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Development of lipid nano particles RNA vaccine against hepatitis B surface antigens of hepatitis B virus

***Mohammed Mahmoud Shawky Kassab** *[1]*

1- *PHD of microbiology and immunology*

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ABSTRACT

Background: Hepatitis B is a severe overwhelming infection. Hepatitis B is a viral infection that attacks liver cells, causing inflammation and damage to the liver. Infection is usually confined to hepatocytes. Over 2 billion people have been infected with hepatitis B virus at some point. Immunity arises from natural infection or from administration of vaccines. **Methodology**: In the present study, bioinformatics were victimized to engineer an RNA vaccine from hepatitis B surfactant antigen. Lipid nanoparticles with a particle size of 150 nanometers were utilized as a vaccine delivery system. **Result**: The vaccine has shown an efficacy of 81% in preclinical animal studies and an efficacy of 79% in human clinical studies (Phase 1/2 clinical trials). It exhibited moderate biological activity and had fewer side effects than other standard vaccines. The effect lasted for a long time. **Conclusion**: The present mRNA-HBV vaccine was a promising prophylactic vaccine against fatal hepatitis B viral infection.

Introduction

Hepatitis B virus (HBV) is a prevalent infectious disease worldwide. It is the leading cause of cirrhosis, liver cancer and liver transplantation worldwide. 4 million people become acutely infected and 1 million die each year. 25% of children (under age 7) become carriers and 10% of older children and adults become carriers.1 Features: An enveloped virus with incomplete circular double-stranded DNA. That is, one strand is missing about one-third and the other strand is 'nicked' (not covalently linked).2 Virion DNA polymerase present inside the viral particle. The HBV-encoded polymerase functions as a reverse transcriptase, using the viral mRNA as a template for progeny genomic DNA synthesis.3 Three significant antigens exist.

They are the e antigen (E), core antigen (C), and surface antigen (HBsAg) of hepatitis B. In patient sera, long rods and spheroids, which consist exclusively of HBsAg, predominate. HBV has serotypes based on surface antigens.4

Transmission: It is transmitted through blood, childbirth, and sexual contact.5

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* *Corresponding author:* Mohammed Mahmoud Shawky Kassab
E-mail address: Mohammed.Kassab676@gmail.com
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Pathophysiology: Hepatocyte damage by immune attack by cytotoxic (CD8) T cells. Antigen-antibody complexes cause arthritis, rashes, and glomerulonephritis. About 5% of HBV infections become chronic carriers. Chronic hepatitis, cirrhosis, and hepatocellular carcinoma can develop. Hepatocellular carcinoma may be associated with integration of some viral DNA into hepatocyte DNA.6 Laboratory diagnosis:

HBV does not grow in cell culture. Three serological tests are commonly used. Surface antigen (HBsAg), surface antibody (HBsAb), and core antibody (HBcAb).7 Detectable HBsAg for more than 6 months indicates a chronic carrier state. The presence of the e antigen indicates a chronic carrier that produces infectious viral particles. The presence of the e antigen is an important indicator of contagiousness.8 HBV-infected individuals in whom neither HBs antigen nor HBs antibodies are detectable are in the 'window' phase. Diagnosis in this patient is made by detection of HB core antibodies.9 Management: Alpha interferon and the reverse transcriptase inhibitor lamivudine can reduce the inflammation associated with chronic hepatitis B but cannot cure the carrier state. Prevention: There are three main approaches: Vaccines containing HBsAg as immunogen or hyperimmune serum globulin from donors with high HBsAb titers or educating chronic carriers about precautions.10 mRNA-HBV vaccine immunogenicity studies: The reaction from antigens and antibodies is highly specific.11 Antigens only react with antibodies elicited by themselves or closely related antigens.12 Due to their high specificity, reactions between antigens and antibodies can distinguish one from the other. This is the basis of the serological response.13 The results of many immunological tests are expressed as a titer expressed as the highest sample dilution. Such as serum that tests positive.

