian TLR3, TLR4, TLR7, and TLR8 were obtained as described elsewhere [22-24].

Statistical analysis

The data obtained were representative of three repetitive experiments and shown as mean ± SEM. The data were further analyzed for the analysis of differences using one way ANOVA, t-test using Graph Pad Prism (Version5). Significant level was considered only when p value was less than 0.05.

RESULTS

Effect of Belladonna 200C in TLR gene expression in JEV infected CAM of enbryonated egg

It is evident from the study that JEV infection increases the expression of TLR3 in CAM (Fig 1a). However, pre-treatment with Belladonna 200C significantly increased the expression compared to other groups ($p<0.05$, t-test). Likewise, viral infection significantly induced the TLR4 expression in CAM. However, there was significantly higher expression of TLR4 in the direct infection group compared to the medicine treated groups (Fig 1b).

TLR7 expression was enhanced by all the virus infected groups. However, Belladonna 200C pre-treated group showed significantly higher expression of TLR7 than the other groups (Fig 1c). Similar patterns were observed for the TLR8 expression both in the CAM of the chick embryo (Fig 1d).

Effect of Belladonna 200C in TLR gene expression in brain of enbryonated egg

Considering the neurological manifestation of JEV infection, brain tissues from embryo from different groups were collected for analysis of TLR expressions. TLR3 expression was significantly higher in the Belladonna pretreated group compared to other infection group ($P<0.05$, in all combination). Strikingly, there was significant down regulation of TLR4 in Belladonna 200C pretreated group (Fig 2b) compared to other test groups. Down regulation of TLR4 was found only in brain of the embryo pretreated with Belladonna 200C.

JEV infected brain also showed upregulation of TLR7 and TLR8. However, pre-treatment with Belladonna 200C further induce the expression for both the TLRs compared to other groups.

Changes of viral load under the influence of Belladonna 200C in JEV infected CAM and brain of chick embryo

There were significantly higher viral load in the infection control group and by the alcohol 200C treated group in both the tissues. However, Belladonna 200C pre-treated chicks showed lowest viral load as measured in brain and CAM tissue.

Modulation of type I IFNs and their regulatory factors by Belladonna 200C in JEV infection

It was found that viral infection significantly reduced the expression of IRF3 and 7 and further IFN-α and IFN-β gene expression in CAM. However, pretreatment with Belladonna 200C overcome the situation and significantly upregulates these expressions (Fig. 4). Similar pattern of these mRNA expression was observed in brain tissues where Belladonna 200C preteated group showed enhanced type I IFNs response which correlated with their regulatory factors (Fig. 5). There were significant changes ($P<0.05$) for IFNs and IRFs both in CAM and brain tissue among the four groups, as analyzed by One-way ANOVA.

Figures

Figure 1

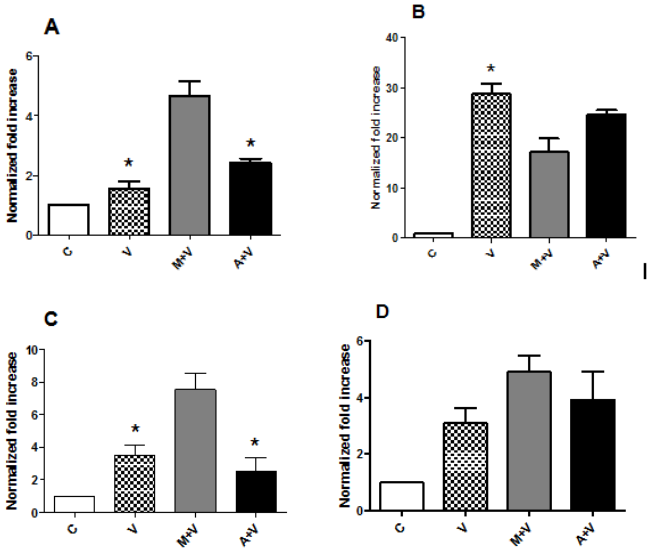

Figure1. Changes of TLR gene expression in CAM. Change of (A) TLR3 (B) TLR4 (C) TLR7 (D) TLR8 gene expression. (C: Control, V: infected with JEV, A+V: pretreated with alcohol 200C followed by infection, M+V: Pretreated with Belladonna 200C followed by infection). Data are expressed as mean ±SEM (n=6). * Indicates $P < 0.05$ as compared to M+V.

