Original Article

Changes in viral load in different organs of Japanese Encephalitis virus-infected chick embryo under the influence of *Belladonna* 200C

Urmita Chakraborty[1], Shailja Katoch[2], Moonmoon Sinha[1], Debadatta Nayak[3], Anil Khurana[3], Raj K. Manchanda[3], Debabrata Sarkar[1], Satadal Das[1]*

[1]Department of Virology, Dr. Anjali Chatterjee Regional Research Institute for Homoeopathy, Kolkata, West Bengal, [2]Department of Livestock Product Technology, College of Veterinary and Animal Sciences, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, Uttar Pradesh, [3]Central Council for Research in Homoeopathy, Ministry of AYUSH, Govt of India, New Delhi, India

Abstract

Background: Japanese encephalitis(JE) is highly prevalent in many states of India. *Belladonna* 200C is widely used in the prevention and treatment of JE.The effect of *Belladonna* 200C in virus replication inside different tissues has not been studied. **Objective:** To study the effect of *Belladonna* 200C in virus replication inside different tissues utilising chick embryo model. **Materials and Methods:** Twelve-day-old fertilised eggs of Black Australorp were inoculated with JE via chorioallantoic membrane (CAM) route in different experimental sets: infection, *Belladonna* 200C treated and vehicle control, keeping matched blank sets. All experimental sets were incubated for 48 hours. After incubation, viable eggs were sacrificed humanly and different tissues were observed and collected for viral load determination by real-time-polymerase chain reaction (PCR). **Results:** The control group showed visible pocks over the CAM; brains were liquefied due to haemorrhagic liquefactive necrosis and white patches were found over the liver. However, the medicine-treated group was apparently normal; there were no visible changes in the brain and the liver was healthy like control. Real-time-PCR results showed high viral load in CAM and brain with absence of viral RNA in liver of the virus-infected group. Pre-treatment with *Belladonna* 200C significantly reduced the overall load ($P < 0.05$) in CAM and brain which correlated with the morbid pathological changes of the organs. **Conclusion:** Although *Belladonna* 200C did not completely inhibit JE viral replication in the brain, it reduced the severity of JE by diminishing the viral loads in this tissue.

Keywords: *Belladonna* 200C, Chorioallantoic membrane, Japanese encephalitis

Introduction

In spite of the availability of vaccine against Japanese encephalitis (JE), it is not possible to immunise a large group of population in the endemic area like India due to socioeconomic and other constrains. The mortality rate among the diseased people is approximately 20%–30%, and 20%–50% of survivors develop permanent neurological disorders.[1-4] Some studies on the antiviral role of *Belladonna* 200C against JE virus (JEV) have already been done, which are based on the Chick Chorioallantoic Membrane (CAM) and mouse model of infection.[5] However, the viral replication potency in different organs, particularly in brain, under the influence of *Belladonna* 200C, has not been studied till now. Considering the importance of the neuropathological squeal of the JE, in this study, we aimed to find out changes in the viral load in different tissues, particularly in the brain under the influence of *Belladonna* 200C.

Embryonated chicken egg is an excellent *in vivo* platform for a range of viral infection study including JE. The highly vascularised environment, with immature immunity, imparts the system as an ideal model for host pathogen interaction that mimics the actual pathogenesis during infection. After application of the virus, it replicates in the outermost layer of CAM, the ectoderm and then evades the mesoderm (the stromal layer).[6]

Access this article online

Quick Response Code:

Website: www.ijrh.org

DOI: 10.4103/ijrh.ijrh_17_18

***Address for correspondence:** Dr. Satadal Das, Department of Virology, Dr. Anjali Chatterjee Regional Research Institute for Homoeopathy, Kolkata, West Bengal, India.
E-mail: drsatdas@hotmail.com

Received: 06.03.2018; **Accepted:** 01.06.2018

How to cite this article: Chakraborty U, Katoch S, Sinha M, Nayak D, Khurana A, Manchanda RK, *et al*. Changes in viral load in different organs of Japanese Encephalitis virus-infected chick embryo under the influence of *Belladonna* 200C. Indian J Res Homoeopathy 2018;12:75-80.

