Primary *Tupaia Javanica* Hepatocyte Culture as an *In Vitro* Model for Human Hepatitis B Virus Infection

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ABSTRACT

Background: *Hepatitis B virus (HBV) infection is still one of the biggest health problems in the world, which could lead to chronic hepatitis, cirrhosis and hepatocellular carcinoma. Treatment for HBV infection has not yet achieved a functional cure. More studies are needed to investigate human HBV (HuHBV), but the scarcity of animal models for HuHBV infection became a barrier. Recently, many studies have shown that Tupaia are suitable for the study of HuHBV. The purpose of this study was to develop a primary Tupaia hepatocyte (PTH) culture from T. javanica, a species of Tupaia found in Indonesia, and to prove that HuHBV can replicate in the PTH.*

Method: *In vitro experimental study using PTH isolated from five wild adult T. javanica in Primate Research Center, IPB University. HuHBV was taken from humans with HBsAg and HBV-DNA (+). PTH cells then were infected with HuHBV after reaching 80% confluence. Observation on PTH cells was done every day for 20 days. Qualitative and quantitative HBsAg were measured using a CMIA while HBV-DNA and cccDNA were measured by RT-PCR.*

Results: *A cytopathic effect was seen on day post-infection (DPI)-16. HBsAg and HBV-DNA were detected from DPI-2 until DPI-18, with HBV-DNA level peaked on DPI-12. cccDNA concentration was fluctuating from DPI-2 until DPI-20 with the highest level on DPI-16.*

Conclusion: *Development of PTH culture from T. javanica showed good result with evidence that HuHBV could replicate in the PTH. This finding supports T. javanica as a potential animal model for HuHBV-related researches.*

Keywords: *hepatitis B virus, primary Tupaia hepatocyte, T. javanica*

ABSTRAK

Latar belakang: *Infeksi virus Hepatitis B (VHB), masih menjadi salah satu masalah kesehatan terbesar di dunia, yang dapat berkembang menjadi hepatitis kronik, sirosis, dan karsinoma hepatoseluler. Terapi VHB yang ada saat ini belum dapat mencapai functional cure. Penelitian terkait VHB manusia (human HBV, HuHBV), terhalang oleh sedikitnya hewan model yang dapat digunakan. Studi literatur terkini menunjukkan bahwa hewan Tupaia cocok dijadikan sebagai hewan model untuk HuHBV. Penelitian ini bertujuan untuk mengembangkan kultur primer hepatosit (KPH) T. javanica, spesies Tupaia yang ditemukan di Indonesia dan untuk membuktikan bahwa HuHBV dapat bereplikasi pada KPH T. javanica*

Metode: *Penelitian eksperimental in vitro menggunakan kultur primer hepatosit Tupaia dari lima ekor T. javanica dewasa di Pusat Studi Satwa Primata (PSSP) Institut Pertanian Bogor. HuHBV diambil dari manusia dengan HBsAg dan DNA VHB (+), kemudian diinfeksikan ke ke KPH setelah KPH mencapai konfluens 80%. Observasi dilakukan setiap hari selama 20 hari. HBsAg kualitatif dan kuantitatif diperiksa menggunakan metode CMIA sementara DNA VHB dan cccDNA diukur dengan RT-PCR.*

Hasil**:** *Efek sitopatik terlihat pada hari pascainfeksi (HPI-16). HBsAg dan DNA VHB terdeteksi pada HPI-2 sampai HPI-18, dengan kadar DNA VHB tertinggi pada HPI-12. Konsentrasi cccDNA berfluktuasi dari HPI-2 sampai HPI-20 dengan kadar tertinggi pada HPI-16.*

Simpulan: *Pengembangan KPH dari T. javanica pada HuHBV menunjukkan hasil yang baik dengan dibuktikannya bahwa HuHBV dapat bereplikasi di KPH dari T. javanica. Hal ini menunjukkan bahwa T. javanica dapat menjadi hewan model yang potensial untuk penelitian HuHBV.*

Kata kunci: *virus hepatitis B, kultur primer hepatosit Tupaia, T. javanica*

INTRODUCTION

Hepatitis B is still a major health problem in the world. It is estimated that there are 240 million people with chronic infection of hepatitis B virus (HBV) worldwide.[1] In Indonesia, The Ministry of Health's 2013 Basic Health Research survey estimated that the proportion of hepatitis was 1.2%, with HBV as the most prevalent cause (21.8%).[2] People chronically infected with HBV can progress to chronic hepatitis, liver cirrhosis (LC), hepatocellular carcinoma (HCC) and death. HBV infection causes approximately 786,000 deaths each year from acute liver failure, LC and HCC.[3,4] Treatment for chronic HBV currently cannot achieve a curative level, as all the available drugs only control virus replication and cannot eliminate HBV from the human body.[5]

