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INVITRO STUDY FOR THE OPTIMAL PRODUCTION OF HEPATITIS B VIRUS SUBVIRAL PARTICLES FOR MEDICAL PURPOSES

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ABSTRACT

During replication of hepatitis B virus (HBV), HBV infected cells secrete infectious viral particles and large quantities of subviral particles which are assembled from surface protein without a viral genome. For the current study, the cell line of HepG2.2.15 genetically integrated with the HBV surface region was cultured as an expression system for in vitro production of hepatitis B virus subviral particles (HBVsvp) at different time for commercial purposes. Recorded Results showed that culturing and incubation of HepG2.2.15 cells over 7 days incubation period resulted in continuous secretion of HBVsvp in the supernatant. ELISA's quantitative measurement of hepatitis B surface antigen reported that the highest titer of virus was day seven. Quantitative detection of HBsAg by ELISA indicated that the highest virus titer was at day seven. According to their size, subviral spherical particles recognized by a diameter of 22 nm. In conclusion, *in vitro* cultivation and incubation of HepG2.2.15 for secretion of HBsAg may be used in studying HBV replication thus, opening a new era for various studies from basic virology, in vitro diagnosis, to drug development against HBV genotypes.

Keywords: Hepatitis B virus subviral particles; HepG2.2.15 cell line; Polyethylene glycol; Virion-based vaccine candidate

1. INTRODUCTION

Hepatitis B virus (HBV) is a member of the family *Hepadnaviridae* and is a small, enveloped DNA virus whose tropism is limited to the hepatocyte causing hepatocellular necrosis and inflammation [1]. HBV infection can be either acute or chronic, and the associated illness ranges in severity from asymptomatic to symptomatic, progressive disease [2]. Data from the World Health Organization reveals that an estimated 257 million people (3.5%) globally are living with chronic HBV infection, 1.34 million deaths (a number comparable to deaths caused by tuberculosis bacteria and more than infection those caused by HIV) due to chronic liver disease (720,000 deaths due to cirrhosis) and liver cancer (470,000 deaths due to hepatocellular carcinoma) [3].

Hepatitis B virion has three various structures, Dane particle (42 nm), spherical (20

nm) and filamentous (22 nm) particles [4]. All three particles sharing a common HBsAg as an envelope [5]. The spherical and filamentous particles (termed by subviral particles) are composed of HBsAg and host-derived lipids without the HBV genome; thus, they are non-infectious while the Dane particle is a complete infectious sphere HBV virion with 42 nm [5]. HBsAg exists in three forms: small (S), medium (M) and large (L). The S domain is common to all three proteins, while the M protein has an additional N terminal domain (PreS2) and L, two additional domains (PreS1 and PreS2). L-HBsAg is a minor component of SVP, but a main component of VP [6].

During the acute phase of the viral infection, non-infectious subviral particles can be present in the serum of infected individuals at up to 100,000 folds over the complete virion (at 1014/mL) [7]. Whereas the main function of complete virions is to deliver the viral genome

to a newly infected cell, The role of subviral particles in infection with the hepatitis B virus was largely ignored but it has been proposed that HBsAg in SVP serves as decoy to reduce the antiviral effects of neutralizing antibodies increasing the ability of the infectious particles to reach susceptible cells and thus lead to the immune tolerance needed to sustain a long-term chronic infection [8]. It has also been shown that artificial SVP produced by transfected Huh7 cells had little impact on viral entry subsequently, did not inhibit the HBV to infect primary human hepatocytes [9]. A study of duck hepatitis B virus SVPs showed that the SVPs acting to either enhance or inhibit infection based on the ratio of SVP-to-infectious-particles [10].