Figure 2.

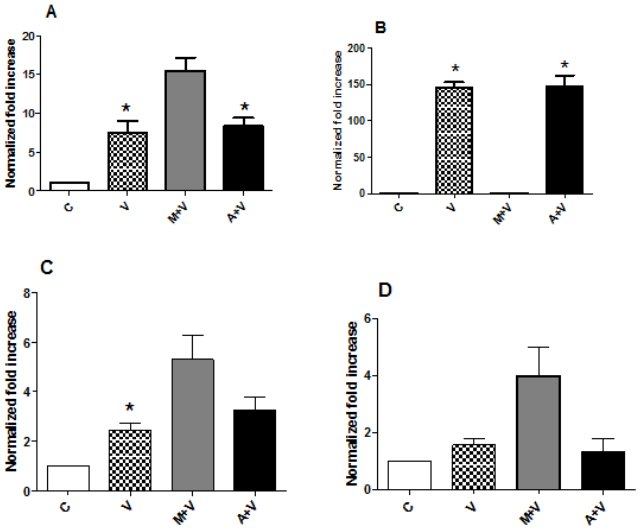

Figure 2. Changes of TLR gene expression in brain. Change of (A) TLR3 (B) TLR4 (C) TLR7 (D) TLR8 gene expression. (C: Control, V: infected with JEV, A+V: pretreated with alcohol 200C followed by infection, M+V: Pretreated with Belladonna 200C followed by infection). Data are expressed as mean ±SEM (n=6). * Indicates $P < 0.05$ as compared to M+V.

Figure 3

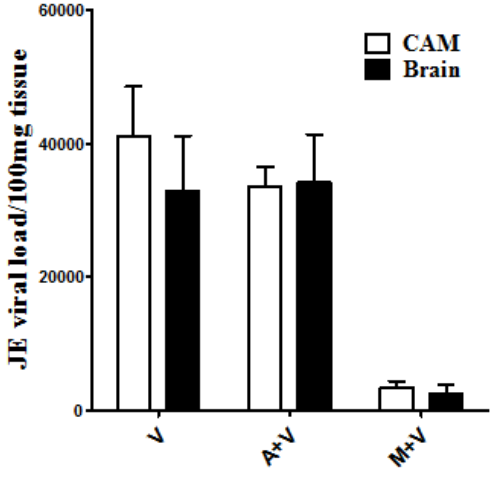

Figure 3. Changes in viral load in CAM and brain tissue of chicken in different experimental groups. (V: infected with JEV, A+V: pretreated with alcohol 200C followed by infection, M+V: Pretreated with Belladonna 200C followed by infection).

Figure 4.

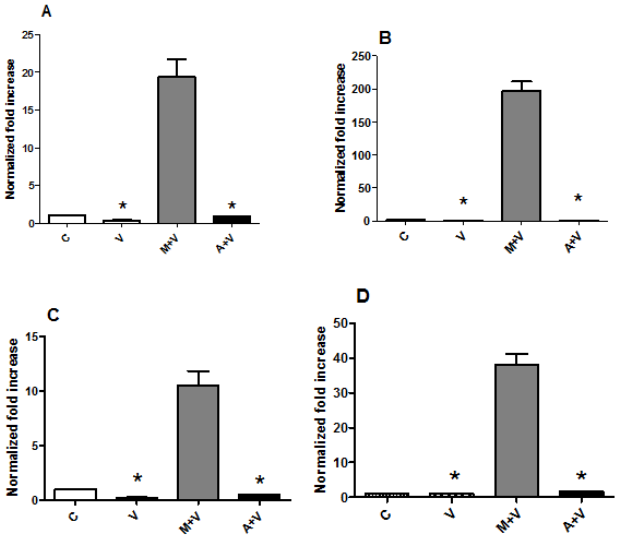

Figure 4.Changes of type I IFNs and IRFs in CAM: A. IFN-α, B. IFN-β, C. IRF-3 and D. IRF-7. (C: Control, V: infected with JEV, A+V: pretreated with alcohol 200C followed by infection, M+V: Pretreated with Belladonna 200C followed by infection). Data are expressed as mean ±SEM (n=6). * Indicates P < 0.05 as compared to M+V.

Figure 5.