It is imperative to conduct more researches regarding human HBV (HuHBV) in order to develop a functional cure. However, there are some obstacles, such as restricted animal models, non-permissive permanent cell lines and limited human hepatocyte resources.[6,7] One animal model suitable for HuHBV is chimpanzees, but there are some difficulties in conducting research using them, including the fact that they are an endangered species, long-lived (more than 50 years) and require high costs of maintenance.[7,8] Rodents are most commonly used as animal models in different kinds of studies, but it is sometimes difficult to apply the results of such studies to humans because of methodological flaws and the critical disparity between the two species, and therefore many research centers are trying to develop models using other animals.[9] Recently, many studies have shown that *Tupaia* are suitable for the study of HuHBV. *Tupaia* are more closely related to nonhuman primates than rodents are based on the overall genomic profile.[10] Prior studies showed that primary hepatocytes from *Tupaia belangeri* can be infected with HuHBV and can transiently be infected in vivo.[11] *T. belangeri* can also be chronically infected with HuHBV and display hepatic histopathological changes similar to humans with chronic HBV.[12,13]

Tupaia belangeri are found in Bangladesh, Bhutan, Cambodia, China, Laos, Malaysia, Myanmar, Thailand and Vietnam, while *T. javanica*, *T. chrysogaster*, *T. glis*, *T. minor*, *T. salatana*, *T. dorsalis*, *T. longipes*, *T. tana*, *T. picta*, *T. montana*, *T. splendidula*, *T. gracilis*, *T. hypochrysa* and *T. ferruginea* originate in Indonesia. Most *Tupaia* in Indonesia are found on Borneo Island – only *T. javanica* and *T. hypochrysa* are found on Java Island.[14,15] A previous study has shown that primary *Tupaia* hepatocytes (PTH) from *T. javanica* can be infected with orangutans HBV (ouHBV) and gibbons HBV (giHBV). This study also showed that sodium taurocholate co-transporting polypeptide (NTCP) was expressed as a receptor for HBV on the surface of infected PTH.[16] This result opens up an opportunity to explore the suitability of *T. javanica* for *in vitro* and *in vivo* animal models for HuHBV, as has been previously shown in *T. belangeri*. The purpose of this study was to develop a primary *T. javanica* hepatocyte culture and provide proof that HuHBV can replicate in these cells by measuring laboratory parameters of HuHBV replication and observing the morphological changes of PTH infected by HuHBV.

METHOD

This is an in vitro experimental study using primary *Tupaia* hepatocyte (PTH). In this study, we analysed the level of laboratory parameters of HuHBV replication, including qualitative HBsAg, quantitative

HBsAg, HBV-DNA and cccDNA. We also observed the morphological changes of PTH infected by HuHBV. The animal procedures and observation had been done in Primate Research Centre, IPB University (PRC-IPB) laboratory.

All procedures in this study were reviewed and approved by PRC-IPB Institutional Animal Care and Use Committee (IACUC, number PRC-IPB-15-B004) for the animal procedures.

Five wild adult *T. javanica* were maintained in the quarantine facility of the PRC-IPB. The procedures were performed by experienced veterinarians at the PRC-IPB.[16]