For instance, a patient's serum with a titer of 1/64 will contain more antibodies or have a higher titer than serum with a titer of 1/4.14 Enzyme-linked immunosorbent assay (ELISA): This method can be utilized for quantification of antigens or antibodies in affected samples. It is based on covalently linking an enzyme to a known antigen or antibody, allowing the reaction of the enzyme-linked material with a patient sample, adding a substrate for the enzyme and assaying for enzymatic activity. This method is nearly as sensitive as radioimmunoassay (RIA), but does not demand special equipment or radiolabels. Fluorescence-activated cell sorting (flow cytometry): This test is ordinarily exploited to measure the number of various kinds of immunologically active blood cells. In this test, the patient's cells are labeled with monoclonal antibodies against proteins specific to the cells of interest such as CD4 protein when measuring the number of T helper cells. Monoclonal antibodies are labeled with fluorescent dyes such as fluorescein and rhodamine. A beam of laser light is passed through individual cells and the number of fluorescent cells is counted using a machine known as a fluorescence-activated cell sorter (FACS).15 The development of HBV vaccine has begun since 1980. The best way to manage HBV infection is with hepatitis B vaccination. Hepatitis B surface antigen (HBsAg), which was isolated from the plasma of asymptomatic HBsAg carriers, was used to create the first licenced hepatitis B vaccine. Then, the recombinant hepatitis B vaccine was created by using recombinant DNA technology. Three vaccination doses given in a row can provide protection for up to 30 years. Infection rates in offspring of carriers with negative hepatitis B e antigen (HBeAg) and 5–10% infection rates in children of mothers with positive HBeAg have been significantly decreased by using hepatitis B immunoglobulin and the hepatitis B vaccination at the same time.16

The global incidence of HBsAg in children under the age of five has dropped drastically thanks to the adoption of the universal hepatitis B vaccination programme by 189 nations by the end of 2018—from 4.7% in the pre-vaccine period to 1.3% in 2015. Although more than half of newborn children do not receive the recommended timely birth dose of the hepatitis B vaccine, certain locations have subpar implementation of the vaccine.17 In the present study, a vaccine based on lipid nanoparticles RNA technology was aimed at developing to combat HBV lethal infection globally.

Material and methods

Ethics Statement:

Current research followed all applicable and institutional international and/or national guidelines for people's care and animal use during all processes run in the study. Animals were approved by the local government CU- IACUC (Institutional Animal Care & Use Committee) Faculty of Pharmacy, Cairo University, Egypt with Weatherall Report Primate Recommendation

number P-6-4-2020. To minimize the number of people, animals exploited and their suffering during the study, all efforts were carried out. All animal experiments were complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

Material:

All chemical and biochemical materials were purchased from Algomhoria pharmaceutical company,Cairo,Egypt.

Source of animal models: They were obtained and legalized from pharmacology and toxicology department of faculty of pharmacy, Cairo university, Egypt.

The type of the study: Screening experimental study. Inclusion criteria for animal models are: Adult animal, can be induced by hepatitis B infection such as transgenic mice. Transgenic mice humanized by liver human cells for increasing expression of viral proteins and evoking strong humoral and cell-mediated immunity included adult male mice weighing 40–50 g that was successfully induced by hepatitis infectious diseases after inoculation with HBV.

Exclusion criteria are: Young animal, pregnant female animal. Place and date of the study: This study was done in faculty of pharmacy, Cairo university during 2021.

Methods:

The potential open reading frames for hepatitis B surface antigens were identified by bioinformatics. RNA from hepatitis B surface antigen was then expressed and purified by organic extraction. In order to increase the amplification of the gene of interest, the gene of interest (cDNA) was cloned using the polymerase chain reaction (PCR). It was then digested with the restriction endonucleases BamHI and SphI and introduced into the pUC18 expression vector. The ligase enzyme was then used to ligate the molecules. Linearization of the pDNA template was completed with restriction enzymes EcoRI, Eam11041 and Lugul (obtained from ThermoFisher Scientific Company, USA). Linearized DNA templates were transcribed into mRNA in vitro in a mixture containing recombinant T7 RNA polymerase and nucleoside triphosphates, RNase inhibitors, and 5X transcription buffer (obtained from ThermoFisher Scientific Company, USA). mRNA transcripts of the gene of interest were obtained; as well as crowned by transcription though the addition of Cap analogs such as the dinucleotide m7G(5 i)-PPP-(5 i)G (obtained from ThermoFisher Scientific Company, USA as a regular Cap analogue),. It was modified via mRNA tailing by poly(A) polymerase (obtained from ThermoFisher Scientific Company, USA). DNase I and protein kinase K (from ThermoFisher Scientific Company, USA) were added for in vitro post-transcriptional purification. Purification was completed by an mRNA extraction procedure. Purification of mRNA was accomplished by an organic extraction procedure termed guanidinium acid thiocyanate-phenol-chloroform extraction. It is a liquid extraction technique for isolating RNA. It is characterized by high purity and recovery of RNA. The chloroform solution consisted only of 96% chloroform and 4% isoamyl alcohol (isoamyl alcohol was observed to cut down foaming and inactivate RNases), mixed with an equal volume of phenol to obtain 25:24:1 solution. For mRNA purification, pH was maintained at ~5. This favorably retained the mRNA in the aqueous phase. This work was based on phase separation by centrifuging a mixture of an aqueous sample and a solution comprising phenol and chloroform saturated with water, followed by separation into an upper aqueous phase and a lower organic phase (predominantly phenol). A chaotropic factor, guanidinium thiocyanate, was introduced into the organic phase to assist in the denaturation of RNases that degrade mRNA. mRNA was separated into the aqueous phase and protein was separated into the organic phase. Below acidic conditions (PH 5), DNA settled into the organic phase and mRNA remained in the aqueous phase. mRNA was refined from the aqueous phase by precipitation with 2-propanol. At that point, the propanol was washed with ethanol and the pellet was briefly air-dried and dissolved in RNase-free water. The ratio of absorbance at 260 nm and 280 nm was exploited to assess the purity of mRNA transcripts. The quantity and size of recombinant mRNA transcripts were further evaluated by Northern blotting. mRNA transcripts were separated by agarose gel electrophoresis, were denatured, and were transferred to nitrocellulose membranes where they were cross-linked with radiolabeled probes.

Purified mRNA was entrapped in a vaccine delivery system consisting of dimethyldioctadecylammonium bromide (DDAB) lipid nanoparticles that form lipid vesicles

surrounding the recombinant mRNA transcript. The particle size of the lipid nanoparticle vaccine delivery system was approximately 150 nm. The lipid nanoparticles were successfully synthesized utilizing hot micro-emulsion method.

Formulation:

The Lipid Nanoparticles-mRNA-HBV Vaccine was a sterile suspension for intramuscular injection. Each 1 mL dose incorporated 20 µg of hepatitis B surface antigen, 50 µg of dimethyldioctadecylammonium bromide lipid (DDAB), and 0.5 mg of aluminum hydroxide, 4.5 mg sodium chloride and 0.62 mg sodium dihydrogen phosphate dihydrate.

Immunogenicity evaluation:

Immunogenicity was evaluated in an animal model by injecting purified mRNA into transgenic mice via the intraperitoneal route. Transgenic mice were mice that had been genetically modified by recombinant DNA technology. Integrated genes or DNA sequences from external sources/ other species were performed by human design.18 100 transgenic mice were injected with the vaccine. They gave each mouse two doses 28 days apart. The first dose was half the second booster dose. Protection tests(active or passive) were utilized to determine vaccine efficacy. Active: After immunization with the vaccine, groups of transgenic mice were challenged by increasing doses of virus. The lowest dose of organisms that killed 50% of the animals(i.e., LD50) was Determined and compared to LD50 of unvaccinated animals to measure the protective efficacy of vaccines. Passive: Graded amounts of serum from immunized subjects were transferred to normal mice and challenged with the infectious agent. The highest serum dilution effective to protect 50% of animals(ED50%) was dictated as a criterion of vaccine efficacy. minimum infectious dose of HBV was 1,049 copies/ ml.

Phase 1-2 Human Clinical Trials:

Three groups of human volunteers were included in the present study. Each group consisted of 100 of subjects. Group1(negative control group) received placebo injections. Group2(positive control group) received a standard HBV subunit vaccine intramuscularly.

Group3(test group) received the tested mRNA-HBV vaccine intramuscularly. The three groups were challenged two weeks later with graded doses of the infectious organism to allow for the development of protective neutralizing antibodies. After 21 days, booster doses were administered intradermally to the three groups. The protective power of the trial vaccine was evaluated over a two-year period. Protective antibodies were detected using an enzyme-linked immunosorbent assay (ELISA); while protective cell-mediated immunity was assessed using a flow cytometry assay. Detection of neutralizing antibodies against mRNA-HBV was performed using ELISA method. Flow cytometry technology (Invitrogen Attune Cytpix flow cytometer, USA) was used to detect and count mRNA HCV vaccine-specific CD+4 and CD+8 T lymphocytes.