The motile blood cells, mainly the macrophages present in this layer, are responsible for the dissemination of the virus and infection to other body parts, especially the brain, by evading the Blood–Brain Barrier (BBB).[7,8] The virus then multiplies in the brain and is responsible for the neuropathological outcome. This situation may mimic the pathology with other hosts including human for JE viral infection and confers the embryonated eggs as a potential system for *in vivo* studies.

The JEV infects both the Central Nervous System (CNS) and non-CNS organs.[9] The present study is focussed on the antiviral role of *Belladonna* 200C against JE by highlighting the altered viral load in different organs of chick embryo mainly in CAM, brain and liver.

Materials and Methods

Chick eggs

One-day-old embryonated chicken eggs of Black Australorp were procured from State poultry farm, Tollygunge, Kolkata, India. The eggs were incubated at 37°C with 60% relative humidity. From the 3rd day onwards, the eggs were turned 3–4 times/day till twelfth day and candled to determine the live/dead status of the embryonated chicken eggs.

Virus strain

JEV was procured from the National Institute of Virology (NIV), Pune, India (NIV no. P-20778, M/K no M-52134). The copy number of the virus inoculum was determined (8.23×10^7/ml) by real-time-Polymerase Chain Reaction (PCR) (Path JEV real-time PCR kit, Primer design) according to the manufacturer's instruction.

Ethical permission

The study was approved by the Institutional Ethics Committee of Dr. Anjali Chatterjee Regional Research Institute for Homoeopathy, Kolkata.

Experimental design

Lethal $dose_{50}$ dose was first determined for the inoculums standardisation.[10] On the twelfth day, live eggs were divided into four groups, with each group containing 10 eggs. Group I (infection) was challenged with JEV (5.6×10^7/ml, LD^{50} dose) through CAM route.[11] Briefly, the surface of the eggs was disinfected with 70% alcohol followed by puncturing on the air space and on the lateral side using egg punch with moderate pressure. Slight suction with rubber teat was applied over the hole at the air space (blunt end), which creates a depression over CAM on lateral surface of the egg. 50 µl of the JEV inoculum was applied over the CAM through the lateral hole using tuberculin syringe. Group II (Treatment) and Group III (Alcohol control) eggs were similarly punched and 50 µl of *Belladonna* 200C (alcoholic extract) and potentised alcohol 200C, respectively, were applied over CAM followed by JEV infection after 15 min. Among the Group IV (control) eggs, 50 µl of bovine albumin phosphate saline was applied. After CAM inoculation, the holes were sealed with wax and the eggs were incubated horizontally at 37°C for 48 h. The eggs were candled on every 12 h up to 48 h to determine the viability and after 48 h the live eggs were humanely killed by chilling on ice for 30 min. CAM and embryo were harvested from all dead or sacrificed eggs after disinfecting the shell, using sterile forceps and scissors. Brain and liver were then studied macroscopically and collected separately for further analysis.

RNA isolation and determination of viral load

The total RNA from CAM, brain and liver was extracted using trizol reagent following manufacturer's guidelines.[12]

The viral load in the samples was determined using real-time PCR kit (Genesig, Primer Design, Watchmoor point, Camberley, UK) on CFX96 Real-Time System (Bio-Rad, USA) following manufacturer's instruction.

Statistical analysis

Each experiment was replicated at least thrice and the experimenters were blinded towards the identity of all groups. The data are represented as mean ± standard deviation. Graph Pad Prism (Version 5, California, USA) was applied for analysis with one-way analysis of variance (ANOVA) and *t*-test.