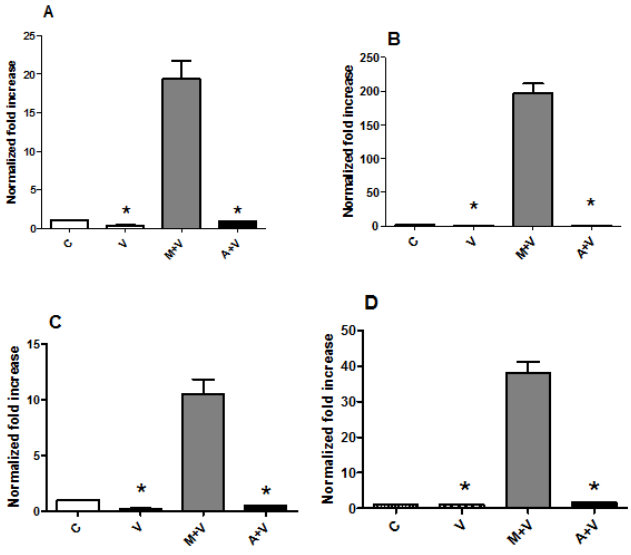

Figure 5.Changes of type I IFNs and IRFs in brain: A. IFN-α, B. IFN-β, C. IRF-3 and D. IRF-7. There were significant changes among these three different infection groups both in CAM and brain as analyzed by One-way ANOVA (p<0.05). (C: Control, V: infected with JEV, A+V: pretreated with alcohol 200C followed by infection, M+V: Pretreated with Belladonna 200C followed by infection). Data are expressed as mean ±SEM (n=6). * Indicates P < 0.05 as compared to M+V.

Figure 6.

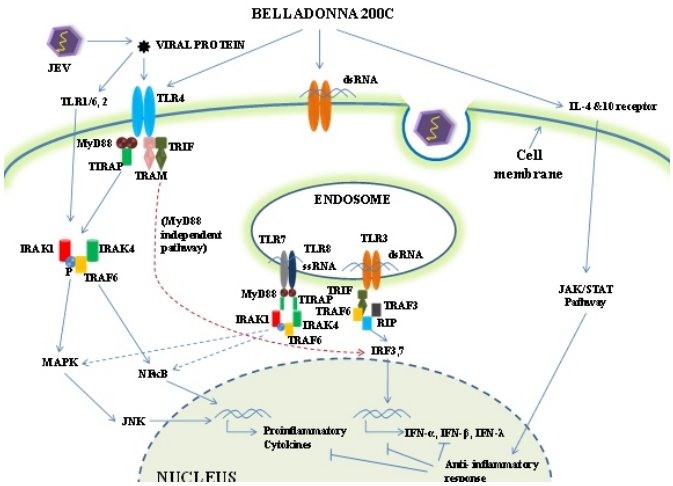

Figure 6. Proposed intracellular signalling pathway influenced by Belladonna in modulating the TLRs during JEV infection. Details are given in the text. (JEV: Japanese encephalitis virus).

DISCUSSION

TLRs are present in the cell membrane and endosomes and act as the first line of innate immune receptors for pathogens. Several studies have been done for the understanding of innate immune responses exerted by TLRs in flavivirus infection. TLR3 is one of the most important pathogen recognition receptor (PRR) for Flaviviridae. Earlier studies have showed a strong association between the antiviral immunity with TLR3 in mice [16]. In the present study, TLR3 expression was enhanced by Belladonna 200C pre-treated chicks which showed lesser viral load, while the infection group showed comparatively lesser TLR3 and higher viral load. So this study supports antiviral role of Belladonna through induction of TLR3 expression in the experimental chick embryo model. Activation of TLR3 induces the activation of innate as well as adaptive immune cells. Activation of TLR3 by recognising the dsRNA leads to the further activation of adaptor protein TRIF [25, 26]. This TLR3-TRIF signalling induces the translocation of transcriptional factors NF-κB and IRF3/7 into the nucleus leading to the production of cytokines importantly, such as type I Interferons (IFN) [27].

