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AN *IN VITRO* STUDY: INDIAN STRAIN OF JAPANESE ENCEPHALITIS VIRUS INFECTION IN PORCINE STABLE KIDNEY CELL USING ^{1}H NMR SPECTROSCOPY

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ABSTRACT

Japanese encephalitis (JE) is a common mosquito borne flaviviral encephalitis which is one of the leading forms of viral encephalitis worldwide with prevalence mostly in the eastern and southern Asia. The effects of Japanese encephalitis virus infection in cell line derived from porcine stable kidney cell (PK) were studied using optical microscopy and ^{1}H NMR spectroscopy. The controls of PK cells were consumed glucose and produced lactate, acetate and formate as extracellular metabolites whereas the infected with JE virus cells consumed significantly higher glucose and produced lactate, acetate and formate as extracellular metabolites. At the onset of infection, extracellular metabolites lactate was converted to acetate, whereas its gradual increase was observed till 72 hours in uninfected cells. In the intracellular metabolites of infected cells showed significantly high concentration of valine, leucine, isoleucine, histidine, phenylalanine and tyrosine respectively in comparison to uninfected cells. Progressive breakdown and consumption of all cellular components were observed as the infections were increases. The Cell Lipid extraction shows that Cellular metabolites except lipid components gradually decreased and disappeared during 12–72 hours of viral infection. The study is useful for monitoring the cellular metabolic changes upon virus infection. This indicates the possible application of cell-virus interaction studies using cellular metabonomics.

Keywords: ^{1}H NMR, Optical microscopy, Japanese Encephalitis Virus, PK cells, Cell–virus interaction.

INTRODUCTION

Arthropod-borne viruses of the genus *Flavivirus* (family *Flaviviridae*) cause a range of serious diseases in humans, the prevention and treatment of which are global public health priorities. Unfortunately no suitable antiviral is available [1]. The disease affects all age groups though the target population being children below the age of 15 years [2]. The mortality in acute encephalitic stage varies from 20-40% in various parts of the India. Among the survivors neurological morbidity has been noted in over 50% of cases [3]. About 50- 60 % of the survivors suffer from serious long term neurological sequelae manifested as convulsions, tremors, paralysis, ataxia, memory loss, respectively impaired cognition behavioral disturbance and other such symptoms [4].

Japanese encephalitis is a single stranded positive sense RNA of approximately 11kb in length who's epidemiological and structural aspects have been extensively reviewed [5]. The flavivirus causes acute encephalitis associated with damage to central nervous

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system [6]. Japanese encephalitis virus is transmitted by mosquitoes and is widely distributed in the Asia, including Japan, China, Taiwan, Korea, Philippines, far-eastern former Soviet Union, all of the Southeast Asia, and India. There are about 50,000 cases and 10,000 deaths reported annually throughout Asia, but these numbers are believed to be greatly underestimated. Structural elements of the E protein are also involved in the viral attachment, fusion, penetration, host range and cell tropism, and virus virulence and attenuation [7].

Upon viral infection, the delivery of the viral nucleic acid into the host cell involves a complex series of tightly–regulated events where the entering virus utilizes the biosynthetic components of the host cells to reach its goal and alter the cell metabolism. Depending on the host cell and virus type, different mechanisms have been proposed [8]. Virus infection requires entry of the virus into the host cell. The viral genome can enter the cytoplasm by penetrating the plasma membrane at the cell surface, or at the endosome after endocytosis. The process of the early phase of the flavivirus infection is not clearly understood; however, there are reports that flaviviruses enter the cell by receptor-mediated endocytosis [9]. Clathrin-dependent and caveola-dependent pathways were both reported to the virus entry mechanism [10]. Studies on virus–cell interactions have proven to be valuable in elucidating viral cellular processes [11-16].

A number of post–genomic technologies are being widely applied to understand the metabolism and molecular basis of human diseases [17]. Metabolomics is a new approach that enables multivariate profiling of the integrated metabolic responses of complex systems to pathophysiological stress and provides complementary information to genomics and proteomics [18]. The use of high-resolution nuclear magnetic resonance (NMR) spectroscopy in the study of metabolomics in biological fluids, tissues and cells, and in exploring the biochemical consequences of human diseases, is rapidly evolving [19-21]. This approach appears to be attractive in the study of cell metabolism owing to its unique non-invasive characteristics which can generate information on the multiple pathways to be monitored simultaneously in a single step.