Results

JEV-infected CAM showed visible pock lesions [Figure 1d], with highly congested blood vessels. Similar observation was also found in the alcohol (200C) control group [Figure 1c]. Severe haemorrhages were found all over the body of embryo and in the brain of these two groups [Figure 2]. Moreover, in these two groups, colliquative necrosis with severe blood clots was found in the brain. However, in the *Belladonna* 200C-treated group, although no such haemorrhages and pock

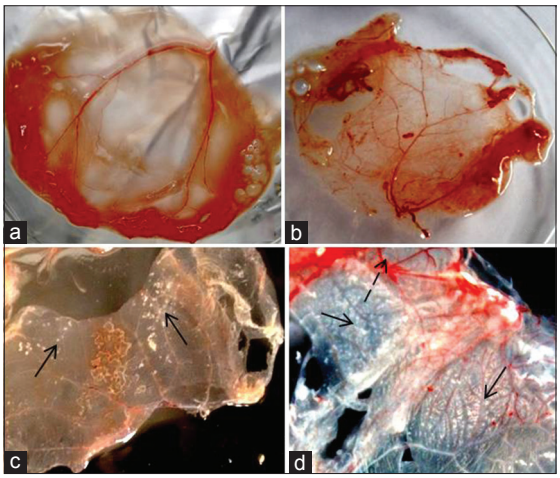

Figure 1: Representative photographs of chorioallantoic membrane tissues from (a) Control and Japanese encephalitis virus-infected groups (b) pre-treated with *Belladonna* 200C, (c) Pre-treated with potentised alcohol (200C), (d) Only virus infected. Control chorioallantoic membrane shows clear blood vessels. *Belladonna*-treated chorioallantoic membrane is apparently healthy. Chorioallantoic membrane from alcohol-treated and virus-infected groups show huge pock lesions (solid arrow) with congested blood vessels (broken arrow)

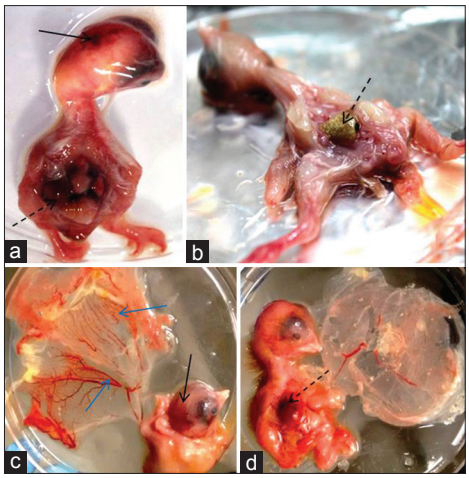

Figure 2: Morbid pathology of chick embryo infected with virus showing haemorrhages in brain (black solid arrow, a,c), yellowish and enlarged liver (broken arrow, b,d), congested blood vessels (blue arrow, c) and haemorrhages throughout the body

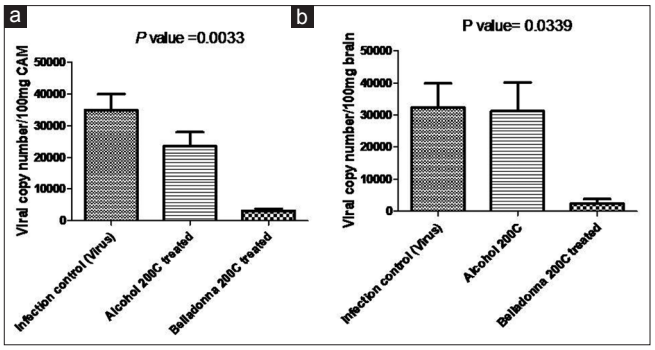

Figure 3: Changes in viral load in (a) Chorioallantoic membrane and (b) brains of chicken embryo infected with virus and treated with potentised alcohol (200C) and *Belladonna* (200C). There were significant changes among these three different infection groups both in chorioallantoic membrane and brain as analysed by one-way analysis of variance

lesions were present, congestions of the blood vessels were observed in few areas of CAM [Figure 1b]. Overall, the CAM was clear and healthy as control [Figure 1a] with intact brains in this medicine-treatedgroup.