In the present study the role of Belladonna 200C was also evaluated in TLR4 expression, which generally interacts with the viral proteins. Previous studies have showed the negative role of TLR4 which favours JE viral replication in mice [16]. In our study, TLR4 expression was highest in the virus infected chicks showing higher mortality. TLR4 expression favours the viral replication opposing the antiviral immune response of host. Interestingly Belladonna 200C pre-treated embryo showed significantly lower TLR4 expression especially in brain. Viral load was also found to be lowest in CAM and brain tissues in the Belladonna 200C pre-treated embryo. Thus, viral load and the expression of TRL4 may be

associated in this experimental model of JEV infection. Other studies with TLR4 knockout mice or using the TLR4 antagonist have shown the lower viral load and proinflammatory response with reduced lung pathology in influenza virus infection [28-29]. It has been found that TLR4 deficient mice show enhanced type I IFN expression as well as JEV specific IgG and IgM enhancement which are essential antiviral innate as well as adaptive immune factors for the host. In this study the reduced viral load among the Belladonna 200C treated chicks is presumably by the altered TLR4 gene expression and subsequent immune signalling in JE.

Other TLR including TLR7 and 8 also play important role in flaviviral infection. Stimulation of TLR7 or TLR8 are essential for activation of signaling pathways through TRIF and MyD88-dependent manner ultimately for pro-inflammatory cytokines, chemokines, and type I IFNs. It has been found earlier that infection with JE upregulated TLR7 expression [17]. TLR7 mediated induction of type I interferon after viral infection are important for antiviral response by the host [30]. In our study increased TLR 7 was also found in the CAM and brain tissue of the virus infected chicks. However, pre-treatment with Belladonna 200C significantly stimulated TLR7 expression compared to virus infected group of chicks. Although, it has been demonstrated that TLR7 deficiency has no effect on the survival rate in JEV infection, increased viral load was found in the brain tissue of these mice showing the important role of TLR7 in regulation of the viral dissemination and propagation. This fact also correlates with our study among the Belladonna 200C treated group showing lesser viral load with higher TLR7 expression. It has been demonstrated that there was no significant change in TLR8 expression, where TLR7 expression has highly enhanced by the JEV infection in mice model. However, TLR7 deficient mice showed significant enhanced expression of TLR8 in JE. In our study both TLR7 and 8 were induced by JEV as found in brain and CAM tissue which were further enhanced among the Belladonna 200C pretreated chick embryo. The variation in the TLR8 expression in our study may be due to the use of different experimental models with varied pathogenesis and experimental conditions.

CONCLUSION

The present study has shown the effect of Belladonna 200C in the alteration of TLR signalling as well as limiting the viral replication in JEV infected CAM model. Lower viral load in the Belladonna 200C treated chicks showed higher TLR3, TLR7 and TLR8 and lower TLR4 expressions ultimately reflecting in the interferon expression (Figure 6). This finding may be a lead for immune therapy against JEV by targeting specific molecules of TLR signalling pathway in future other than use of this medicine for prevention and treatment of JE.

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by CCRH, Ministry of AYUSH, Govt. of India.

REFERENCES

[1] Dutta K, Kumawat KL, Nazmi A, Mishra MK, Basu A. Minocycline differentially modulates viral infection and persistence in an experimental model of Japanese encephalitis. J. Neuroimmune Pharmacol. 2010; 5:553–65..

[2] Chen CJ, Ou YC, Lin SY, et al. Glial activation involvement in neuronal death by Japanese encephalitis virus infection. J. Gen. Virol. 2010; 91:1028–37.

[3] Ghoshal A, Das S, Ghosh S, et al. Proinflammatory mediators released by activated microglia induces neuronal death in Japanese encephalitis. Glia. 2007; 55:483–96.

[4] Chakraborty U, Katoch S, Sinha M, et al. Changes in viral load in different organs of Japanese encephalitis virus-infected chick embryo under the influence of belladonna 200C. Ind J Res Homoeopath, 2018; 12:75-80.

[5] Biziagos E, Crance JM, Passagot J and Deloince R. Inhibitory Effects of Atropine, Protamine, and Their Combination on Hepatitis A Virus Replication in PLC/PRF/5 Cells. Antimicrob Agents and Chemother, 1990; 34(6): 1112-1117.

[6] Özçelik B, Kartal M and Orhan I. Cytotoxicity, antiviral and antimicrobial activities of alkaloids, flavonoids, and phenolic acids. Pharmaceut Biol, 2011; 49(4): 396–402.

[7] Razani-Boroujerdi S, Behl M, Hahn FF, et al. Role of muscarinic receptors in the regulation of immune and inflammatory responses. –J Neuroimmunol. 2008 Feb; 194(1-2): 8388.