Historically, the abundance of subviral particles has been very useful as a convenient marker for monitoring HBV infection, screening of blood donors, and measuring antiviral treatment response [11]. Similarly, the Au antigen particles (contains HbsAg particles), collected directly from infected patients' pooled plasma, formed the basis of the first generation of HBV vaccine, which proved to be highly effective [12]. Due to the potential safety concerns surrounding human plasma use, this highly successful vaccine was replaced by the current recombinant (2nd generation) HBV vaccine containing only one of the HBV envelope proteins, S without capsid protein. While in most cases it has been proven safe and efficient, the recombinant S vaccine does not induce an adequate response in about 5–10 % of normal individuals [13]. Also, since the recombinant vaccine mainly elicits an antibody response targets a single specific epitope, HBV may develop mutations in this epitope to escape the antibodies induced by the vaccine [14].

To overcome the limitations of the current vaccine escape issue, it is urgently needed to develop a possible 3rd generation HBV vaccine which can be achieved by producing these subunits in a very high quantities including different forms of SVPs instead of one type and at the same time more safe than SVPs obtained from the sera of infected individuals which

contain infectious virus as well as SVPs. There is also a need for highly concentrated amounts of HBsAg to develop a sensitive and effective immunoassay to detect antibodies to HBs either they formed against native HBV infection or from vaccination [15].

So, according to the vital role of HBsAg, the main purpose of this study was in vitro propagation of HBsAg in a highly concentrated amount by using HepG2.2.15 cell lines transfected with HBV Surface region as an expression system for antigenic subviral particles. For this purpose, we optimized the conditions for highest production rate within a specific time frame then investigate morphological and serological characters of secreted HBsAg that may prove to be beneficial as diagnostic markers and possibly, as an SVPs-based vaccine candidate that finally contributed to improved control of chronic HBV infections.

2. MATERIALS AND METHODS:

2.1. HepG2.2.15 cell line maintenance

Human hepatocyte carcinoma HepG2.2.15 cell line incorporated with HBV S domain obtained from a culture collection, (ECACC). Maintenance of the cells was carried out initially by culturing in 10 ml of complete Williams medium (Lonza, Swiss), supplemented with 10 % (v/v) fetal bovine serum (FBS) (Serolab, Brazil), 100 U/ml Penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (Lonza, Swiss), 250 µl insulin (5µg/ml) (Sigma, China), and 250 µl hydrocortisone (Sigma, China). Then, HepG 2.2.15 were incubated overnight. To assessed 80 % confluency degree of cultivation, cell monolayer washed twice, cells were harvested by trypsinization, centrifuged at 1500 rpm for 5minutes, the supernatant discarded, the cells pellet resuspended in 1ml fresh growth medium for counting. The total number of cells per milliliter and percent of viability was determined using a hemocytometer, and trypan blue vital stain. The number of viable (seen as bright cells) were counted [16].

2.2. Growth curve

For evaluating the growth characteristics (cell viability and activity) of a HepG 2.2.15 cell line, a growth curve was generated which was constructed from samples taken at different time intervals (from day one to day eight) throughout the growth cycle depending on enzyme-based assay using MTT colorimetric method that measure mitochondrial dehydrogenase activities in the living cells [17]. It was described as the following:

HepG2.2.15 initially cultured in 75 cm² flasks at a seeding density of 3×10^5 viable cells/ml. after assessing the >80 % of confluency for HepG2.2.15, density and viability of the cells using a hemocytometer was (19×10^6). After counting, cells were distributed at serial two-fold dilutions of cells (1.25×10^3 to 640×10^3) in 200 μ l culture medium per well in duplicate for 7 days, cells were incubated for 24 hours at 37 °C. A 200 μ L of MTT Reagent added to each well of each day separately. The plate incubated in CO₂ incubator for 2 to 4 hours at 37 °C. The cells examined for the appearance of punctate, intracellular precipitate using an inverted microscope. When the purple precipitate was clearly visible under the microscope, 200 μ l of detergent reagent (DMSO) added to all wells, left in the dark for 2 to 4 hours at room temperature for dissolving formazan salt. Eventually, the absorbance was measured in each well by ELISA at a wavelength between 570 and 590 nm with a reference wavelength of 650 nm.