Dynamic studies using magnetic resonance spectroscopy of viral infection on animal models in order to understand the mechanisms of infection are reported [22-24]. Cell culture is widely used as an in vitro model that allows better experimental control of physiological parameters than tissues or in vivo studies. Thus far, there have been no reports on dynamic studies of flaviviral infection in cell lines using NMR spectroscopy. This paper presents a study of JE virus infection in porcine kidney (PK) cell line in acute, subacute and chronic stages of infection using high resolution 1H NMR spectroscopy.

In the present study, to analyze the early phase of JEV infection in a porcine kidney cell line, (PK). This study attempts the question of when the JE virus enters into the cells which metabolites released by early stage of infection with the help of Nuclear magnetic resonance spectroscopy. This study provides an insight into the interaction between JEV and vertebrate cells.

MATERIALS AND METHODS

Virus and Cell line

GP- 78 (non neuroinvassive) strain of Japanese encephalitis virus and porcine stable kidney (PK) cell line used in this study was kindly gifted by Dr. S. Vrati (National Institute of Immunology, New Delhi, India). The virus was passaged in the PK Cell [25].

Virus titer determination

Virus titer will be determined by standard plaque assay. In brief, PK cells monolayer will be grown in 60-mm six well tissue culture plates. Tenfold serial dilution of virus stock was being added to 90% (monolayer) confluent PK cell line. After 1hour incubation at 37ºC with 5% CO_2 unabsorbed virus will be removed by gentle washing with PBS (phosphate saline buffer) and an overlay medium (1% agarose in MEM with 1% FBS) will be added on to it. After 4 days incubation the cells will be fixed with 10% formaldehyde and stained with 0.5% crystal violet and plaques will be counted.

Other component

Eagle's minimum essential medium, HEPES buffer, sodium bicarbonate, phenol red, deuterium oxide (D_2O) and trimethylsilylpropionic acid sodium salt (TSP) (all from Sigma Aldrich, USA), penicillin, streptomycin, fetal bovine serum (FBS), L-glutamine, phosphate buffer saline, fungizone, trypsin, trypan blue stain, Non essential amino acid and tryptose phosphate broth (TPB) (all from GIBCO, USA) and cell culture flask 75 cm2 (Falcon, USA) were used in this study.

Cell culture

Fresh stocks of the PK cells were seeded at a density of $1.9x10^6$ cells/75 cm^2 flask in six identical sets (each set having two flasks). The cells in each flask were grown in 20ml. Eagle's minimum essential medium (MEM, 9.4 g/ l) containing FBS (10%), L-glutamine (3%), sodium bicarbonate (7.5%), HEPES (1 M), Fungizone (250mg/ml), penicillin (100 units/ml) and streptomycin (10 mg/ml), Non essential amino acid and tryptose phosphate broth (TPB) and phenol red (4%) supplemented with under sterile conditions at 37ºC in a 90% humidified and 5% CO_2 atmosphere. After 3 days, the growth medium was replaced with fresh growth medium and the cells were allowed to grow further for 2 days during that the cells were grown with 100% confluence. Confluence was measured by optical microscopy.

Infection of JE Virus in cells

After the cells were grown with 100% confluence, the growth medium (MEM with 10% FBS) was replaced with maintenance medium (MEM with 2% FBS) from all the flasks. The cells from one flask from each set were inoculated with GP-78 strain of JE virus ($1x10^{10}$ Pfu/ml), while the other flask served as a control. After 12 h, one set of flasks both control and infected was taken out from the incubator, photographs of the cells were taken under a Nikon inverted microscope at a magnification of X 400 and the maintenance medium was taken out and stored at -80ºC until 1H NMR experiments were performed for analyzing the extracellular metabolites from the medium. The cells were harvested by trypsinization, stained with trypan blue and counted, washed twice with ice-cold PBS 1X (4ºC, pH=7. 4) and suspended in buffered D_2O (50 ml PBS 10X and 450 ml D_2O) for 1H NMR experiments of the whole cells. Virus infection in the remaining five sets of flasks was continued for a 24, 36, 48, 60 or 72 hour period and, at the end of each period, photographs of cells from one set of flasks both control and infected were taken, the maintenance medium was taken out for ^1H NMR experiments and the cells were harvested, counted and suspended in buffered D_2O as described above for flasks with 12 hours infected and uninfected cells.