Tupaia were anaesthetised with ketamine (5 mg/100 g body weight, BW) and xylazine (1 mg/100 g BW). Blood was collected by exsanguination and the livers were collected by laparotomy. The collected liver was pre-perfused with pre-perfusion media (500 mL phosphate buffer saline, PBS, 2.5 ml ethylene glycol tetraacetic acid, 1M EGTA (5mM) and penicillin-streptomycin 2%), then perfused with perfusion media (50 mL Dulbecco's Modified Eagle Medium, DMEM, 500 µL 100× $CaCl_2$, 1% collagenase) until the liver looked pale. Pre-perfusion media (10 mL) was injected into the liver using a 10 mL syringe five times (total 50 mL pre-perfusion media) then 10 mL of perfusion media was injected into the liver. Next, the liver was sliced and minced, incubated at 37°C for 15 minutes and centrifuged at 2000 rpm for 15 minutes. The supernatant was removed and 10 mL pre-perfusion media was added. The cells were counted and 10^5 cells per well were added to a 12 well tissue culture plate and to a T25 flask. Hepatocyte culture medium (HBM/CC-3199 and HCM/CC-4182 from Lonza with 20% foetal bovine serum/FBS) was added to the well and flask. The plate and flask were incubated at 37°C with 5% CO_2.The HuHBV used for infection was obtained from a human with chronic HBV infection, serologically positive for HBV surface antigen (HBsAg) and HBV e-antigen (HBeAg), and with Hepatitis B virus deoxyribonucleic acid (HBV-DNA) viral load of > 5.75×10^8 IU/mL. HuHBV was resuspended in HBM media, then the HuHBV suspension was added to the hepatocyte culture on day 14 when the cells reached 80% confluence. HuHBV suspension was added to each well at 10^6 IU/mL and 5×10^6 IU/mL HuHBV suspension was added to each flask and then the incubation was conducted for 16–20 hours at 37°C and 5% CO_2. After the incubation period ended, the cells were washed using PBS and a culture medium was added. The second incubation was done for 24 hours at 37°C and 5% CO_2.

Supernatant from the plate, supernatant from the flask and cells from the plate were collected on day 2, 4, 6, 8, 10, 12, 14, 16 and 18 post-infection (DPI). Qualitative HBsAg, quantitative HBsAg, HBV-DNA and cccDNA were assessed to detect HBV replication. Qualitative and quantitative HBsAg were analysed using a chemiluminescent microparticle immunoassay (CMIA). The concentration of HBV-DNA and cccDNA was measured by real-time polymerase chain reaction (PCR, m2000 Abbott RealTime HBV Assay). Data collected from the measurements were processed using Microsoft Excel.

RESULTS

Daily observations of the wells showed that *Tupaia* hepatocytes have a polygonal shape (Figure 1. A and B), are epithelial-like and reach 80% confluence on day 14 after isolation. After the cells reached 80% confluence, they were infected with HuHBV. After infection, there was no change in PTH shape by DPI-10 (Figure 1. C and D), but starting from DPI-16 a cytopathic effect (CPE) was observed: the size of the cells was expanded, syncytia were formed up to 5 nuclei, and displayed nuclear inclusions representing viral replication compartments (Figure 1. E and F). Figure 1 also showed that the number of hepatocyte colonies decreased over time. Figure 2 showed a comparison between the normal morphology of PTH on DPI-0 and cellular changes that occurred due to the cytopathic effect of HuHBV infection on DPI-20.

Samples were collected from both flask and plate on DPI-2, -4, -6, -8, -10, -12, -14, -16 and -18. Qualitative HBsAg, quantitative HBsAg, HBV-DNA, and cccDNA were measured from the samples. On DPI-2, qualitative HBsAg, HBV-DNA, and cccDNA could be detected in samples from both flask and plate and remained positive until DPI-18, but quantitative HBsAg level was less than 0.5 IU/mL from DPI-2 until DPI-18.

HBV-DNA could be detected in the supernatant and the cells as early as DPI-2. The HBV-DNA underwent a spike on DPI-12 then continuously declined until DPI-18. As seen in Figure 3, the concentration of HBV-DNA was highest in the supernatant (7.08×10^3 IU/mL, DPI-12) and lowest in the cells (< 20 IU/mL, DPI-12. Meanwhile, cccDNA could also be detected in the cells as early as at DPI-2 and latest at DPI-20 with fluctuations of concentration over the observation period. The highest cccDNA concentration was 4451 copies/mL on DPI-16 (Figure 4).