The morbid pathologic observation also correlates well with the viral load as determined by viral RNA copy number among different test groups. There were significant changes of JE viral copy number both in the CAM tissue [P = 0.0033, one-way ANOVA, Figure 3a] and in the brain tissue [P = 0.0339, one-way ANOVA, Figure 3b]. There were significantly high viral load in the CAM tissues of infection control (P = 0.0017, one tailed t-test) and alcohol-treated group (P = 0.0052, one-tailed t-test) compared to the *Belladonna* 200C-treated group. Similar pattern was found in the brain tissue of infection control (P = 0.0086, one-tailed t-test)- and alcohol-treated group (P = 0.0165, one-tailed t-test) compared to the *Belladonna* 200C-treated group.

Discussion

Homoeopathy is the cheapest and preferred way of controlling the JE infection in a country like India where poor adherence, negligence and other socioeconomic constraints prevail. Vaccination does not give 100% protection to JE. *Belladonna* derived from the plant *Atropa Belladonna* contains many active components which have effects on central and peripheral nervous systems and have been found effective against JE.

Apart from tannins, flavonoids and coumarines, tropane alkaloids are the main constituents of *A. Belladonna.* Being a neurotrophic virus, JE evades the CNS via transmigrating phagocytic cells and crosses the BBB.[13] *Belladonna* alkaloids can not only cross the BBB, but it may pass through placenta and may be found in the milk.[14]

In contrast to the previous investigations on *Belladonna* 200C with adult mouse model,[5] chick embryo model is more advantageous due to the immune immaturity. The developed adaptive and innate immunity prevent the adult mice being infected with JEV.[15] Moreover, the cost-effectiveness, handling procedure and easier ethical procedures with this *in vivo* embryonated egg model is one of the preferred models for host pathogen interaction tools.

The virus was inoculated through CAM route. However, the presence of the virus in huge number in the brain indicates the dissemination through the blood via BBB. There is only one study which has demonstrated the replication of JEV in different organs of experimentally infected pigs through intravenous route and highlighted the tissue tropism both in the CNS and non-CNS organs such as liver.[9] However, in the present investigation, viral loads were almost similar both in the brain and CAM. Although no JEV was observed in the liver, white patches and yellowish colouration which were observed may be due to reflections of general sickness [Figure 2b]. In this study, with chick embryo, the JEV showed tissue tropism mainly on the brain. The presence of virus in the brain in the medicine-treated group indicates that *Belladonna* 200C cannot totally restrict the viral entry and its replication in the brain. However, pre-treatment of the medicine significantly reduced the replication of the virus both in CAM and brain. Very few congested blood vessels in the CAM and absence of haemorrhages with colliquative necrosis in the brain were found in the medicine-treated group, which indicates the role of *Belladonna* 200C in reducing the complications and severity of the disease.

Conclusion

Belladonna 200C has the antiviral property against JE. Pre-treatment of *Belladonna* 200C may reduce the viral entry, its propagation in different organs particularly in the brain and thus may reduce the pathological sequel of the disease.

Financial support and sponsorship

The work was supported by CCRH, Ministry of AYUSH, Government of India.

Conflicts of interest

None declared.

