Invivo evaluation of *Withania somnifera* based Indian traditional formulation (*Amukkara Choornam),* against chikungunya virus induced morbidity and arthralgia

Abstract

Chikungunya viral fever results in extreme morbidity and arthralgia in affected individuals. Currently, modern medicines providing symptomatic relief for the acute febrile phase and the chronic arthritic phase are only options available. Traditional Indian medical system, however, uses specific formulations for treatment of this infection; one such polyherbal formulation used to treat the post pyretic phase of chikungunya is amukkara choornam. The current study was undertaken to study the efficacy of amukkara choornam in the treatment of chikungunya in C57BL/6J mice. The formulation when administered to chikungunya infected mice relieved morbidity and joint swelling. Analysis of virus clearance in brain and joint tissues upon formulation treatment revealed a direct correlation of viral load in brain to morbidity during infection; likewise, joint swelling receded prior to complete viral clearance explaining possible immune-modulatory effect of amukkara choornam. This study provides insight to the possible mode of action of amukkara choornam during chikungunya.

Keywords: Polyherbal; Siddha formulations; Chikungunya; post pyretic phase; *In vivo*

Supplementary information

***In vitro* antiviral activity assays**

Material and methods

Development of Mice model for evaluating amukkara choornam during chikungunya virus infection

8 weeks old C57BL/6J mice were used for this study. Virus was prepared and was checked using plaque assay using previously standardized protocols (Lee et al., 2013). $1X10^6$ pfu of virus was injected in the hind left footpad of eight mice. Mice were observed for 15 days and 2 mice each were sacrificed at Day 3, Day 5, Day 7 and Day 15. A total of 14 organs were recovered from their body namely Joint, Brain, Muscle, Liver, Spleen, Skin, Intestine, Bone marrow, Serum, Heart, Thymus, Lungs and Kidney. Organs from both animals were pooled and viral nucleic acid (VNA) was extracted from the stored samples using High Pure Viral Nucleic Acid kit (Roche, Germany Cat No. 11858874001). Estimation of Viral copy number in the organs was done using QuantiTect® Reverse Transcription kit (Qiagen, Germany, Cat No.205310), as per the manufacturer's instructions. Using laboratory generated reference strain in serial dilution of 100ng to 1pg, exact copy number of CHIKV from viral RNA isolated from patient sera.

Results

Development of Mice model for chikungunya virus infection

C57BL/6J mouse has been widely used as a promising chikungunya model (Morrison et al., 2011; Yeo & Chu, 2013) for testing potential CHIKV inhibitors (Kuo et al., 2016; Varghese et al., 2016). This mouse is capable of hosting replication of CHIKV administered in the footpad of the hind limbs and succumbs to it at high challenge doses. For the establishment of mice model for chikungunya virus infection $1X10^6$ virus particles were injected in the footpad of 8 mice and 3 mice were mock infected with PBS alone and used as a control group. Mice were observed for the difference in their morbidity conditions based on Morton & Griffiths (1985) scale (Morton & Griffiths, 1985) and two mice were sacrificed after Day 3, Day 5 Day 7 and Day 10 respectively. It was observed that morbidity started to increase after Day 2 post infection and continued deterioration occurred till Day 7 thereafter mortality was observed at Day 8 and Day 10 in the remaining mice, Mice were sacrificed and Organ wise viral load was detected for every mouse (Figure 2S).