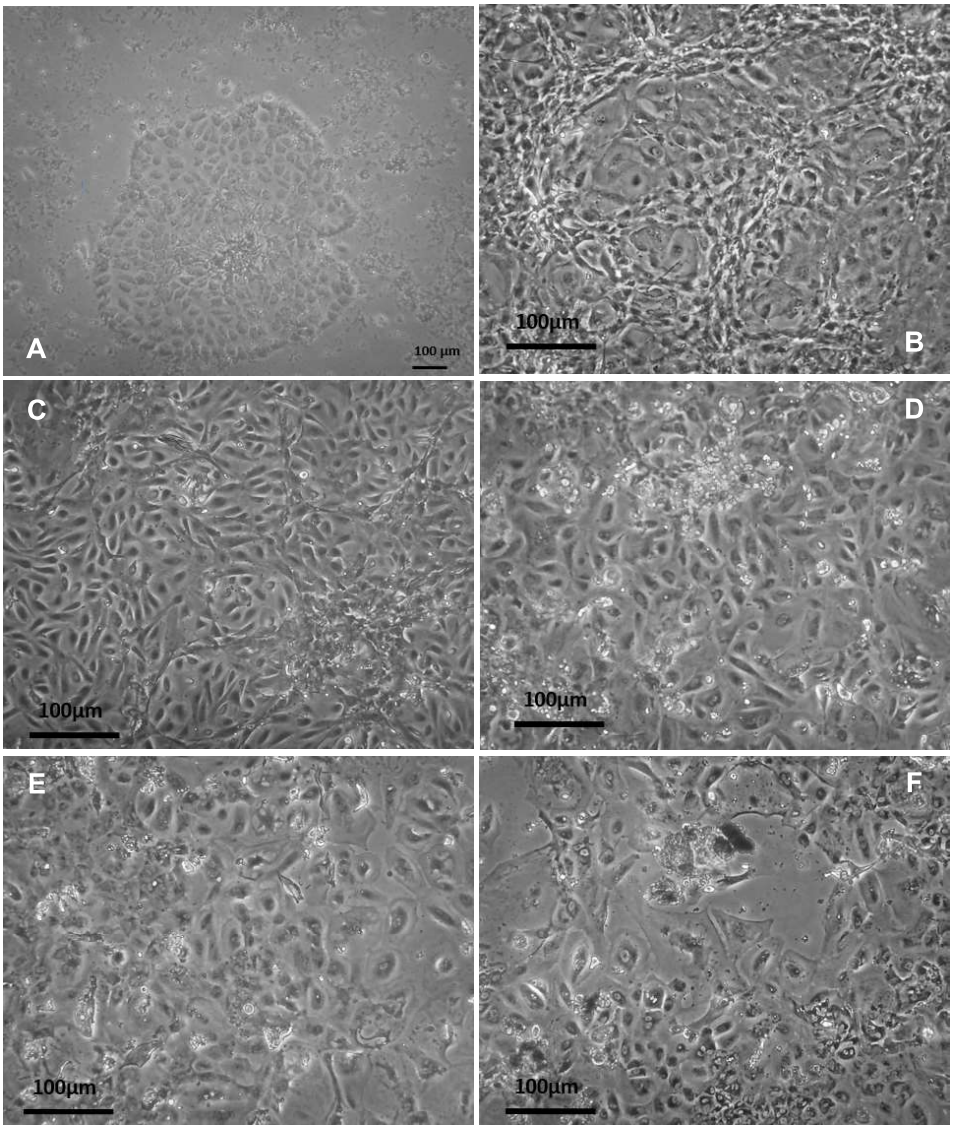


Figure 1. Primary Tupaia hepatocyte, hepatocyte colonies decreased over time. A. Culture on Day 4. B. Culture on Day 14. C. DPI-4. D. DPI-10. E. DPI-16. F. DPI-20. DPI: Days post-infection.