NMR experiments

All one and two dimensional NMR experiments were performed on a Bruker Biospin Avance 400 MHz NMR spectrometer using a 5mm broadband inverse probe equipped with z-gradient. For quantitation and chemical shift reference, a reusable coaxial insert containing TSP in D_2O was used. D_2O present in coaxial insert used for locking the spectrometer. Before using this coaxial, the concentration of TSP was calibrated with a solution of known concentration of glycine. The infected and uninfected cells were sonicated under ice cold condition. The sonicated cells were diluted up to 500µl with phosphate buffer prepared in D_2O and taken into an NMR tube along with coaxial insert. All one-dimensional ^1H spectra were recorded using one pulse sequence with the suppression of the water signal by a presentation during the relaxation delay. Typical acquisition parameters used were: spectral width 8012Hz, time domain data points 32768, a flip angle of 45°, relaxation delay 6 s to ensure maximum recovery of magnetization to the equilibrium between the scans, acquisition time 2.044 s and typically, 128 transients were accumulated for each sample. The resulting data were Fourier-transformed after multiplication by the exponential window function using a line-broadening function of 0.3 Hz and zero filling of 16k data points. The manual phase correction was performed followed by an automatic baseline correction. In order to confirm the assignments of resonances in cell extracts and extracellular media, a ^1H–^1H two-dimensional Gradient correlated spectroscopy (COSY-GP) experiment was also performed by suppressing the residual water signal by the presentation.

The typical NMR parameters used for the two-dimensional experiment were: spectral width 8012 Hz in both dimensions, time domain data points 2048, number of free induction decays (FIDs) with 256 t_1 increments, relaxation delay 2.5 s and number of transients 80. Phase-sensitive data were obtained by the time-proportional phase increment (TPPI) method. The resulting data were zero filled to 1024 points in t_1 dimension and Fourier transformed along both dimensions after multiplying the data by the sine-bell squared window function shifted by 90°C.

Extracellular metabolites

The extracellular media from infected cells and their uninfected counterparts were thawed, an aliquot of 500µl from each medium was drawn separately into 5mm NMR tubes followed by insertion of coaxial insert and ^1H NMR spectra were recorded. For comparing the metabolite patterns excreted by infected cells with uninfected cells, all spectra were recorded under identical experimental conditions.

Intracellular metabolites

Water-soluble intracellular metabolites of all infected and uninfected whole cells were extracted into phosphate buffer prepared in D_2O, by sonicating the cells under ice-cold conditions. The whole cell lysate was thoroughly mixed by vortexing the sample and make up it to total volume of 500µl. Then the whole cell lysate containing water-soluble cellular metabolites were taken in 5mm NMR tubes and one-dimensional ^1H spectra were recorded under identical conditions by suppressing the residual water by the presentation. After recording the spectra, all samples were stored in -80° C for lipid extraction.

Cell lipids

The cell lysate, which was already stored in -80°C after recording the proton NMR spectra, was subjected to lipid extraction following Folch's extraction procedure [26], to study cell lipid components. The supernatant and residue was lyophilized and then powdered. Briefly, the residual cell pellet was mixed with 5.0 ml of chloroform, methanol and saline water in a ratio of 2.0:1.0:0.18. The resultant mixture was sonicated for 3 min under ice cold conditions and centrifuged at 2400 rpm for 10 minutes to allow clear separation of the aqueous and non-aqueous layers. Subsequently, the aqueous (upper) layer was taken out and subjected to re-extraction of the remaining lipids, if any, by treatment with 2.0ml of chloroform and methanol in a ratio of 2:1, sonicating and centrifuging for 10 minutes. The non-aqueous (lower) layers from both extraction steps were pooled and dried by passing a jet of nitrogen gas into the solution. The residue obtained was dissolved in 500ml $CDCl_3$:CD_3OD mixtures in a ratio of 2:1 and ^1H NMR Spectra were recorded.

RESULTS

The number of cells after 12 hours and 24 hours virus infection was slightly less compared with the number of uninfected cells under similar conditions. This reduced number of cells indicates the beginning of infection of cells due to virus. However, morphologically no cytopathic effects were observed in 12 hours and 24 hours infected cells under the inverted optical microscope (Fig. 1a, 1b and 1c) – infected and uninfected cells show identical consistency. However, when the virus infection time was 36 hours or more, a noticeable cytopathic effect was observed (Figs. 1d). At 36 hours infection, rounding of the cells accompanied with detachment from the flask surface was observed (Fig. 1d), and this detachment increased as well as time increases. While at 72 hour infection all cells detached from the surface (Fig.1g).