2.3. Optimizing the Production of SVP

To address the optimum conditions for determination of the highest productivity of Hepatitis B Subviral Particles (HBsvp) over 7 days cells culturing. The HepG2.2.15 cell suspension produced from cell culture was distributed into three groups. Each group contains seven flasks (25 cm²), each of which represents 7 days. A 0.5×10^6 cell/flask were added as initial seeding density of cell suspension for all groups then incubated for up to 7 days. Subsequently, the production of

HBsvp was determined for different days by ELISA including the following days 1, 2, 3, 4, 5, 6, 7 for optimizing the optimal viral production.

3. Characterization of Subviral Particles of Hepatitis B

3.1. HBsvp Concentration

Cell culture supernatant (containing HBsvp) at separate days (from 1 to 7) was harvested for viral concentration by polyethylene glycol (PEG) protocol [18]. The concentration starting with incubation for all samples with 40% Polyethylene glycol PEG (Nice, India) at 4 °C for overnight. Then, the medium was centrifuged for two hours at 4 °C at 4000 rpm on the second day, pellet have been resuspended in a mix of (75 %) 1x PBS and FBS (25 %), subsequently incubated at 4 °C for second night. The isolates were collected on the third day, and centrifuged at 4000 rpm at 4 °C for 20 minutes. The isolates (which contain the concentrated HBsvp) were collected after centrifugation and stored at -20 °C for quantifying by ELISA.

3.2. Serological characterization of HBsvp

HBsvp containing supernatant (before and after concentration with PEG) were assayed for qualitative and quantitative detection of HBsAg by ELISA (Cam Tech Medical). Following the manufacturer's instructions [19], The loading plate stage began with the addition of 50 μ l of (Negative Control, Positive Control, 21 specimens (represents 3 groups) and Anti-HBsAg conjugate into each well used. The plate incubated at 37 °C for 30 min, washed 6 times with the working washing solution, 50 μ l of Substrate solution distributed on each well and incubated for 10 minutes at 37 °C. Finally, the reaction was stopped by adding 50 μ l of Blocking Reagent to each well and the color developed on the microplate reader readied at 450 nm.

3.3. Morphological characterization

For estimating the size and shape of the different viral structures (Dane, spherical and filamentous structures), chronic HBV patient

positive samples and HBsVp containing supernatant was examined through transmission electron microscopy TEM (JEOL 1010, nanotechnology center, Egypt) at 200 KV volt. The image is created after staining viruses with negative stain onto the carbon-coated copper grid, dried and loaded onto a specimen holder for analysis by TEM [20].

4. RESULTS

4.1. Examination of HepG2.2.15

The HepG2.2.15 cell were morphologically observed as elongated cylindrical HepG2.2.15 with an increase in proliferation rate starting from in day 1 till day 7 (**photograph 1**).

4.2. Characterization of Subviral Particles of Hepatitis B

Concentration of the SVPs by ELISA



Photograph 1 shows the morphology HepG2.2.15 cell line under inverted phase contrast light microscope with 400x. day 1 (A); day 3 (B); day 5 (C); day 7(D).

confirmed that the highest secretion of the virus was observed on day 7 after culturing and incubation of each different three groups. The average titre of virus was 0.943, 1.283, 1.426 (OD) for the first, second, and third groups (**Figure 2**).

4.3.Characterization of Subviral Particles of Hepatitis B

Concentration of the SVPs by ELISA confirmed that the highest secretion of the virus was observed on day 7 after culturing and incubation of each different three groups. The average titre of virus was 0.943, 1.283, 1.426 (OD) for the first, second, and third groups (**Figure 2**).