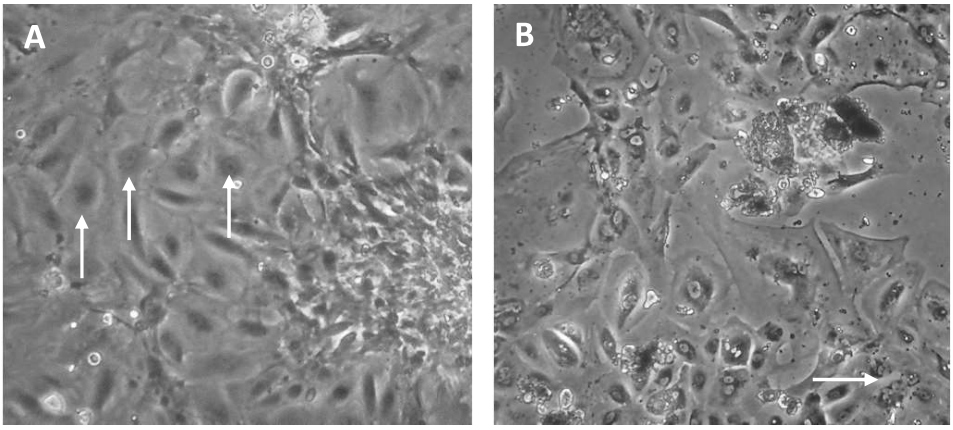
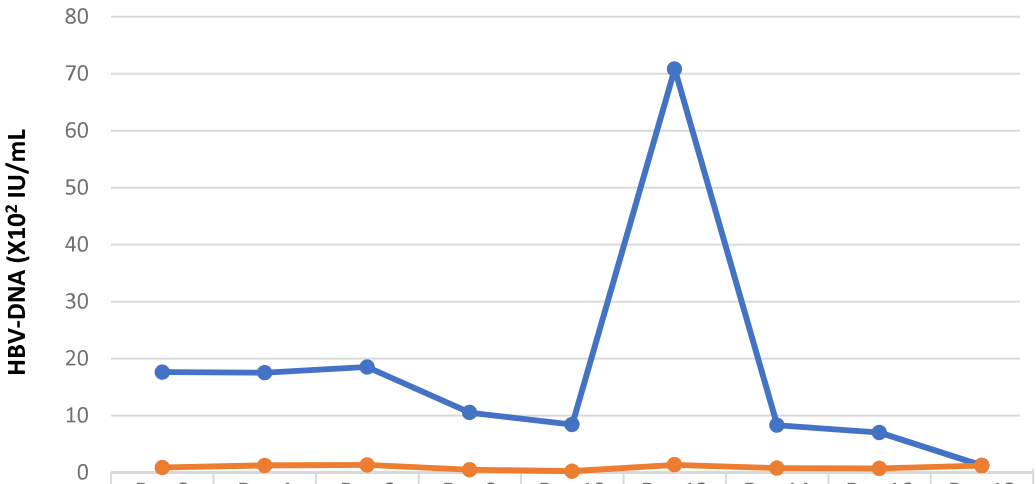
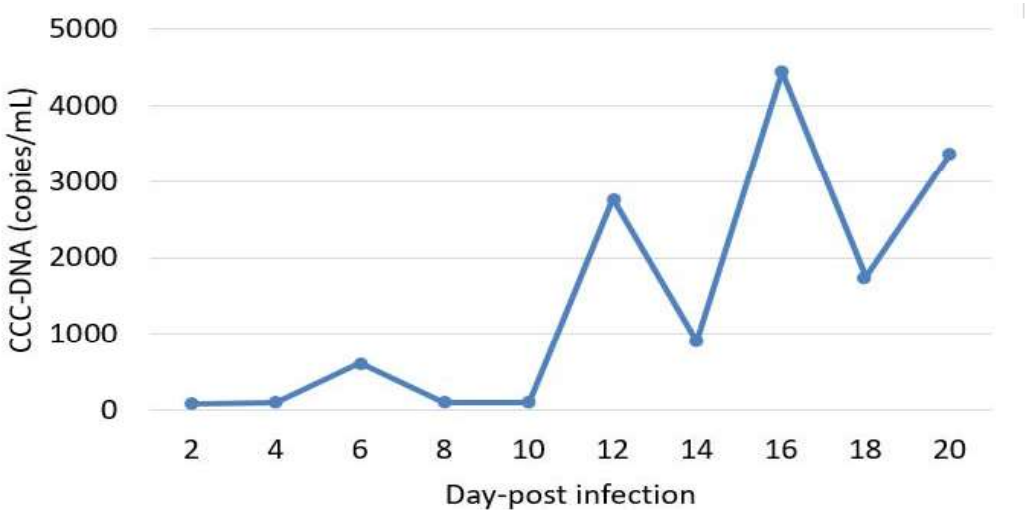


Figure 2. A. DPI-0: normal morphology of primary Tupaia hepatocytes was epithelial-like (arrow) B. DPI-20: a cytopathic effect in primary Tupaia hepatocytes showed enlarged morphology and formation of syncytia (arrow)



	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18
Supernatant	17.6	17.5	18.5	10.5	8.4	70.8	8.3	7	1.2
Cell	0.85	1.22	1.3	0.46	0.2	1.34	0.75	0.7	1.2

Figure 3. Time course of HBV-DNA concentration in the supernatant and cells after infection of PTH with HuHBV.