Figure 1. Cells in culture flasks monitored under a Nikon inverted microscope at a magnification of _400: (a) uninfected PK cells; (b) –(g) PK cells infected with JE virus for (b) 12 h, (c) 24 h, (d) 36 h, (e) 48 h, (f) 60 h and (g) 72 h

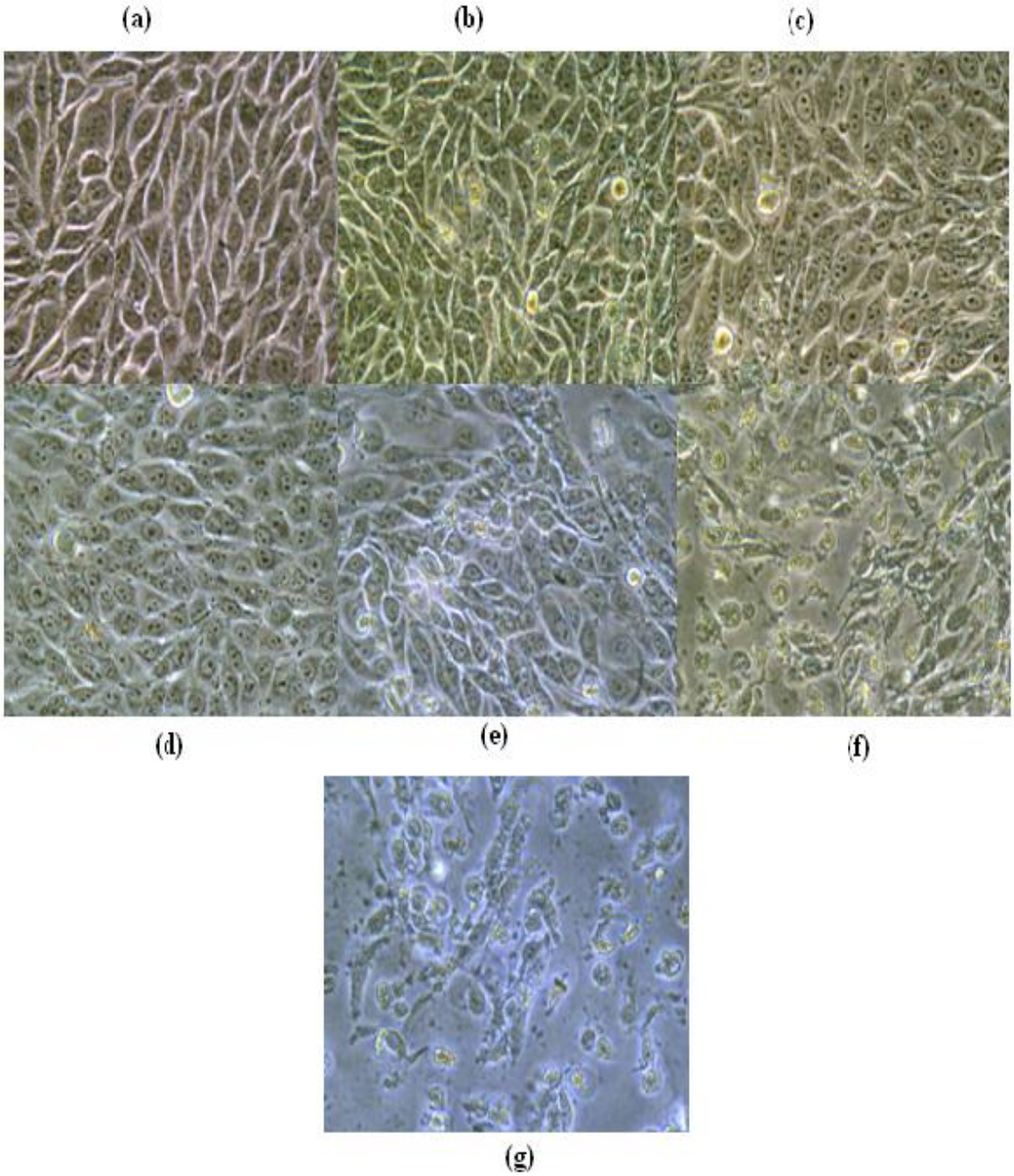


Figure 2. 400 MHz 1H NMR spectra of extracellular media of (a) control cells, (b) 12 h infected cells, (c) 24 h infected cells, (d) 36 h infected cells, (e) 48 h infected cells, (f) 60 h infected cells, (g) 72 h infected cells with JE virus. All spectra were recorded under identical conditions and plotted with the same vertical scale for direct comparison

