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(RESEARCH ARTICLE)

The potential of chitosan nanoparticle as delivery system for HBcAg gene expression with pEGFP-N1 on HeLa cells

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Abstract

Hepatitis B (HB) is a contagious infectious disease that requires special attention in developing countries, including Indonesia. Morbidity and mortality due to complications caused by chronic HB infection are quite significant, which can progress to hepatic cirrhosis and carcinoma. Vaccination is considered to have good effectiveness and efficiency to prevent the burden of this disease, so it is necessary to develop a vaccine. One of the methods is the recombinant DNA technology approach through transformation of Escherichia coli DH5α bacteria and nanoparticle delivery agents into target cells. The aim of this study was to determine the effectiveness of chitosan nanoparticles as a delivery system for HBcAg VHB gene expression using pEGFP-N1 in HeLa cells as eukaryotic host cells. The methods in this study were recultured transformant bacteria from previous studies, isolation of recombinant DNA plasmids, plasmids restrictions, plasmid PCR, sequencing, transfection of HeLa cell culture and fluorescence observation of EGFP-HBcAg protein. Based on the PCR results, the transformant bacteria E. coli DH5α managed to carry pEGFP-N1-HBcAg plasmids. The restriction plasmid showed the formation of two bands. The results of transfection of HeLa cell culture showed that there was a green glow (fluorescent) in the treatment using the chitosan nanoparticle delivery agent. The conclusion is that chitosan can be used as a candidate for delivery agents in HB DNA vaccines using pEGFP-N1 vectors.

Keywords: Chitosan; HeLa; Hepatitis; pEGFP-N1; Vaccine

1. Introduction

Hepatitis B (HB) is a contagious infectious disease that attacks hepatocytes due to the Hepatitis B Virus (HBV) (1). Apart from being a global problem, HB is also a health problem that requires special attention in developing countries, including Indonesia. Basic Health Research by the Ministry of Health of the Republic of Indonesia in 2018 found a hepatitis prevalence figure of 0.39% or around 1 million people affected by hepatitis, as reported by Pusdatin Ministry of Health of the Republic of Indonesia. Morbidity and mortality due to complications arising from chronic Hepatitis B (HB) infection are quite significant, that it can progress to hepatic cirrhosis and hepatocellular carcinoma. Management of HB can be through health promotion activities, special protection, provision of immunizations, surveillance, control of risk factors, early detection, and/or case handling.

Immunization is considered to have good effectiveness and efficiency, so it is necessary to develop vaccines that are both preventive and curative. The HB vaccine has been available commercially since 1981 in the form of an inactivated

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virus (Heptavax) and was proven to be quite safe and effective until a recombinant DNA vaccine was developed in the following years (2). Recombivax HB vaccine was the first vaccine produced using the recombinant DNA method in 1986 (3). Research and development of next generation vaccines against HBV generally uses new adjuvants for recombinant viral antigens as well as DNA vaccines (3,4). The basic principle of making DNA vaccines is by inserting DNA or target gene sequences into eukaryotic expression vectors which are expected to code for proteins that are immunogenic in the human body (5). Much research related to DNA vaccines has been carried out to increase efficacy through optimizing DNA plasmids, improving delivery methods, selecting target antigens, and using immunological adjuvants.

In addition to the DNA vaccine vector and target antigen used, the DNA vaccine delivery method is also important. The low expression of DNA vaccines and the vaccine's resistance to passing through cell membranes means that only a small amount of the vaccine can reach the antigen presenting cell to induce an immune response. Natural and synthetic polymers are options for vaccine delivery systems because they are biodegradable, biocompatible, non-toxic and easily modified to become carriers of genetic material (6). Based on these characteristics, chitosan is considered to have great potential as an effective vaccine delivery agent. Chitosan has a positive electrical charge so it can bind to DNA which has a negative charge and prevent nuclease degradation (7). This research aims to determine the potential of chitosan nanoparticles as a delivery system for HBcAg gene expression using the pEGFP-N1 vector in HeLa cells.