References

1. WHO Position paper on Japanese encephalitis vaccines. Wkly Epidemiol Rec 2006;81:331-9.
2. Fischer M, Hills S, Staples E, Johnson B, Yaich M. Japanese encephalitis prevention and control: Advances, challenges, and new initiatives. In: Scheld WM, Hammer SM, Hughes JM. editors. Emerging Infections 8. Washington, DC: ASM Press; 2008. p. 93-124.
3. Vaughn DW, Hoke CH Jr., The epidemiology of Japanese encephalitis: Prospects for prevention. Epidemiol Rev 1992;14:197-221.
4. Endy TP, Nisalak A. Japanese encephalitis virus: Ecology and epidemiology. Curr Top Microbiol Immunol 2002;267:11-48.
5. Bandyopadhyay B, Das S, Sengupta M, Saha C, Raveendar C, Chakravarty R, *et al*. The role of 'Belladonna 200' in the prevention of Japanese encephalitis (JE) virus infection. In: Adoga MP, editor. Molecular Virology. Ch. 7. U.K.: InTech; 2012. p. 111-24.
6. Yuan YJ, Xu K, Wu W, Luo Q, Yu JL. Application of the chick embryo chorioallantoic membrane in neurosurgery disease. Int J Med Sci 2014;11:1275-81.
7. Gupta N, Rao PV. Transcriptomic profile of host response in Japanese encephalitis virus infection. Virol J 2011;8:92.
8. Cooper MD, Chen CL, Bucy RP, Thompson CB. Avian T cell ontogeny. Adv Immunol 1991;50:87-117.
9. Ricklin ME, Garcìa-Nicolàs O, Brechbühl D, Python S, Zumkehr B, Posthaus H, *et al.* Japanese encephalitis virus tropism in experimentally infected pigs. Vet Res 2016;47:34.
10. Reed LJ, Muench H. A simple method of estimating fifty percent end points. Am J Hyg 1938;27:493-7.
11. Härtl A, Hillesheim HG, Künkel W, Schrinner EJ. The candida infected hen's egg. An alternative test system for systemic Anticandida activity Arzneimittelforschung 1995;45:926-8.
12. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-9.
13. Diamond MS. Evasion of innate and adaptive immunity by *Flaviviruses*. Immunol Cell Biol 2003;81:196-206.
14. The European Agency for Evaluation of Medicinal Products. Committee for Veterinary Medicinal Products. Veterinary Medicines Evaluation Unit. Summary Report. *Atropa Belladonna*. EMEA/MRL/540/98/FINAL; December, 1998.
15. Ogata T, Miura T, Ema M, Matsuda R. Influence of age on the susceptibility of mice to Japanese encephalitis virus infection (author's transl). Nihon Eiseigaku Zasshi 1975;30:411-6.

बेलाडोना 200सी के प्रभाव में जापानी मस्तिष्ककोप से संक्रमित भ्रूण के विभिन्न अंगों में वायरल लोड में परिवर्तन

उद्देश्यः जापानी मस्तिष्ककोप भारत के कई राज्यों में अत्याधिक प्रचलित है। होम्योपैथी में, *बेलाडोना* 200सी का प्रयोग व्यापक रूप से जापानी मस्तिष्ककोप से बचाव और उपचार में किया जाता है जिसे पहले ही इन–विट्रो और इन–विवो प्रयोगों में स्थापित किया गया है। हालांकि विभिन्न ऊतकों में वायरस प्रतिकृति में बेलाडोना 200सी के प्रभाव का अबतक अध्ययन नहीं किया गया है। प्रस्तुत लेख जापानी मस्तिष्ककोप संक्रमण में चूजे के भ्रूण के मॉडल का प्रयोग करते हुए *बेलाडोना* 200सी के इसी पहलू को रेखांकित करता है।

सामग्री व विधिः ब्लैक ऑस्ट्रार्लोप के बारह दिन पुराने उर्वरक अडों को विभिन्न प्रयोगात्मक सेटोः संक्रमण, *बेलाडोना* 200सी से उपचारित और वाहन नियंत्रण मिलान किए गए रिक्त सेटों को बनाए रखा गया, में कोरियोलाटॉयिक झिल्ली मार्ग के माध्यम से जापानी मस्तिष्ककोप दिया गया।

सभी प्रायोगिक सेट 48 घंटो के लिए सेते गये। ऊष्मा प्रदान करने के बाद, जीवक्षम अंडों को मानवीय रूप से समाप्त किया गया और रियल टाइम पॉलीमेरेज चेन रिएक्शन द्वारा वायरल लोड निर्धारण के लिए विभिन्न ऊतकों को देखा गया व एकत्र किया गया।