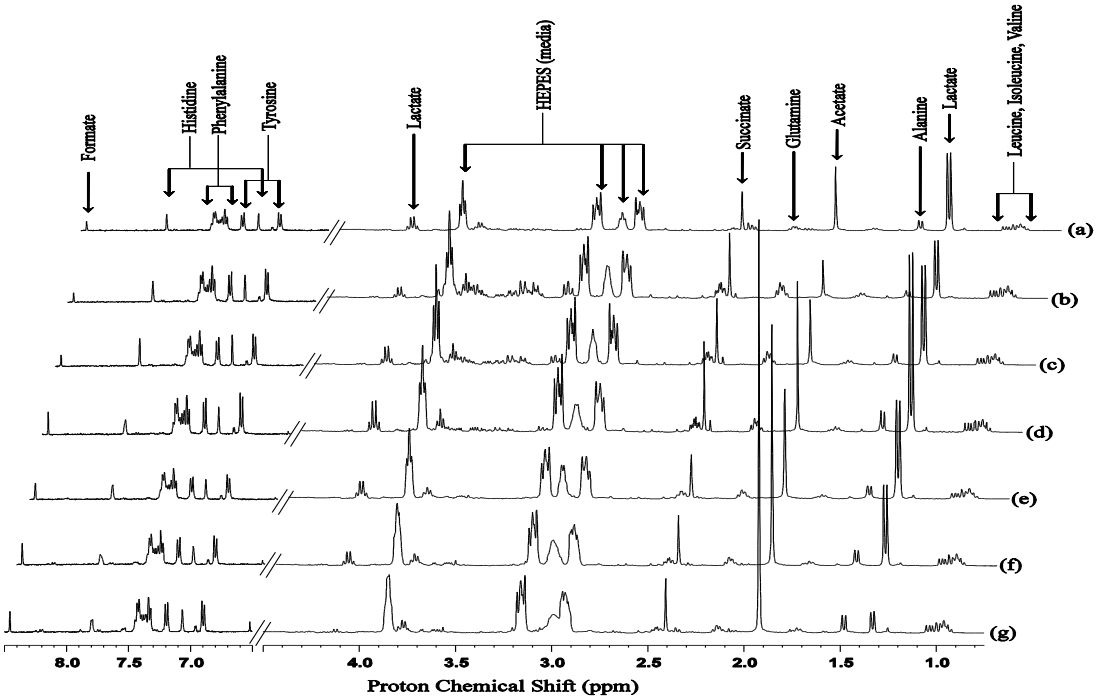


Figure 3. Portions of 400MHz 1H NMR spectra of PK cells: (a) control cells uninfected) and (b) –(g) cells infected with JE virus for (b) 12 h, (c) 24 h, (d) 36 h, (e) 48 h, (f) 60 h and (g) 72 h. All spectra were obtained under identical conditions and plotted with identical vertical scale for direct comparison. The peak marked '#' refers to methanol as an impurity