HBsVp concentration

After propagation of HBsVp containing

supernatant, poly ETG protocol was used for concentration of HBsvp in three groups among 7 days, and quantitative detection of HBsAg were assayed by ELISA, the resulted optical density for samples showed that the highest viral concentration was day 7 concentrated sample (2.327 O.D.) after cultivation of HepG2.2.15 for seven days compared to concentrated SVP in day one

4.1. Growth curve for HepG 2.2.15 Adherent Cell Lines

Absorbance measurements for HbsAg with different cell number cultured for seven days indicated that the highest concentration was at

day seven (Figure 4).

4.2. Electron microscopy: HBsvp morphological characterization

Observation by transmission electron microscopy (JEOL 1010, nanotech center, Egypt) at 200 KV evaluate both the shape and size of chronic HBV patient control sample and SVP secreted from HepG2.2.15 cell line. Control sample appeared as two form of spherical particles (Dane particle 42 nm) and spherical (20 nm), while the SVP produced from HepG2.2.15 observed as spherical (20 nm) particles confirming the absence of complete infectious Dane particles (Figure 5)

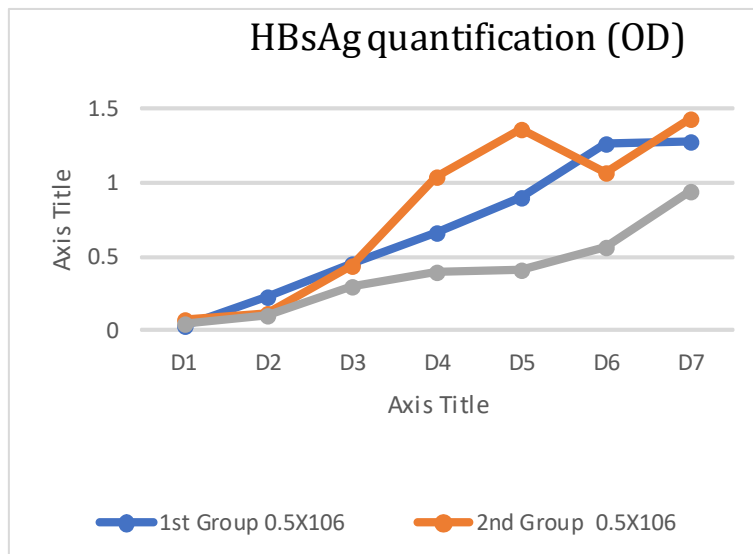


Figure2. shows ELISA measurement of HBsAg titers in the HepG2.2.15 supernatant for 3 groups at different days (D1:D7). The figure indicates the Highest Secretion of Virus (day seven) and the lowest Secretion (day one).



Figure 3. Shows ELISA measurement for HBsAg before and after concentration for different supernatant samples

5. DISCUSSION

100,000-fold over the complete virions

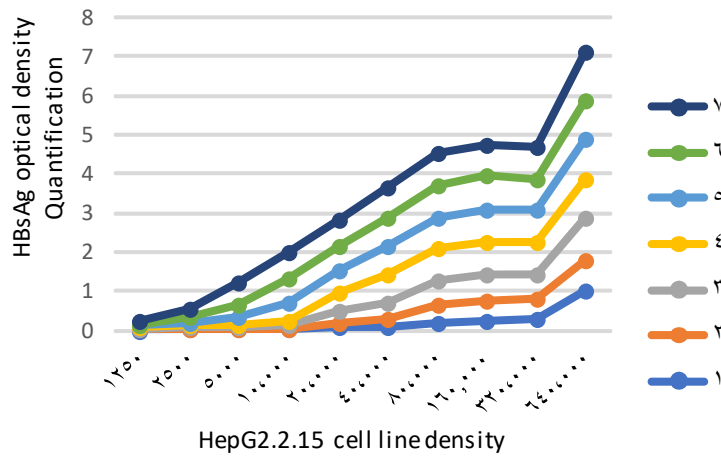


Figure 4: Showed ELISA measurement for HbsAg with different cell density for seven days.