[8] Larena M, Lobigs M. In: Flavivirus Encephalitis. Croatia RD, editor. 2011. Immunobiology of Japanese encephalitis virus; pp. 317–338. InTech.

[9] Larena M, Regner M, Lee E, Lobigs M. Pivotal role of antibody and subsidiary contribution of CD8+ T cells to recovery from infection in a murine model of Japanese encephalitis. J Virol. 2011; 5(11):5446–5455.

[10] Monath TP, Guirakhoo F, Nichols R, et al. Chimeric live, attenuated vaccine against Japanese encephalitis (ChimeriVax-JE): phase 2 clinical trials for safety and immunogenicity, effect of vaccine dose and schedule, and memory response to challenge with inactivated Japanese encephalitis antigen. J Infect Dis. 2003; 188(8):1213–1230.

[11] Randall RE, Goodbourn S. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J Gen Virol. 2008; 89:1–47.

[12] Samuel CE. Antiviral actions of interferons. Clin Microbiol Rev. 2001; 14:778–809.

[13] Perkins DJ, Vogel SN. Space and Time: New Considerations About the Relationship Between Toll-like Receptors (TLRs) and Type I Interferons (IFNs). –Cytokine. 2015; 74(2): 171174.

[14] Kawai T, Akira S. "Signaling to NF-kappaB by Toll-lik receptors". Trends in Molecular Med. 2007; 13 (11): 460–9.

[15] Heil F, Hemmi H, Hochrein H, et al. "Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8". Science. 2004; 303 (5663): 1526–9.

[16] Zhang Y, El-Far M, Dupuy FP, et al. "HCV RNA Activates APCs via TLR7/TLR8 While Virus Selectively Stimulates Macrophages Without Inducing Antiviral Responses". Scien Reports. 2016; 6: 29447.

[17] Han YW, Youn CJ, Uyangaa E, Kim SB and Kim JH. Distinct Dictation of Japanese Encephalitis Virus-Induced Neuroinflammation and Lethality via Triggering TLR3 and TLR4 Signal Pathways. PLOS Path. 2014; 10(9): e1004319.

[18] Awais M, Wang K, Lin X, et al. Tlr7 Deficiency leads to Tlr8 compensative regulation of immune response against JEV in Mice. Frontiers in Immunol. 2017; 8 (160).

[19] Aleyas AG, George JA, Han YW, et al. Functional modulation of dendritic cells and macrophages by Japanese encephalitis virus through MyD88 adaptor molecule-dependent and –independent pathways. J Immunol. 2009;183: 2462–2474.

[20] Nowak-Sliwinska P, Segura T and Iruela-Arispe M L. The chicken chorioallantoic membrane model in biology, medicine and bioengineering. Angiogen. 2014; 17(4): 779–804.

[21] Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques. 1993;15(3):532-4, 536-7.

[22] Pieper J, Methner U, Berndt A. Heterogeneity of avian γδ T cells. Vet Immunol and Immunopath. 2008; 124: 241–252.

[23] Jie H, Lian L, Qu LJ, Zheng J X, et al. Differential expression of Toll-like receptor genes in lymphoid tissues between Marek's disease virus-infected and noninfected chickens in chickens. Poultry Sci. 2013; 92 :645–654.

[24] Philbin VJ, Iqbal M, Boyd Y, et al. Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8. Immunol. 2005; 114: 507–521.

[25] Barton GM. Viral recognition by Toll-like receptors. Semin Immunol. 2007; 19: 33–40.

[26] Yamamoto M, Sato S, Hemmi H, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science. 2003; 301: 640–643.

[27] Green AM, Beatty PR, Hadjilaou A, Harris E. Innate immunity to dengue virus infection and subversion of antiviral responses. J Mol Biol. 2014; 426: 1148–1160.

[28] Nhu QM, Shirey K, Teijaro JR, et al. Novel signaling interactions between proteinase-activated receptor 2 and Toll-like receptors in vitro and in vivo. Mucosal Immunol. 2010; 3: 29–39.

[29] Shirey KA, Lai W, Scott AJ, et al. The TLR4 antagonist Eritoran protects mice from lethal influenza infection. Nature.2013; 497: 498–502.

[30] Nazmi A, Mukherjee S, Kundu K, et al. TLR7 is a key regulator of innate immunity against Japanese Encephalitis Virus infection. Neurobiol Dis. 2014; 69:235-47.