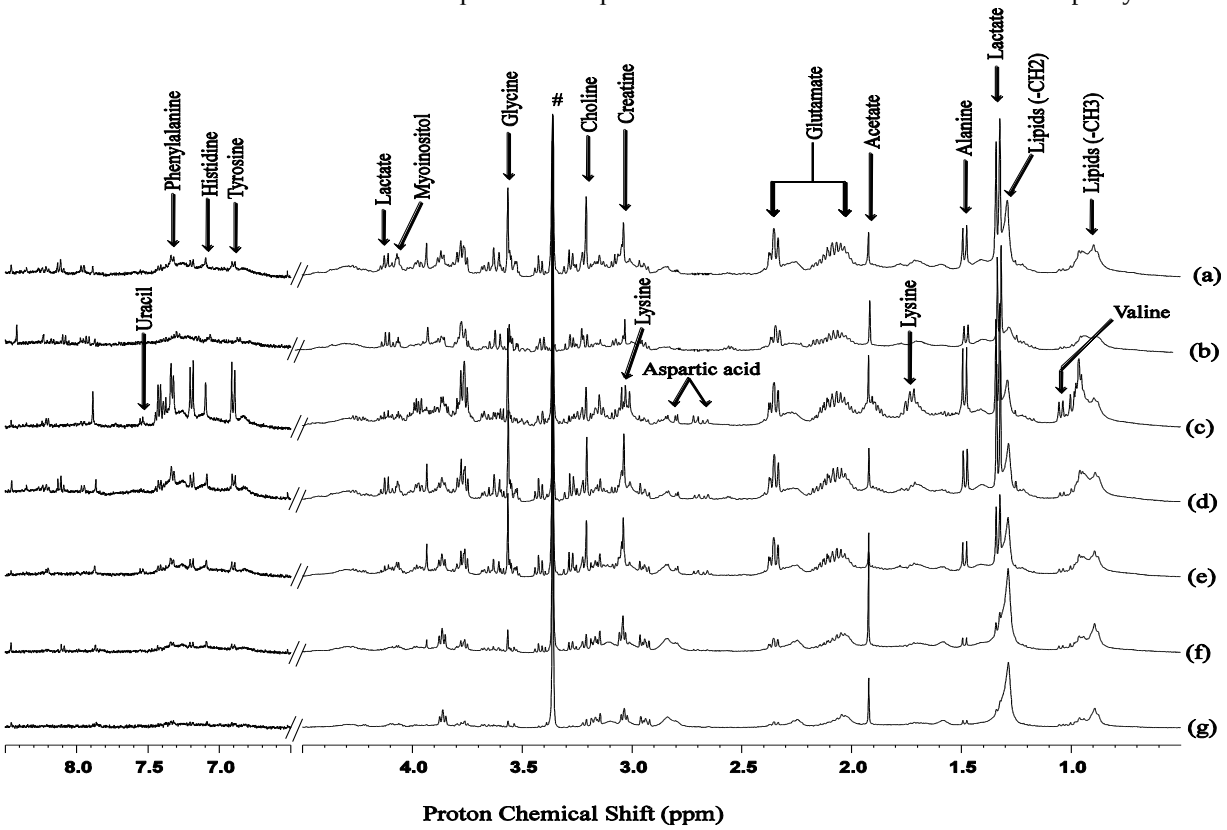


Figure 4. Portion of a 400MHz COSY spectrum of water-soluble intracellular metabolites of PK cells infected with JE virus for 24 h: Ala, alanine; Lac, lactate; Asp, aspartic