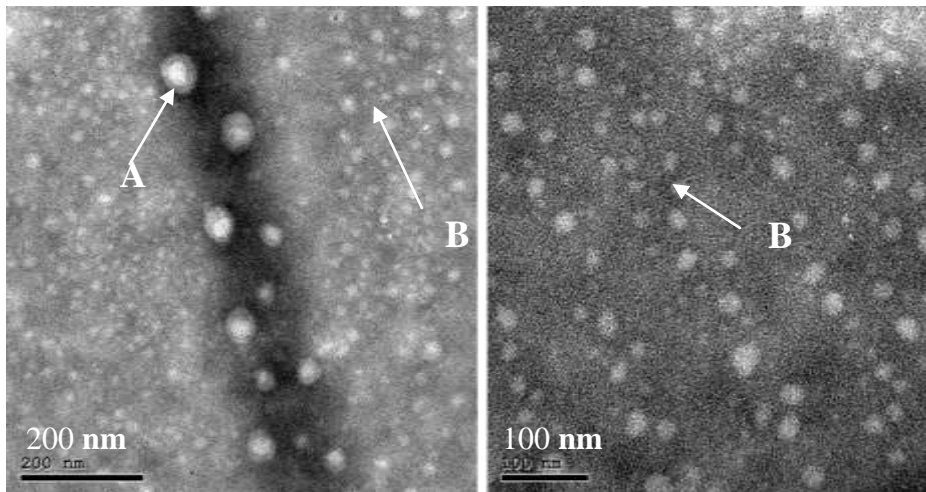


Figure 5. shows transmission electron photographs represent the size and morphology of HBV And HBVsvp derived from cell line HepG2.2.15. I) The positive sample for HBV serum includes (A: 42nm Dane particle), (B: 22nm subviral spherical particle. II) The HepG2.2.15 cell line supernatant includes (B: 22nm subviral spherical particle II).

A major characteristic of HBV-replicating cells is the secretion of large amounts of complete and incomplete viral particles. complete virion are spheres, 42 nm in diameter, and usually present in the blood of infected patients at 109/mL [21]. By contrast, incomplete subviral HBV particles, of which two main types are identified, either lack the genome and capsid, or just the genome. The first type is the typical 20 nm diameter HBsAg spheres and filaments (HBsAg particles), which are constructed exclusively from of surface antigenic proteins and with a ratio up to

(1014/mL) [22]. The second type is the newly described empty (genome-free) virions, which have been assembled from outer surface proteins surrounding the viral capsid without genome and usually present in the blood of infected people at 100 times higher rates than full virions (1011/mL) [23].

In the present study, HepG2.2.15 cell culturing in 10 % complete williams medium supplemented with growth factors hydrocortisone and insulin for 8 days resulted in an increase in proliferation rate till day 7 compared to day 1, day 8 represent the

stationary state for the cells without further division, cells were morphologically observed as elongated cylindrical cells.

optimization of SVP production starting with distribution of HepG2.2.15 cell suspension into three groups (each group contains seven flasks) represents 7day with initial seeding density of cell suspension for all groups 0.5×10^6 cell/flask, the highest production rate of HBsvp after incubation for up to 7days in 7ml of cell culture supernatant (containing HBsvp) was determined quantitatively by ELISA. Our findings showed that the highest titer of the virus was day7 with average virus titre 1.217 (OD) for first, second, and third groups by 95.83 % increase compared to 4.17 % day 1 production rate that represent the lowest virus titre, with average virus titre 0.053 (OD) for the first, second, and third groups. We concentrated the SVP in a high titer. In this experiment each run was performed on average for different supernatant obtained. Also, for run 7, the highest concentrations of SVP were at day 7 with 2.327 (OD).

In comparison to positive HBV samples which exhibit two particle types : Dane particle in 42 nm and subviral spherical particle in 22 nm, transmission electron photographs shows that HBVsvp secreted from HepG2.2.15 cell line observed as incomplete HB subviral sphere particles with 22 nm diameter size. This sphere type of svp represent the classical HBsAg (either lacks both genome and capsid or just the genome) and found in abundant manner.