परिणामः संक्रमण नियंत्रण समूह में कोरियोएलांटोयिक झिल्ली में छाले दृष्टिगत थे, मस्तिष्क हायमोरैझिक लिक्विफैक्टिव नैकरोसिस के कारण तरलीकृत हो गया था और यकृत पर सफेद निशान भी पाए गए। जबकि औषधि द्वारा उपचारित समूह साधारण पाया गया, मस्तिष्क में कोई परिवर्तन दृष्टिगत नहीं था व यकृत नियंत्रण समूह की तरह स्वस्थ था। रियल टाइम पॉलीमेरेज़ चेन रिएक्शन से कोरियोएलांटोयिक झिल्ली व मस्तिष्क में वायरल लोड अधिक पाया गया। वायरस संक्रमित समूह में यकृत में वायरल आरएनए की अनुपस्थिति थी। *बेलाडोना* 200सी से पूर्व उपचार करने पर कोरियोएलांटोयिक झिल्ली व मस्तिष्क में कुल लोड (पीढ0.05) में महत्वपूर्ण कमी आई जोकि अंगों की विकृत रोगजनक परिवर्तन से सहसंबंधित है।

निष्कर्षः बेलाडोना 200सी मस्तिष्क में जापानी मस्तिष्ककोप के वायरल प्रतिकृति को रोक नहीं पाई, परंतु इससे इस ऊतक में वायरल लोड को कम करके जापानी मस्तिष्ककोप की तीव्रता में कमी आ जाती है।

Évolutions de la charge virale dans différents organes d'embryons de poulet infectés par le virus de l'encéphalite japonaise sous l'influence de Belladonna 200C

Contexte: L'encéphalite japonaise (EJ) est très répandue dans de nombreux états d'Inde. Belladonna 200C est couramment utilisée dans la prévention et le traitement de l'EJ. Les effets de Belladonna 200C sur la réplication virale dans les différents tissus n'ont pas été étudiés.

Objectif: Étudier les effets de Belladonna 200C sur la réplication virale dans les différents tissus en utilisant le modèle des embryons de poulet.

Matériels et méthodes: Des œufs d'Australorp noir, fertilisés depuis douze jours, ont été inoculés avec l'EJ par la membrane chorioallantoïde (MCA) dans le cadre de différents ensembles d'expérience : infection, traité avec Belladonna 200C et contrôle du vecteur, en gardant des ensembles vierges appariés. Tous les ensembles d'expérience ont été incubés pendant 48 heures. Après l'incubation, les œufs viables ont été tués par une personne et les différents tissus ont été observés et prélevés pour déterminer la charge virale au moyen de l'amplification en chaîne par polymérase en temps réel (PCR).

Résultats: Le groupe témoin avait des pustules visibles sur la MCA. Le cerveau s'était liquéfié du fait de la nécrose hémorragique provoquant la liquéfaction et des taches blanches ont été constatées sur le foie. Cependant, le groupe traité à l'aide de médicaments semblait normal. Il n'y avait pas de changement visible dans le cerveau et le foie était sain comme dans le groupe de contrôle. Les résultats de l'amplification en chaîne par polymérase en temps réel ont montré une forte charge virale dans la MCA et le cerveau et l'absence d'ARN viral dans le foie des membres du groupe infecté par le virus. Un prétraitement avec Belladonna 200C a fait baisser de manière significative la charge virale globale ($P < 0,05$) dans la MCA et le cerveau correspondant aux changements pathologiques morbides des organes.

Conclusion: Bien que Belladonna 200C n'ait pas empêché complètement la réplication virale de l'EJ dans le cerveau, elle a réduit la gravité de l'EJ en y faisant baisser les charges virales.

Cambios en la carga vírica en diferentes órganos de embriones de pollo infectados por el virus de la encefalitis japonesa bajo la influencia de *Belladonna* 200C

Objetivos: La encefalitis japonesa (EJ) es sumamente prevalente en muchos estados de la India. *Belladonna* 200C es ampliamente utilizado en la prevención y el tratamiento de JE. El efecto de *Belladonna* 200C en la replicación del virus dentro de diferentes tejidos no ha sido estudiado. **Objetivo:** Estudiar el efecto de *Belladonna* 200C en la replicación del virus en diferentes tejidos utilizando el modelo de embrión de pollo.