2. Material and Methods

This research is a laboratory experimental study with a Posttest Only Control Group research design, because data collection was carried out after treatment and compared with experimental controls. This research is divided into 3 research stages in general. The first stage was the isolation of the recombinant DNA plasmid (pEGFP-N1-HBcAg) which had been constructed by previous researchers and confirmed by plasmid restriction analysis and confirmation of the gene sequence by sequencing using the pEGFP-N1 sequencing primer. The second stage is making a complex with a chitosan delivery system and its formulation (chitosan-pEGFP-N1-HBcAg). The third stage is transfection of the HeLa cell line and expression testing. The ability of the delivery system was observed qualitatively based on fluorescence observations of EGFP-HBcAg protein expression using an inverted microscope. This research has Ethical Clearance with number KE/FK/0929/EC/2022 issued by the Medical and Health Research Ethics Commission, Faculty of Medicine, Public Health and Nursing, Gadjah Mada University.

2.1. Recombinant DNA plasmid preparation

Bacterial colonies were cultured into liquid media to isolate plasmids using the Plasmid DNA Extraction Maxi Kit (Favorgen Biotech Corp) and carried out according to the provided kit protocol procedure. The purity and concentration of the isolated results were then measured using a NanoDrop Spectrophotometer. The isolation results were double digested by adding 1 µL of plasmid which was then added with 1 µL of *Bgl*II enzyme (10 U/µL), 1 µl of *Eco*RI enzyme (20 U/µL), 2 µL of restriction enzyme buffer and 15 µL of Nuclease Free Water (NFW). The mixture was incubated at 37°C for 15 minutes. Then the plasmid was electrophoresed to ensure that the restriction was successful.

Electrophoresis was performed using 1% agarose gel by dissolving 0.5 grams of agarose with 50 mL of 1x TBE buffer. The solution was then heated in the microwave until dissolved. After that, 5 µL of SYBR Safe DNA Gel Stain (Invitrogen) was added to the solution. Then the agarose solution was poured into the gel mold and waited for the gel to harden for ± 20 minutes. After the gel had hardened, the gel was inserted into the electrophoresis chamber which had been filled with 1x TBE buffer. Loading the sample into the gel well by mixing 5 µL of plasmid isolation sample with 5x Gel Pilot DNA loading dye (Qiagen) on parafilm. Electrophoresis was performed using a voltage of 100 V for 60 minutes. Then, the results were visualized with a UV illuminator.

The isolation results were also tested by PCR with 30 µl Gotaq® Master Mixes 2X Solution, 4,8 µl Primer Forward pEGFP-N1 (5' TGGGAGGTCTATATAAGCAGAG 3') 10 µM, 4,8 µl Primer Reverse pEGEP-N1 (5' CGTCGCCGTCCAGCTCGACCAG 3') 10 µM and 17,4 µl Nuclease Free Water. The PCR running was carried out at an annealing temperature of 56.9 °C. The PCR results were then sequenced and the data obtained were analyzed using computer software.

2.2. Chitosan-DNA complex preparation

Chitosan stock solution for the preparation of chitosan-DNA complex was made by the Complex Coacervation method with modification (7). Chitosan stock solution was made by dissolving 20 mg of chitosan (medium molecular weight chitosan/ MMWC, 150-700 kDa with 100 mL of 1% acetic acid (pH 5.0). The solution was sterile filtered using a 0.2 µm filter and stored in a sterile conical tube. A total of 1 µg of pEGFP-N1-HBcAg and several solutions with different concentrations of chitosan in a DNA:chitosan ratio (wt/wt) of 1:0.5; 1:1.0; 1:2.0; 1:3.0; heated separately at 50ºC for 10

minutes. Then the pEGP-C1-HBcAg and pEGP-N1-HBcAg solutions were each mixed with the chitosan solution by vortexing at 2500 rpm for 30 seconds at 4ºC. Because in the first experiment with a ratio of 1:0.5, positive entrapment results were still obtained, a second experiment was carried out with a lower ratio of 1:0.1; 1:0.2; 1:0.3; 1:0.4.