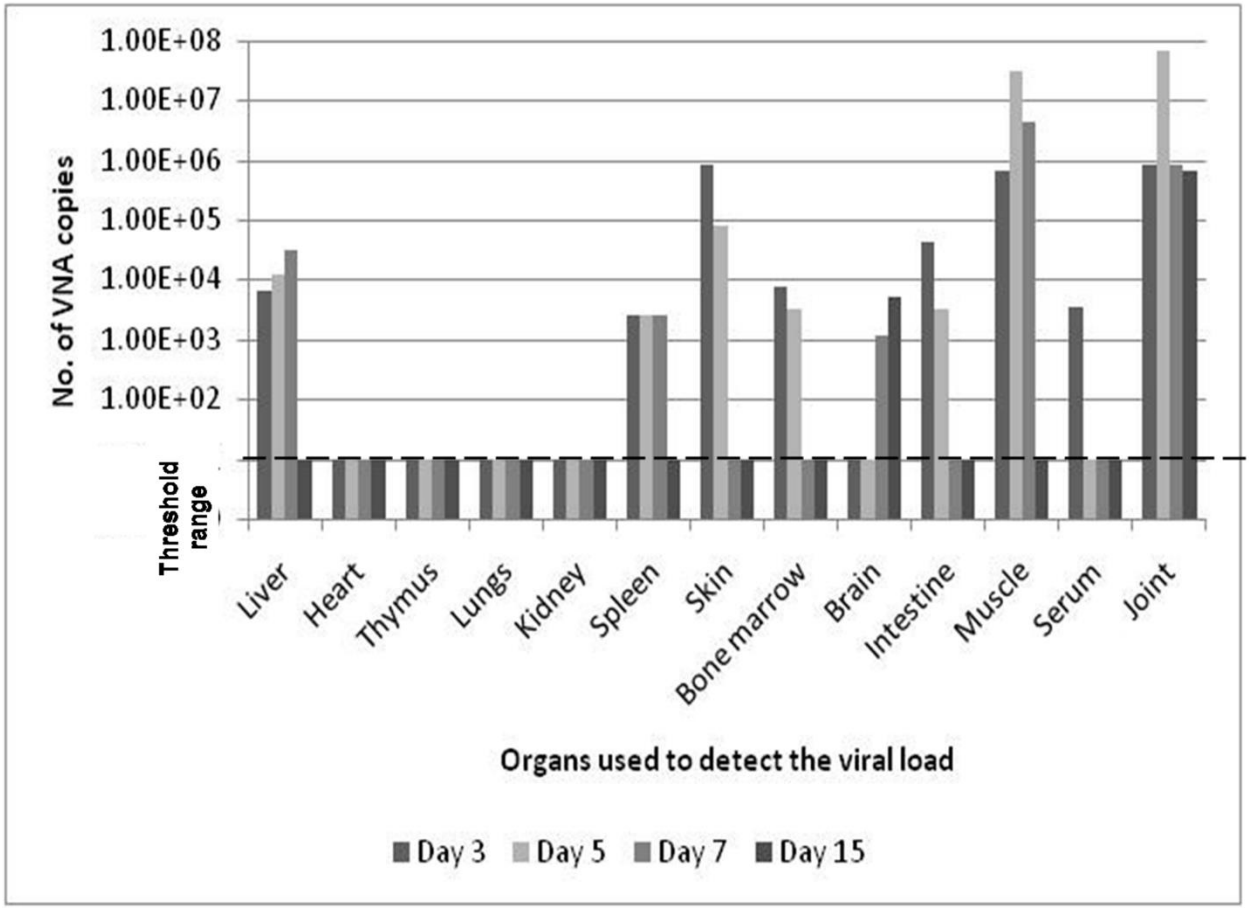

Figure 2S: Organ wise viral load detection in mice at Day 3, Day 5, Day 7 and Day 15 post infection for establishment of animal model upon chikungunya viral infection

We investigated the kinetic of virus replication in tissues of 8-weeks-old mice at D3 and D5 pi and at the onset of symptoms (D7 pi) and at D 15 (or before, depending on the time of death) pi. Infectious virus was detected at low level at D3 pi in serum and at D5 and D7 pi in liver (Figure 2S). Strikingly, at all-time points analyzed, infectious CHIKV was detected very abundantly in muscle, joint and skin and to a lower extent in the brain, whereas in D15 virus was cleared from most organs but was found in brain. It was found that Viral load at Day 3 was highest in the joints followed by skin, muscle, intestine, bone marrow, liver, serum and spleen wherein viral copy number was $9.1X10^5$, $8.2X10^5$, $7.8X10^5$, $4.5X10^4$, $8.2X10^3$, $7.3X10^3$, $4.1X10^3$ and $3.2X10^3$ respectively. Viral load increased till Day 5 post infection in all the other organs except for serum and skin, in these organs viral load decreased 5 days post infection was number of VNA copies decreased to $7.2X10^2$ and $8.4X10^4$ respectively. At Day 7 post infection viral load decreased substantially in all organs except for brain where the viral copy number was detected to be $1.8X10^3$.

Mortality of mice was observed at Day 8 and Day 10 post infection when organ wise viral load was detected in these mice it was observed that in all organs was below detectable range except joint and brain where detected number of VNA was $7.2X10^5$ and $5.4X10^3$ respectively.

References

Kuo, S.-C., Wang, Y.-M., Ho, Y.-J., Chang, T.-Y., Lai, Z.-Z., Tsui, P.-Y., . . . Lin, C.-C. (2016). Suramin treatment reduces chikungunya pathogenesis in mice. *Antiviral research, 134*, 89-96.

Lee, R. C., Hapuarachchi, H. C., Chen, K. C., Hussain, K. M., Chen, H., Low, S. L., . . . Chu, J. J. (2013). Mosquito cellular factors and functions in mediating the infectious entry of chikungunya virus. *PLoS Negl Trop Dis, 7*(2), e2050. doi:10.1371/journal.pntd.0002050

Morrison, T. E., Oko, L., Montgomery, S. A., Whitmore, A. C., Lotstein, A. R., Gunn, B. M., . . . Heise, M. T. (2011). A Mouse Model of Chikungunya Virus–Induced Musculoskeletal Inflammatory Disease: Evidence of Arthritis, Tenosynovitis, Myositis, and Persistence. *The American journal of pathology, 178*(1), 32-40.

Morton, D., & Griffiths, P. (1985). *Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment*: publisher not identified.

Varghese, F. S., Thaa, B., Amrun, S. N., Simarmata, D., Rausalu, K., Nyman, T. A., . . . Ahola, T. (2016). The Antiviral Alkaloid Berberine Reduces Chikungunya Virus-Induced Mitogen-Activated Protein Kinase Signaling. *Journal of Virology, 90*(21), 9743-9757.

Yeo, L. S., & Chu, J. J. H. (2013). Recent developments and challenges in mouse models of Chikungunya virus infection. *Future Virology, 8*(5), 423-426.