Statistical analysis

All cultures were conducted in triplets. Their results were presented by means and standard deviation. One way analysis of variance (p value≤ 0.05) was used as means for performing statistical analysis using excel-spreadsheet. F-test was utilized during this study.

Results

Graph 1 shows that the proportion of patients that are alive increases to 81% following vaccination, compared to 27% in the unvaccinated condition. The absorbance of various serum concentrations of neutralizing antibodies to HBV RNA vaccine surface active antigens is shown in Graph 2 by ELISA. Graph 3 demonstrates that throughout phases 1 and 2 of the clinical evaluation, the protective power of the test RNA HBV was 79%. After receiving the mRNA-HBV vaccine, the CD+4 helper T cell count increased somewhat, as shown in Graph 4. The absorbance of the serum-neutralizing antibody concentrations to the HBV mRNA vaccination by ELISA is shown in Table 2. The immunization against hepatitis virus infection is shown in Table 3. The immunization of transgenic mice with the mRNA-HBV vaccine is shown in Table 4. T. lymphocyte estimate following mRNA-HBV immunization is shown in Table 5. The immunization of transgenic mice with the mRNA-HBV vaccine is shown in Table 4. Table 6 shows the effectiveness of the test RNA HBV vaccine during phases 1 and 2 of clinical trials. Hepatitis B virus LD50% was shown to have a viral concentration of above 60 mcg/ml. The mRNA-HBV vaccine's ED50% was determined to be 13 mcg/ml.

Table 1. List of instruments:.

Instrument	Model and manufacturer
Autoclaves	Tomy, japan
Aerobic incubator	Sanyo, Japan
Digital balance	Mettler Toledo, Switzerland
Oven	Binder, Germany
Deep freezer -70 ^{0}C	Artiko
Refrigator 5	whirpool
PH meter electrode	Mettle r-toledo, UK
Deep freezer -20	whirlpool
Gyratory shaker	Corning gyratory shaker, Japan
190-1100nm Ultraviolet visible spectrophotometer	UV1600PC, China
Light(optical) microscope	Amscope 120X-1200X, China

Table 2. It represents the absorbance of the concentrations of serum neutralizing antibodies to HBV mRNA vaccine via ELISA

Concentration(ng/ml)	**Absorbance**
1.55	0.187
3.07	0.259
6.19	0.349
12.5	0.567
25	1.029
50	1.809
100	3.034

Table 3. It represents the vaccination against hepatitis viral infection:.

Description	**Vaccinated**	**Pre-vaccinated**
CD+4 COUNT	1070	1000
CD+8 COUNT	730	600
Total	**1800**	**1600**

Table 4. It represents the vaccination of transgenic mice with mRNA- HBV vaccine:

Description	**Vaccinated**	**Non-vaccinated**
Alive	81	27
Dead	19	73
Total	**100**	**100**

Table 5. It represents The estimation of T. lymphocytes after mRNA-HBV vaccination:

Description	Vaccinated	Pre-vaccinated
CD+4 COUNT	1070	1000
CD+8 COUNT	730	600
Total	**1800**	**1600**

Table 6: It represents protection power of test RNA HBV vaccine during clinical trials phases 1/2:

	Vaccinated		Non-vaccinated
	test	standard	control
% infected	21	10	90
%noninfected	79	90	10