acid; Val, valine; Leu, luecine; Ile, isoluecine; Lys, lysine; Glu, glutamate; Tyr, tyrosine; Pro, proline; Phala, Phenylalanine; Thr, Threonine; His, histidine; Ura, uracil

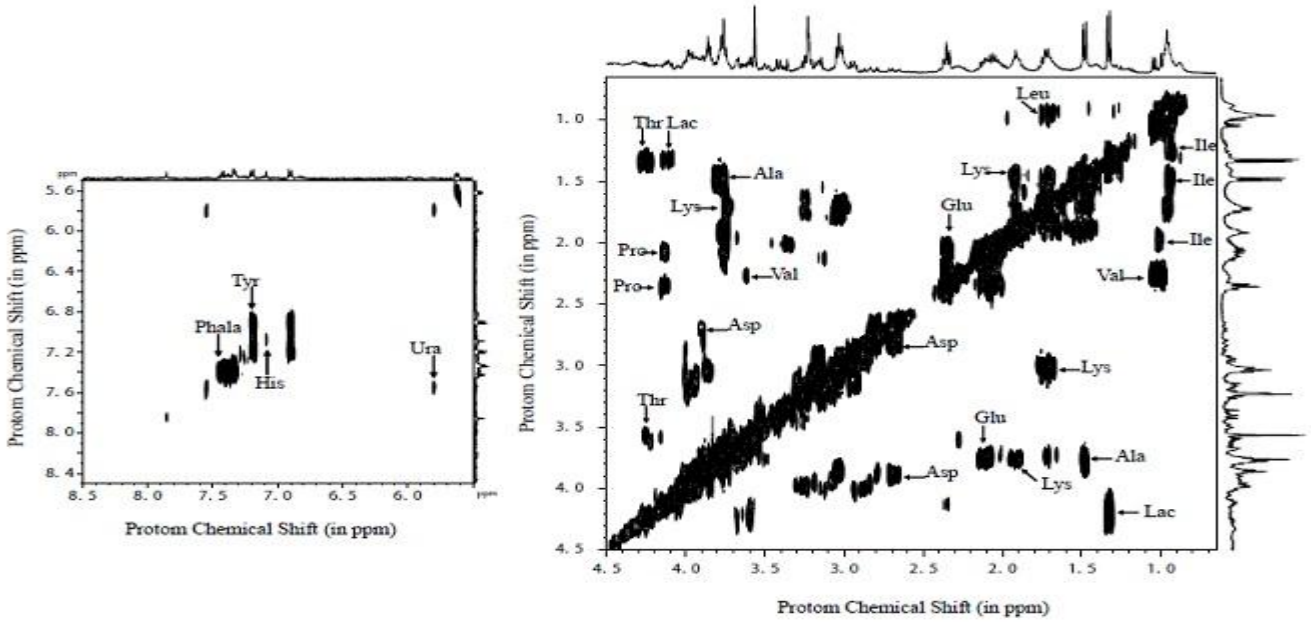
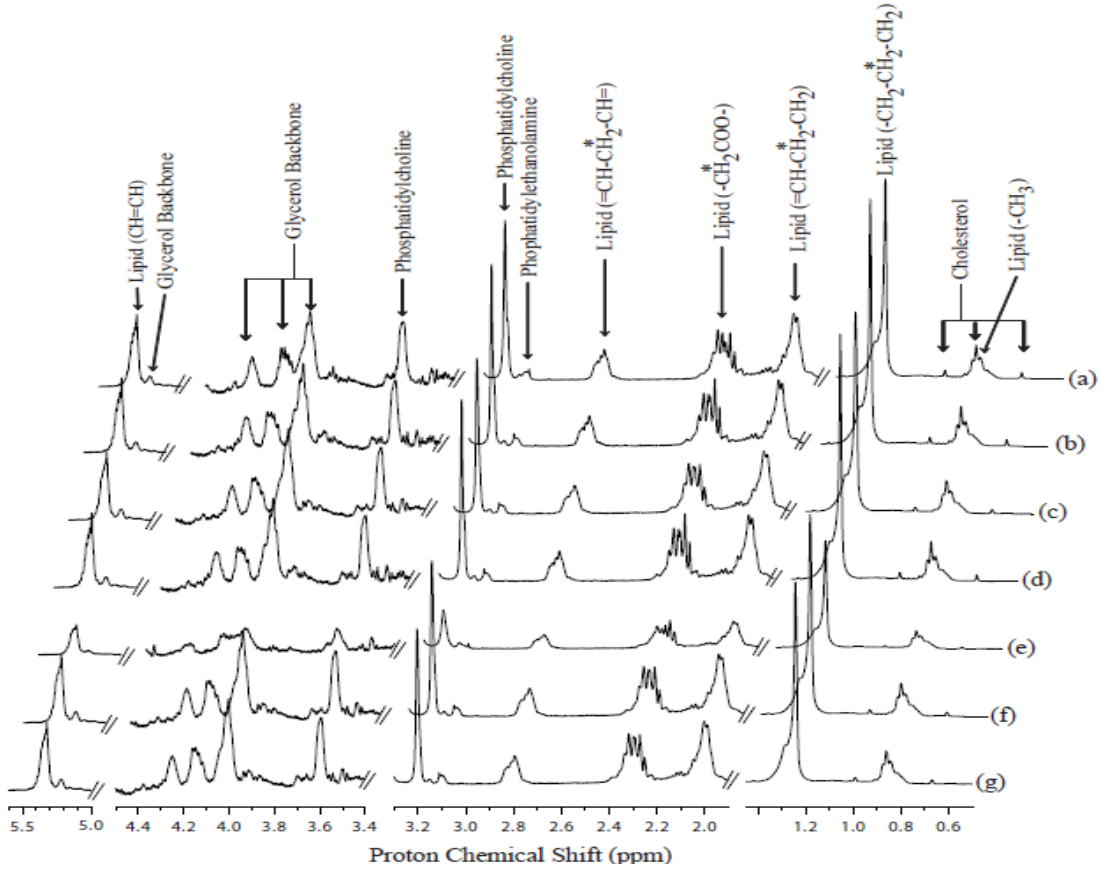