The formulation of chitosan and plasmid DNA complex was also observed by agarose gel electrophoresis. 1% agarose with 10x TBE solvent as much as 50 ml is heated to boiling then SYBR Safe DNA Gel Stain (Invitrogen) is added as much as 5 µL and poured into the gel mold. Electrophoresis is run with a voltage of 100V for 30 minutes then the results are observed under UV light. Chitosan/DNA nanoparticle samples are compared with free DNA samples. In principle, the work of recombinant DNA plasmid molecules that are not encapsulated or form a complex with chitosan nanoparticles will move freely, while recombinant DNA plasmid molecules that are successfully encapsulated perfectly or form a large molecular complex with chitosan nanoparticles will be retained in the well.

Particle size and zeta potential charge were obtained from tests conducted by the Integrated Research Laboratory of the Faculty of Dentistry, Universitas Gadjah Mada. Cytotoxic testing was conducted by the Parasitology Laboratory, Faculty of Medicine, Universitas Gadjah Mada with chitosan nanoparticle concentration levels.

2.3. *In vitro* transfection assay and EGFP-HBcAg protein expression

HeLa cells were cultured in 24-well petri dishes (Nunc™ Cell-Culture Treated Multidishes) with an initial cell density of 1x105 cells/well and a total volume of culture media of 0.5 mL. Then the cells were incubated at 5% CO_2 and a temperature of 37 °C for 72 hours. The culture media was replaced every day. The culture medium consisted of 10 ml of Fetal Bovine Serum (FBS), 2 mL of Penstrep, 500 µl of Fungizone, 1 mL of MEM-NEAA and sufficient DMEM so that the total solution became 100 ml. After the HeLa cell culture was 70-80% confluent with a density of 3.2x106 cells/petri dish, transfection was carried out on the HeLa cell culture with the following treatment sequence:

- Without transfection, only cultured cells (negative control)
- Transfection using only chitosan (negative control)
- Transfection using only recombinant DNA plasmid (negative control)
- Transfection with chitosan nanoparticle complex and DNA plasmid (treatment)
- Transfection with commercial delivery reagent and recombinant DNA plasmid (positive control)

Each treatment was transfected with 8 µg of recombinant DNA plasmid. After transfection, the cells were incubated for 4 hours. Then, it was replaced with new media and continued incubation for another 24 hours to observe the fluorescence using inverted microscope (Nikon Eclipse T2s) with 200x magnification.

3. Result and Discussions

3.1. Recombinant DNA plasmid preparation

Recombinant plasmid isolation was carried out using the Plasmid DNA Extraction Maxi Kit (Favorgen Biotech Corp) according to the procedure stated on the insert kit. The results of 2 samples of recombinant plasmid isolation were visualized using 1% agarose gel electrophoresis (Figure 1). The isolation product then tested for DNA purity and integrity using the NanoDrop UV/VIS spectrophotometer method (Table 1). DNA purity and integrity are indicators of DNA quality that need to be ensured before further analysis is carried out by measuring absorbance at wavelengths of 230 nm, 260 nm, 280 nm.

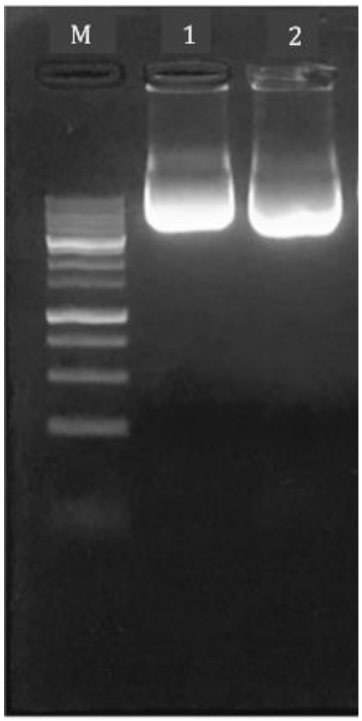

Figure 1 Isolate of pEGFP-N1-HBcAg (1 and 2); Ladder 1000 bp(M)

Table 1 Purity and concentration of DNA plasmid recombinant

No	Sample	$A_{260/230}$	$A_{260/280}$	Concentration (ng/µL)
1	$N1_1$	2,037	1,928	1216,86
2	$N1_2$	2,071	1,888	1302,85