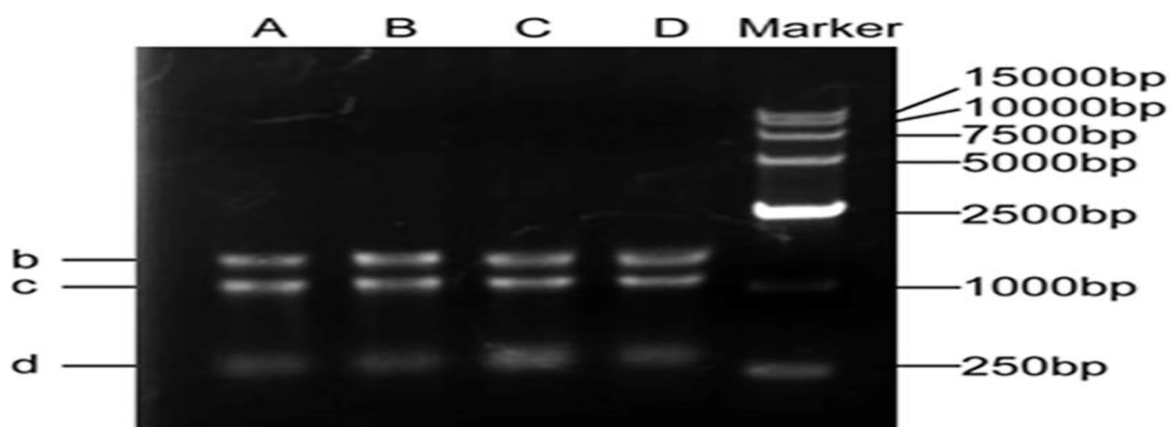
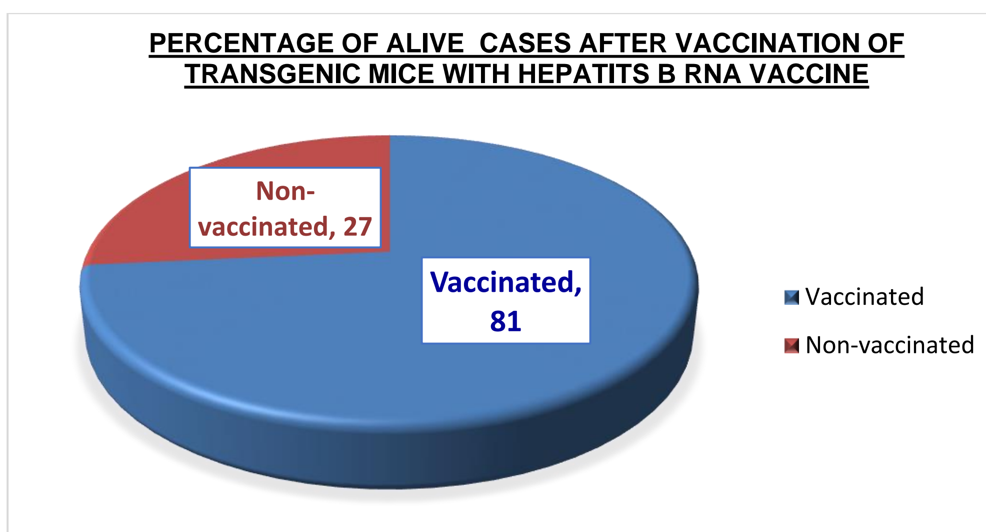
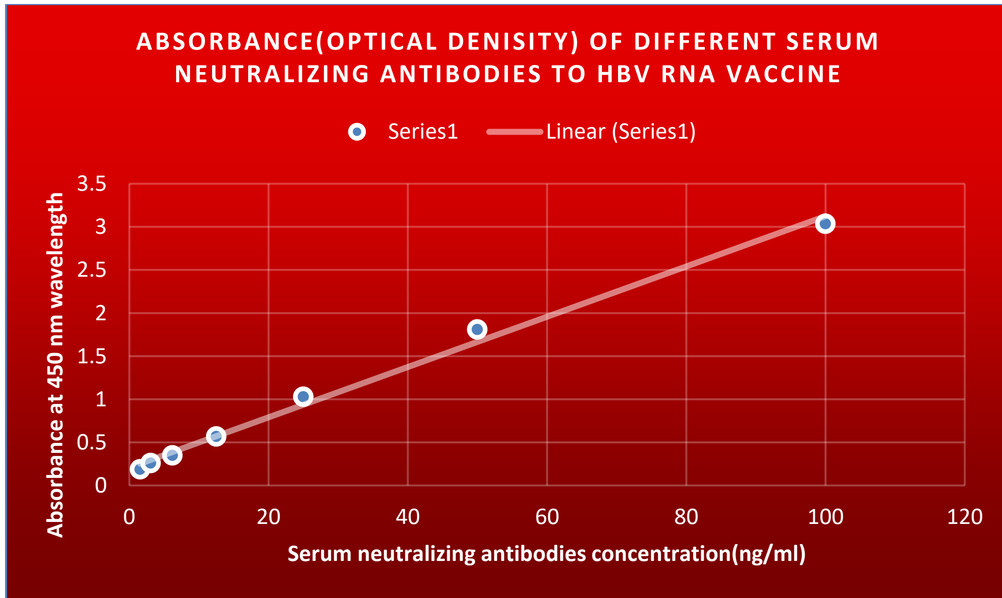