Materiales y métodos: En los huevos fertilizados de Australop negra de 12 días, se inoculó el virus EJ a través de la membrana corioalantoica (MCA) en diferentes conjuntos experimentales: infección, tratados con *Belladonna* 200C y control con vehículo, manteniendo lossets vacíos idénticos. Todos los conjuntos experimentales se incubaron durante 48 h. Tras la incubación se sacrificaron los huevos viables y se observaron los diferentes tejidos. La carga vírica se determinó con la reacción en cadena de la polimerasa en tiempo real (PCR).

Resultados: El grupo de control de infección mostraba cicatrices visibles en la MCA; los cerebros estaban licuados debido a la necrosis licuefactiva hemorrágica y se observaron parches blancos en el hígado. Sin embargo, el grupo tratado con medicamentos fue aparentemente normal; no hubo cambios visibles en el cerebro y el hígado estaba sano como control..Los resultados del PCR a tiempo real mostraron una elevada carga vírica en la MCA y el cerebro con ausencia de ARN vírico en el hígado del grupo infectado con el virus. El pretratamiento con *Belladonna* 200C redujo significativamente la carga global ($P< 0,05$) en la MCA y el cerebro que se correlacionó con los cambios patológicos mórbidos en los órganos.

Conclusiones: Pese a que *Belladonna* 200C no fue capaz de inhibir completamente la replicación vírica EJ en el cerebro, redujo la gravedad de la EJ disminuyendo las cargas víricas e este tejidos.

Veränderungen der Viruslast in verschiedenen Organen des mit dem Japanischen Enzephalitis-Virus infizierten Hühnerembryos unter dem Einfluss von Belladonna 200C

Abstrakt

Hintergrund: Japanische Enzephalitis (JE) ist in vielen Bundesstaaten Indiens weit verbreitet. Belladonna 200C ist weit verbreitet in der Prävention und Behandlung von JE. Die Wirkung von Belladonna 200C auf die Virusreplikation in verschiedenen Geweben wurde nicht untersucht.

Ziel: Untersuchung der Wirkung von Belladonna 200C auf die Virusreplikation in verschiedenen Geweben unter Verwendung des Hühnerembryomodells.

Materialien und Methoden: Zwölf Tage alte befruchtete Eier von Black Australorp wurden mit JE über die Route der Chorioallantoismembran (CAM) in verschiedenen experimentellen Sets inokuliert: Infektion, Belladonna 200C behandelt und Fahrzeugkontrolle, die zusammenpassende leere Sätze behalten. Nach der Inkubation wurden lebensfähige Eier menschlich geopfert und verschiedene Gewebe wurden beobachtet und zur Viruslastbestimmung durch Echtzeit-Polymerasekettenreaktion (PCR) gesammelt.

Ergebnisse: Die Kontrollgruppe zeigte sichtbare Pocken über der CAM; Die Gehirne wurden aufgrund von hämorrhagisch-verflüssigender Nekrose verflüssigt und weiße Flecken wurden über der Leber gefunden. Die medikamentös behandelt Gruppe war jedoch anscheinend normal; es gab keine sichtbaren Veränderungen im Gehirn und die Leber war gesund wie die Kontrolle. Echtzeit-PCR-Ergebnisse zeigten eine hohe Viruslast in CAM und Gehirn mit Abwesenheit von viraler RNA in der Leber der Virus-infizierten Gruppe. Die Vorbehandlung mit Belladonna 200C reduzierte signifikant die Gesamtbelastung ($P < 0,05$) in CAM und Gehirn, die mit den krankhaften pathologischen Veränderungen der Organe korrelierte.

Schlussfolgerung: Obwohl Belladonna 200C die JE-Virusreplikation im Gehirn nicht vollständig inhibierte, reduzierte es die Schwere von JE, indem es die Viruslast in diesem Gewebe verringerte.