Electrophoresis is effective to separate DNA fragments, especially between 100 bp to 25 kb, including plasmids. The phosphate skeleton of DNA and RNA will be negatively charged and the DNA fragments will migrate to the anode which is positively charged. The electrophoresis results showed that there were two bands of recombinant plasmid DNA which are supercoil and circular nick forms, where DNA conformation is one of the factors that can influence the speed of DNA migration even though it has the same molecular weight. As is known, DNA has three types of conformations. They are super-helix circular or also called supercoiled (covalently closed circular) (I), circular-opened (nicked) (II) and linear (III). Form I is a compact form and can be arranged in such a way if the polynucleotide chain is intact. If one of the double strands of DNA is damaged, form I will change its conformation to form II and if both double chains are damaged, the conformation will become form III. Form I is known to migrate faster than form III and form II is the slowest of the three. Other factors that influence the speed of DNA migration are the size of the DNA molecule, the concentration and type of agarose gel, electrical voltage, the content of Ethidium bromide and the composition of the electrophoresis buffer (8). The results of measuring the purity ratio using spectrophotometric principles obtained results within the normal range. DNA purity is expressed as a ratio between absorbance A 260/280. If the ratio is greater than 2.0 then there is a possibility of RNA contamination and if the ratio is smaller than 1.8 there is a possibility of protein, phenol or other organic substance contamination (9).

Plasmid restriction is an approach used to ensure the presence of target gene inserts by utilizing restriction enzymes. General restrictions are carried out before sequencing because the sequencing process is quite time consuming and expensive. The enzymes used in this research were the *Bgl*II and *EcoR*I enzymes to cut the recombinant plasmids pEGFP-C1-HBcAg and p-EGFP-N1-HBcAg at the end of the insert (MCS). These two enzymes are included in restriction endonucleases which can cut DNA at certain sequences, namely the AGACTC (*Bgl*II) sequence and the GAATTC (EcoRI) sequence. Visualization of restriction results using 1% agarose gel electrophoresis (Figure 2).

Plasmids with UC and UN codes are plasmid isolation samples that were not treated with restriction (uncut), similar to the results of 1% agarose gel electrophoresis visualization in Figure 1, namely forming a band between 2000-5000 bp.

The result of this restriction is that two DNA bands appear at 1 and 2 respectively (Figure 2). The first band in the restriction results is between 2000-5000 bp and is estimated to be 4733 bp in size. The second band at slightly above 500 bp is estimated to be 564 bp in size. This is due to the double digestion mechanism by BglII and EcoRI. The size of 4733 bp is the size of the pEGFP-N1 full genome while the size of 564 bp is the size of the HBcAg target insert gene. Based on these results it can be assumed that the target gene has been successfully inserted into the vector plasmid.

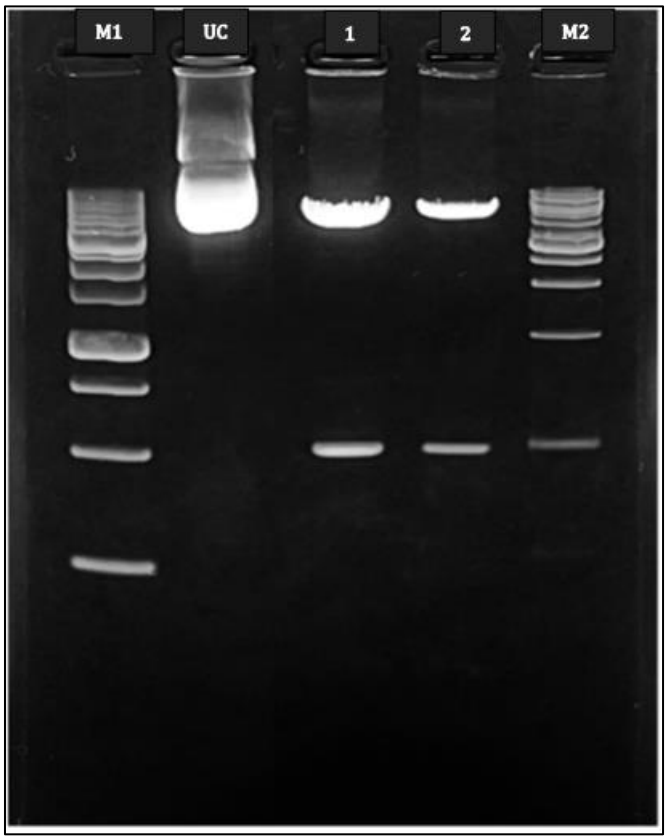

Figure 2 Restriction using *Bgl*II and *Eco*RI enzymes. Unrestricted (uncut) isolated plasmid (U); restricted (cut) isolated plasmids (1 and 2); 1000 bp marker (M1); 100 bp marker (M2)