	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18	Day 2…
CCC-DNA	90	109	617	108	110	2778	910	4451	1746	3373

Figure 4. Level of cccDNA post-infection of HuHBV

DISCUSSION

This study showed that PTH from *T. javanica* can be reproducibly infected with HuHBV, resulting in the synthesis of HBV-DNA from hepatocytes and secretion of HBsAg and HBV-DNA to the supernatant. Furthermore, cccDNA was also detected in this study. The detection of cccDNA indicates that HuHBV-infected PTH from *T. javanica* supports cccDNA formation and intracellular amplification cycle.[17]

Similar to the morphology in a previous study, the PTH in this study had a polygonal shape.[16,18] CPE is a cellular structural change induced by a viral infection and it can form rounding, detachment, clumping, ballooning, formation of syncytium and formation of inclusion body. The cellular structural change is detectable by light microscopy.[19] In this study, CPE was detected on DPI-16 and DPI-20 (Figure 1. E and F). Prior studies showed contradicting results. One study found no morphological differences between infected PTH and uninfected PTH from *T. javanica*.[16] Meanwhile, result from another study that used adenovirus as a vector to carry the HBV genome to

infect PTH showed dose-dependent CPE; higher dose made CPE more macroscopically visible within the first few DPI, eventually leading to cell death.[17]

In the present study, HBV-DNA was detected in both cell and supernatant, meaning that the infected cell is capable of secreting the virus outside the cell. The HBV-DNA in the supernatant decreased gradually but was still detected until the end of observation on day 18 (Figure 3). The decrease was probably caused by limited cell proliferation capability and limited cell life span leading to cell death. Despite the gradual decline of HBV-DNA concentration in the supernatant, the HBV-DNA concentration in the cells was stable throughout the observation period. This could probably be due to reduced hepatocyte secretion capability. The other phenomenon observed was a sudden peak of HBV-DNA in the supernatant on day 12.

In the REVEAL Study, around 60% of patients with HBsAg (+) and HBeAg (+) had HBV-DNA higher than 2×10^7 IU/mL and around 80% of patients with HBsAg (+) and HBeAg (−) had HBV-DNA less than 2×10^4 IU/mL.[20] Having our results compared, we found significantly lower concentration in PTH, only around 10^2 IU/mL, compared to 10^4-10^7 IU/mL in human hepatocytes. This could probably be caused by virions we used which came from human serum were not being purified. Human serum can interfere with HBV binding to hepatocytes, hence reducing infection multiplicity.[21]

We measured HBsAg and HBV-DNA as markers of HBV replication in PTH. HBsAg is a protein located on the surface of HBV which is secreted as an infectious particle or a subviral particle after HBV infects hepatocytes. HBV-DNA contains HBV genome surrounded by a nucleocapsid and a lipid membrane containing HBsAg. HBV-DNA is secreted together with HBsAg as an infectious particle after HBV infects hepatocytes.[5] In the previous study using HuHBV to infect PTH from *T. belangeri*, HBsAg and viral DNA were detected until DPI-12.[11] In our study, both qualitative HBsAg and HBV-DNA were detected as early as DPI-2 and persisted until DPI-18, meaning that PTH isolated from the liver of *T. javanica* can be infected with HuHBV and HuHBV can replicate in PTH isolated from the liver of *T. javanica*. The HBV-DNA level in the supernatant and cells fluctuated until DPI-18, probably meaning that HBV-DNA can be detected longer in the supernatant and cells. Although the HBV-DNA was detected until DPI-18, the level of HBV-DNA was below 10^3 IU/mL, and the quantitative HBsAg level was below 0.5 IU/mL, meaning that HuHBV replication in PTH remained low.

In this study we also measured cccDNA and we can detect cccDNA during the observation period. cccDNA is an episomal viral genome, stable minichromosome, act as a template for all viral transcripts, located in nuclei of infected hepatocyte and responsible for chronicity of HBV infection. The persistence of cccDNA during the observation period in our study demonstrates the stability of cccDNA inside the nuclei of infected PTH. In the end of the observation period, cccDNA tended to increase, this probably due to intrahepatocyte amplification pathway of cccDNA pool. As we know, this is the first study that demonstrates cccDNA detection inside PTH from *T. javanica*. This result is very promising because it will open opportunities to make *T. javanica* a model for HuHBV chronic infection. The study needs development in many aspects. Therefore, in the future we plan to observe the maximum duration of PTH from *T. javanica* after infection with HuHBV, carry out a binding study involving a receptor for HuHBV in PTH, enhance HuHBV replication in PTH and detect viral RNA.

CONCLUSION

In this study, we were able to develop PTH culture from *T. javanica* and it showed that HuHBV could replicate in the PTH culture. This finding supports *T. javanica* as a possible animal model for HuHBV-related researches.

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