HBsvp in vitro production by transfection procedures was important for several reasons. Initially is they being not contagious compared to the isolation from the sera of infected individuals that contains both infectious virus as well as SVP; so, it can be safely handled in accordance with previous reports [24-28]. HBVsvp could also be produced in about absolute quantities, and can also be produced for the different genotypes and subtypes [29]. Additionally, SVPs can be assembled in various ways that contain only the HBV S

protein or those that contain both the S and L proteins[9].

For the present study, selection of the cell line HepG2.2.15 transfected with HBV surface region as it is a stably producing SVPs, has been commonly used for drug screenings, and more favorable than the primary human hepatocytes and dHepaRG cell culture systems which are known by limited availability, high variability, loss of hepatocyte function in culture [30]. Also, invitro cell culture models as HepG2 cells constitute a potent cell model for HBV and has been used in an increasing number of studies [31-33] However, the system has some limitations. HepG2 cells are transformed and have chromosomal abnormalities as well as defects in some intracellular signaling pathways. In addition, HepAD38 cell line produced a relatively small quantities of SVP compared to HBV particles [9].

Since the viral envelope protein structure on the surface of empty virions possibly resembles that of full virions but are non-infectious, because they assemble without containing the genetic material, it would provide the possibility for the use as a prophylaxis before infection or used with non-responders to the current vaccine in agreement with a previous relevant reports [34,35].

6. CONCLUSION

Using HepG2.2.15 cell line as an expression system in 10 % complete williams E medium supplemented with growth factors as hydrocortisone and insulin gives priority in continual secretion of large quantities of main types of HB subviral particles (either lacking the genome and capsid or lacking only the genome) rather than widely used hepatoma cell lines not susceptible to HBV infection. Such results put a spotlight on HBsAg protein that may be useful as diagnostic markers, and maybe as a potential vaccine candidate as the immunogenic agents to induce the defensive antibody (anti-HBs) fighting HBV infection. It is important to note that further improvement

and optimization of the conditions for the hepatoma cell culture are needed.

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Author Disclosure Statement

The authors report no conflicts of interest in this work.

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دراسة معملية للإنتاج الأمثل للجزيئات تحت الفيروسيّة
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الملخص العربي

أثناء تكاثر فيروس التهاب الكبد البائي (HBV) ، تفرز الخلايا المصابة جزيئات فيروسية معدية وكميات كبيرة من الجسيمات تحت الفيروسيّة التي تتجمع فقط من البروتين السطحي دون الجينوم الفيروسي. في الدراسة الحالية، تم استزراع خطوط الخلية من النوع HepG2.2.15 والتي تم دمجها وراثيًا مع منطقة سطح فيروس HBV كنظام تعبير لإنتاج الجزيئات تحت الفيروسيّة لفيروس التهاب الكبد البائي (HBVsvp) في أوقات مختلفة لأغراض تجارية. أظهرت النتائج التي توصلنا إليها أن زراعة وتحضين خلايا HepG2.2.15 خلال فترة حضانة سبعة أيام أدت إلى إفراز مستمر لـ HBsvp في مديا الزرع. كما أن التقدير الكمي لـ HBsAg بواسطة ELISA أظهر أن أعلى معيار للفيروس كان في اليوم السابع. وفقًا لحجمها، وجد أن قطر الجسيمات تحت الفيروسيّة للفيروس البائي ذات الشكل الكروي كان 22 نانومتر. في الختام، يمكن استخدام خلايا HepG2.2.15 وزراعتها معملياً تحت ظروف مثلي لإفراز HBsAg وذلك لدراسة تكاثر فيروس التهاب الكبد البائي وبالتالي فتح حقبة جديدة لدراسات مختلفة من علم الفيروسات ، التشخيص المخبري، إلى تطوير الأدوية ضد الأنماط الجينية المختلفة لفيروس التهاب الكبد البائي