The next stage is to carry out more definite confirmation using the sequencing method. In general, the aim and principle of sequencing is to identify a nucleotide sequence by comparing it to a known nucleotide sequence through sequencing the nucleotide bases in the DNA molecule. As a comparison, this study used the HBcAg sequence from previous research (5). The sequencing method used is the Sanger method, where this method refers to the early termination of DNA synthesis which produces chain-terminating dideoxynucleotides, that is, there is no 3'hydroxyl deoxyribose in the DNA polymerase reaction. The DNA synthesis process is initiated by primers that react at specific sites on the DNA template and is extended with the help of DNA polymerase. There are 4 reactions that run separately but simultaneously, each containing one deoxynucleotide (A, C, G, T) (10). The sequencing work in this research was provided by Genetica Science Corp. The sequencing results show that there are no significant differences in the samples isolated from the recombinant plasmid, so it can be assumed that the recombinant plasmid is similar to the HBcAg target gene sequence (Appendix 2). The preparations made for sequencing are carrying out PCR on the results of plasmid isolation to increase the amplicon fragments of the target gene and detect the target gene isolated. The PCR method was carried out using the pEGFP-N1 universal sequencing primer to confirm that the plasmid isolate obtained was indeed pEGFP-N1-HBcAg. The results of amplification from PCR are products that are between the 700 – 800 bp band which is estimated to be 775 bp in size. There is an increase in product size due to the increase in base pairs belonging to the egfp gene which is located respectively at the N-terminus of the core protein.

Electrophoresis is a separation method with a working principle based on differences in the speed of movement of charged particles when they are in an electric field. The capacity of chitosan to conjugate with plasmid DNA was observed with the help of SYBR Safe DNA Gel Stain (Invitrogen) where SYBR Safe interacts with double-stranded DNA bases which can produce luminescence when observed under UV light (Figure 3).

Figure 3 PCR isolation product of pEGFP-N1-HBcAg (K+: positive control; 1 & 2: Sample; K-: negative control; M: ladder 100 bp)

3.2. Chitosan-DNA complex preparation

Preparation of chitosan-DNA nanoparticle complexes was carried out using the complex coacervation method. The formation of complexes in a ratio of 1:0.1 – 1:0.4 were first observed using a complex migration test using 1% agarose gel electrophoresis or known as the gel retardation assay (Figure 4). In this experiment, only chitosan control and recombinant DNA plasmid control were used.