Figure 5. Parts of 400MHz 1H NMR spectra of cell lipid components extracted with an organic solvent (Chloroform: Methanol): (a) control cells (uninfected) and (b) –(g) cells infected with JE virus for (b) 12 h, (c) 24 h, (d) 36 h, (e) 48 h, (f) 60 h and (g) 72 h. All spectra were obtained under identical conditions and plotted with the same vertical scale for direct comparison



All uninfected and infected cells consumed the glucose from the MEM (2% FBS) and excreted the metabolic end products lactate, acetate and formate. The rate of glucose consumption is very high in infected cell as compared to uninfected cells. Similarly glutamine was also decreased more rapidly in infected cell than uninfected cell. All other amino acids present in the medium were parallel in both uninfected and infected cells. Figure 2 illustrates the spectra of extracellular media of uninfected and infected cells.

It may be noted that, in Figure 2, the absolute quantity of acetate is eminent at 36, 48, 60 and 72 hours post infection in comparison with 12 and 24 hours. On the other hand, the quantity of lactate is started to increase from 12 to 24 hours infection and after that decrease from 36 hours and almost disappeared at 72 hours post infection (see figure 2).

The increase of acetate at 36 and 48 hours may be attributed to the conversion of lactate into acetate. Before 36 hours infection, lactate and acetate, both were smoothly increases. It may because of normal cell metabolism. The spectra of uninfected whole cells showed signals arising from lipids, acetate, lactate, threonine, alanine, glutamate, creatinine, choline and phosphocholine (Fig. 3a). The cells after 24 hours infection showed Aspartic acid, Lysine and Uracil in addition to those observed in uninfected cells (Fig. 3c). A Very high concentration of valine histidine, tyrosine, and phenylalanine was observed as compared to uninfected cell as well as 12 hours infected cell. It showed that the infection started after 24 hrs of virus addition to healthy cells. With further increase in the infection time (from 24 to 72h), the quantities of all the metabolites were gradually decreased and eventually most of the metabolites except lipids and acetate disappeared (Figs. 3c to g). However, the NMR signals were better resolved and more metabolites could be identified, as shown in the COSY spectrum of extract from 24 hours infected cells (Figure 4). The metabolites identified from the two-dimensional spectra were alanine, valine, leucine, isoleucine, lysine, glutamate, tyrosine, lactate, acetate, aspartic acid, creatinine, choline, phosphocholine, phenylalanine, myoinositol, uracil and formate. As in the case of the spectra of infected cells (Fig. 3), all these metabolites gradually reduced to increase in viral infection time and finally disappeared after 72 hours infection. From the figure 3 it is clearly seen that amount of cellular lipids were increased with increase in JE virus infection time. Lactate and acetate were smoothly increases up to 36 hrs. It is only because of the glycolysis and TCA cycle. When the infection was high (i.e. after 36 h) lactate was rapidly converted into acetate (figure 3). With an increasing time of JE virus infection, all cellular components were used up, whereas cell lipid components were increases. Thus, these results clearly indicate that the JE virus does not utilize cell lipids during its viral particle synthesis, in spite of all other cellular components being used up when cellular damage takes place, as observed by optical microscopy (Fig. 1). Interestingly, the intensity of the total lipid signals in the spectra of the cells was similar to the uninfected and 12 hours infected cells, whereas they gradually increase with virus infection time (Fig. 3).

This well correlates to the fact that the total quantity of lipids is the same in all uninfected and gradually increase in infected cells, as determined from subsequent lipid extraction from whole cells (Fig. 5). From figure 5 and figure 3 it is clearly seen that cell lipid were not utilized by the Japanese encephalitis, an enveloped virus. These results reveal early detection of JE virus infection, and may have implications in virus cell interaction using NMR spectroscopy.

DISCUSSION

Cell apoptosis through viruses is associated with inhibition of cellular macromolecular synthesis and alters in cellular morphology. The morphological changes are not direct consequences of viral inhibition of host macromolecular synthesis [27]. 1H NMR spectra of cellular lipids extracted in organic solvent, chloroform, showed signals from lipids, cholesterol, phosphatidylcholine and phosphatidylethanolamine. The intensities of these lipid signals were increases in infected cells as compared to uninfected cells. It may be renowned that JE virus is an enveloped single-stranded RNA virus that possesses a glycoprotein containing lipid envelop in its outer structure which should utilize cell lipids during its viral particle synthesis other than the ^{1}H NMR results indicate an increase in the cell lipids with an increase in infection time. There would be one possible reason for increasing concentration of cell lipid with infection time that is the cell apoptosis which is normally increased in a programmed cell death. The spectra for lipid extracts for typical control cells and all time points infected cells are shown in major differences in the metabolites were observed in infected as well as uninfected cells.

A rounding of the cells when in contact with this virus and, when mixed in suspension, by the inability of cells to extend normally, manifests this phenomenon. Apparently, these effects can be related to changes in cellular membranes and are reflected in the alteration in the lipid metabolism noted. Cells utilized glucose through glycolysis and TCA metabolic pathways and excrete some of the metabolic end-products such as lactate, acetate and formate into the extracellular medium. Such conversion of lactate to acetate, which accumulated in the growth medium, is reported in several bacterial [28-32] and viral system [27].

The present proton NMR spectroscopic study, monitoring of the JE effect on cellular metabolic changes upon virus infection of cells, may be potentially useful in probing the molecular mechanism of virus-cell interactions. The fact that the effect of Japanese Encephalitis virus infection could be observed through metabolic changes at much earlier stages indicates possible application of cell-

virus interaction studies using NMR spectroscopy. Regardless of the actual mechanism of cell shrinkage, it could have significance for other late viral functions. For instance, cell shrinkage may promote virion assembly by bringing virion components closer together.

CONCLUSION

It has been shown that Japanese encephalitis virus infection in PK cells results in the progressive breakdown and consumption of all cellular components except lipids and acetate. The present proton NMR spectroscopic study, monitoring cellular metabolic changes upon virus infection of cells, may be potentially useful in probing the molecular mechanism of virus-cell interactions. The fact that the effect of virus infection could be observed through metabolic changes at much earlier stages indicates possible application of cell-virus interaction studies using NMR spectroscopy.

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