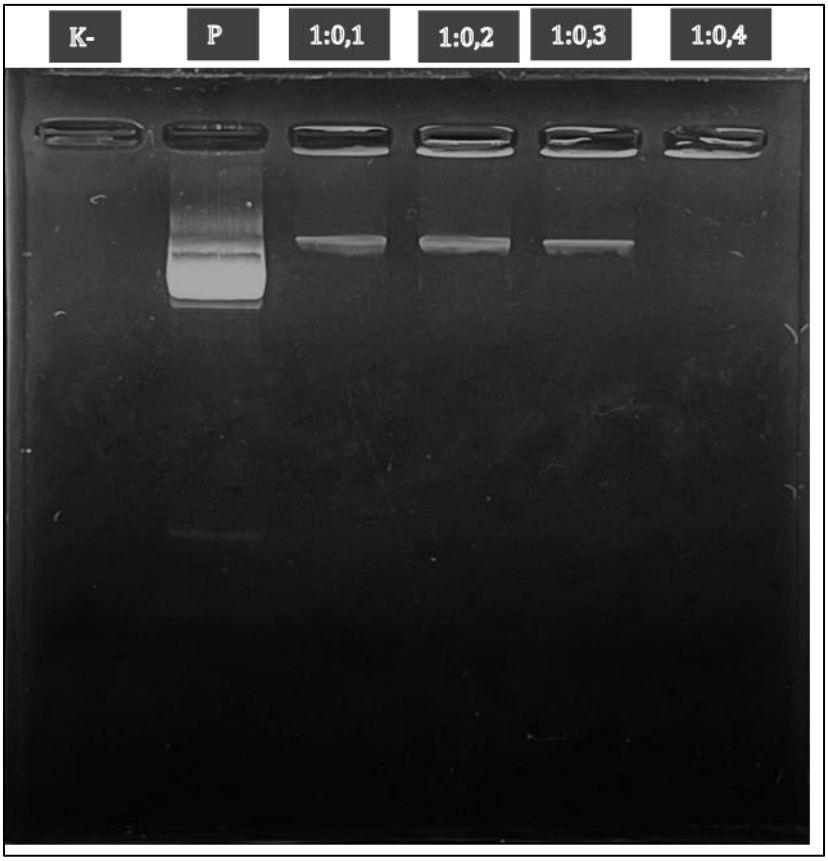

Figure 4 Agarose gel retaradation assay of chitosan-DNA complexes ((K-) chitosan only; (P) plasmid only)

After being observed under a UV transilluminator lamp, DNA with a ratio of 1:0.4 in the chitosan-pEGFP-N1-HBcAg complex was seen to remain in the wells of the agarose gel. The successful conformation made DNA unable to migrate

through the agarose gel. The ionic bonds in this nanoparticle complex are the result of electrostatic interactions between positively charged chitosan and negatively charged DNA. Large molecules have difficulty passing through gel pores so they migrate more slowly through the gel than smaller DNA (8). In P (without chitosan) it was seen that DNA could migrate through the agarose gel and became thinner as the ratio of chitosan was increased.

The following test is the cytotoxic test of the chitosan-DNA complex to ensure that no transfecting agent has toxicity potential for the cell. Based on the results obtained in the test, it can be concluded that the chitosan-pEGFP-N1-HBcAg complex is not toxic to HeLa cells. This is showed from the high percentage of living cells that were 86.3% when given the chitosan-pEGFP-N1-HBcAg complex; 99,2% when given the negative control PBS; and 100% when given negative control of chitosan only (Figure 5).

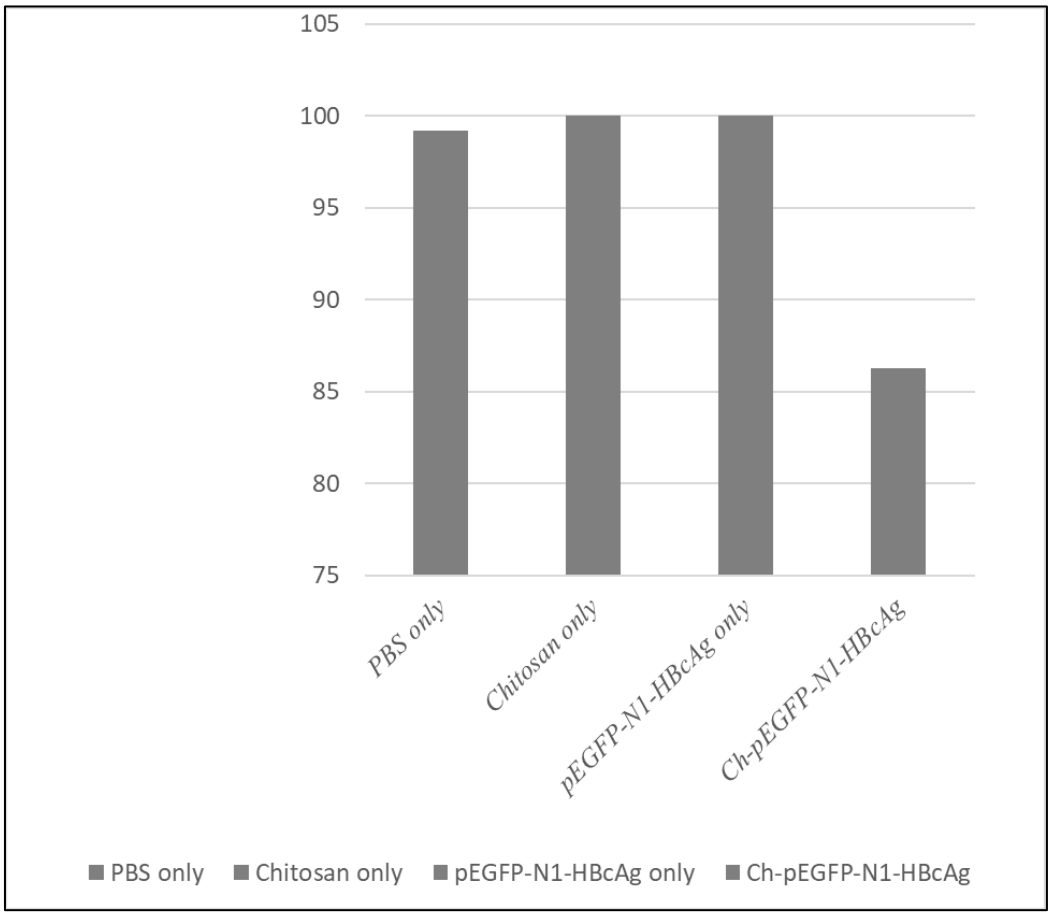

Figure 5 Cytotoxic assay

3.3. *In vitro* transfection assay and EGFP-HBcAg protein expression

Transfection is a series of processes of inserting foreign nucleic acids into eukaryotic cells. This technique is often used in the practice of transferring genetic material into mammalian cells, for example DNA plasmids transfected into HeLa cells. The plasmid structure consisting of a promoter, origin of replication, multiple cloning sites, gene of interest and selection marker supports the transfection process into eukaryotic cells. Plasmid DNA can be in linear or supercoiled form when transfected into host cells, but from previous research it has been found that the supercoiled form of plasmid has higher efficiency because it is more difficult to be degraded by exonucleases. However, the linear form has the advantage of being easily recombined and more stable in the transfection process. Another advantage of using plasmid DNA is that it is less immunogenic than using viral DNA, thereby reducing the risk of pathological integration into host cells. Apart from the advantages mentioned, it is necessary to note the disadvantages of transfection using DNA plasmids, namely lower transfection efficiency and recombinant protein production. This study uses the pEGFP-N1 vector which has the HBcAg gene inserted at the *Bgl*II and *Eco*RI restriction sites so that the HBcAg protein expressed will bind to the EGFP protein expressed by the egfp gene. HeLa cells that had been transfected were then subjected to fluorescence observation using a confocal microscope. The results of fluorescence observations showed that transfection with negative control treatment gave negative results. Meanwhile, the chitosan-DNA complex treatment

and positive control showed positive results (Figure 6). Transfection requires controls to ensure the success and efficiency of the reagents and nucleic acids used. In general, the controls used consist of positive control, negative control, un-transfected control and mock. A positive control is the use of DNA or RNA that has been previously proven to be successful in the transfection process according to the expected expression of a specific target gene.

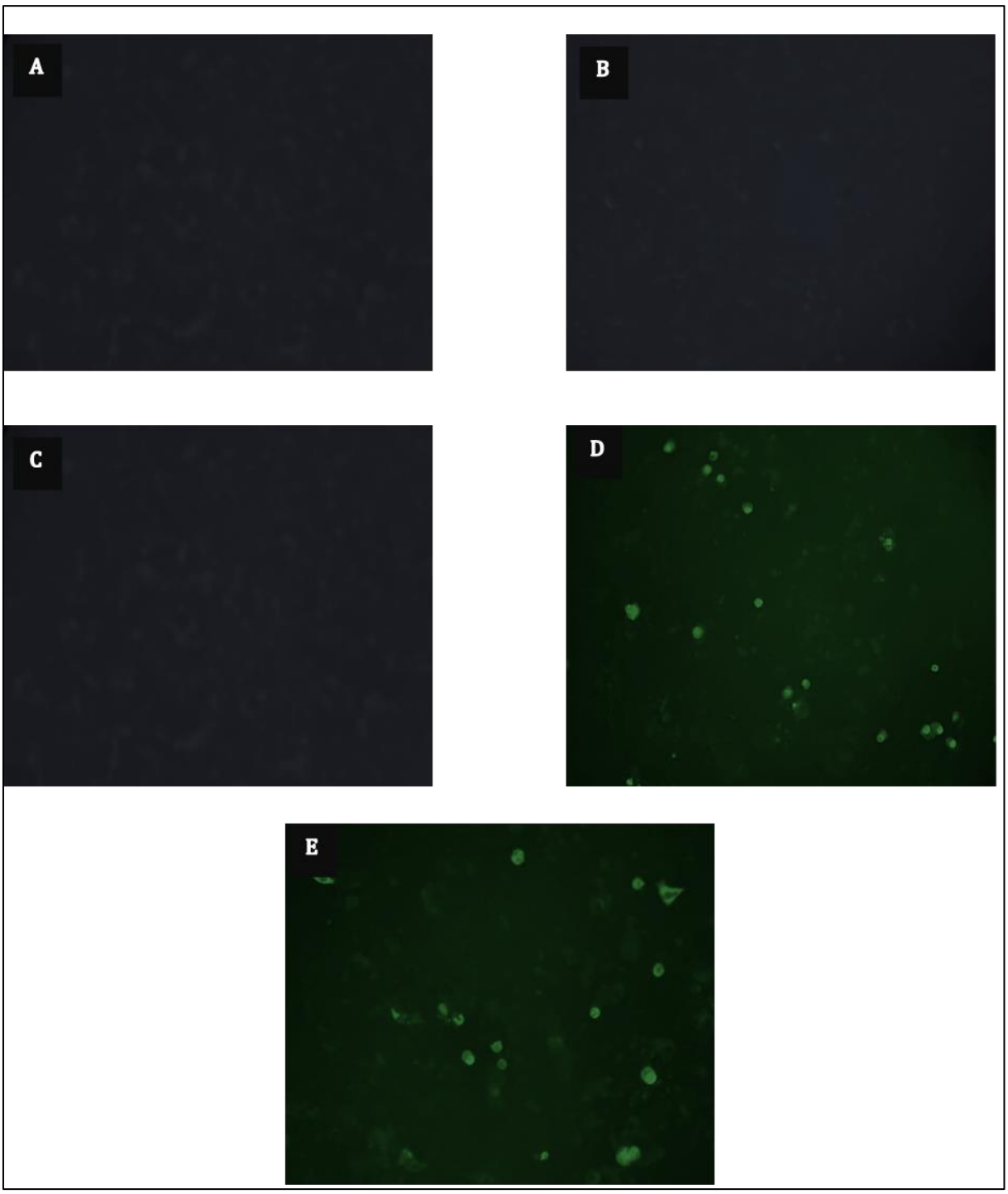

Figure 6 Fluorescence observation. A). Control untransfected HeLa cells only; B). Negative control HeLa cells + chitosan; C). Negative control HeLa + pEGFP-N1-HBcAg cells; D). Chitosan-pEGFP-C1-HBcAg Treatment; E). HighGene-pEGFP-N1-HBcAg Treatment

Conversely, negative controls are necessary to ensure that the expected target gene expression originates from transfection, and not from other causes. For example, negative controls can use host cells alone without adding target DNA and or delivery media. Untransfected controls are controls that only use host culture cells without using

transfection reagents and nucleic acids, functioning as basal information regarding host cells and basal expression levels of target genes without the influence of transfection. Mock control is a transfection without a target gene or nucleic acid, making it possible to observe the effects produced by the transfection reagent, such as the emergence of auto-fluorescence noise in the background. In this study, the commercial transfection reagent HighGene was chosen which has been proven to successfully transfect the HBcAg target gene with the pEGFP-N1 vector as a positive control. As an untransfected control, culture cells alone were chosen without the addition of chitosan delivery nanoparticles and recombinant DNA plasmids and as negative controls were culture cells treatment with the addition of chitosan nanoparticles alone without DNA plasmids and culture cells with the addition of DNA plasmids alone without chitosan nanoparticles (11–13).

4. Conclusion

Based on the research results that have been obtained, it can be concluded that the HBcAg gene was successfully transfected in HeLa cell culture, as indicated by the green glow when chitosan nanoparticles were treated. We suggest for continuing quantitative expression tests as well as in vivo tests which aim to determine the effectiveness of recombinant nanoparticle-DNA complexes as DNA vaccine candidates.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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