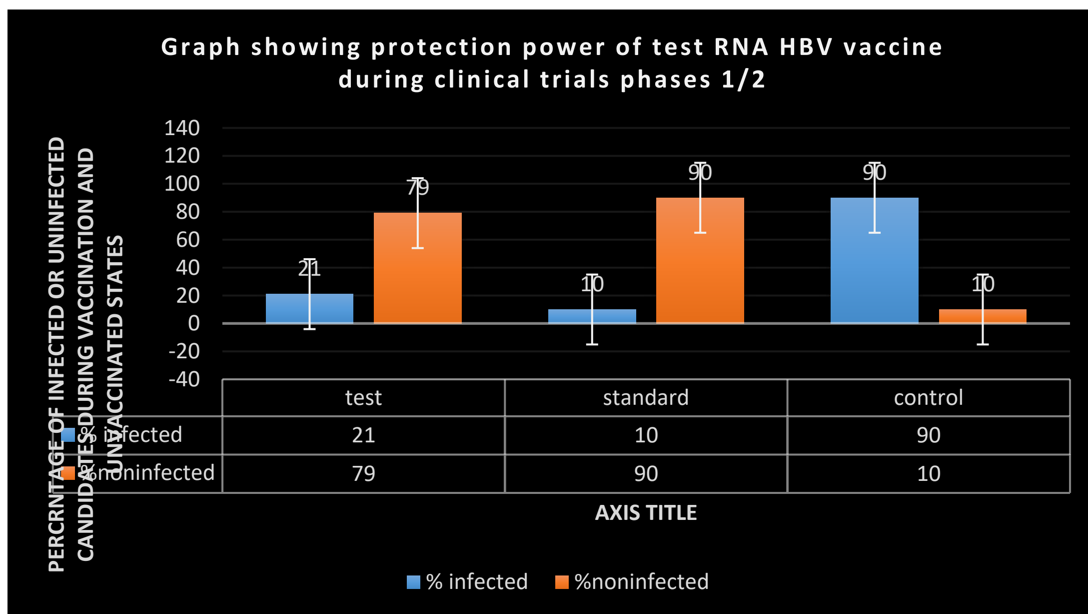
Figure 1. It shows different amounts and sizes of mRNA transcripts of HBV surface active antigens measured via Northern technique.



Graph 1. It demonstrates that the percentage of alive cases after vaccination reach 81%; while, it was 27% during unvaccination state.

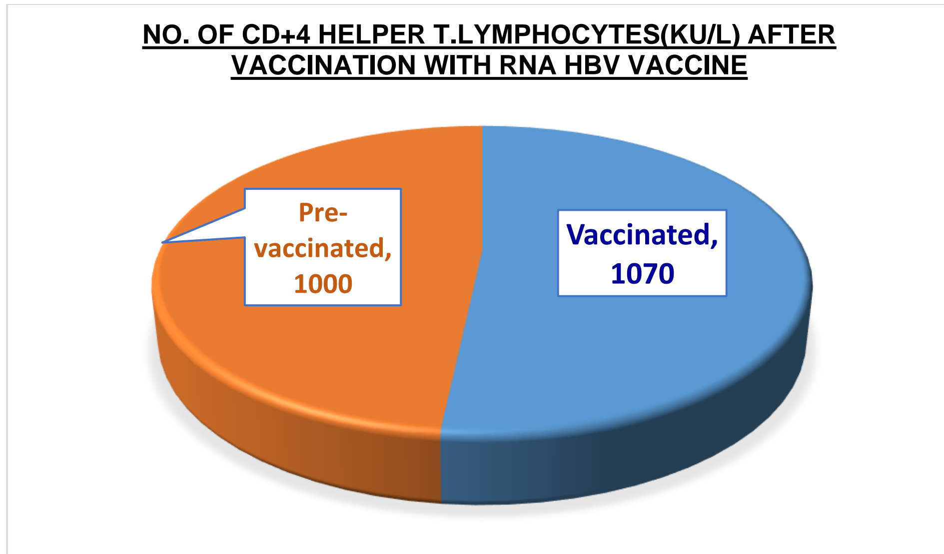


Graph 2. It represents the absorbance of different serum concentrations of neutralizing antibodies to surface active antigens HBV RNA vaccine via ELISA.

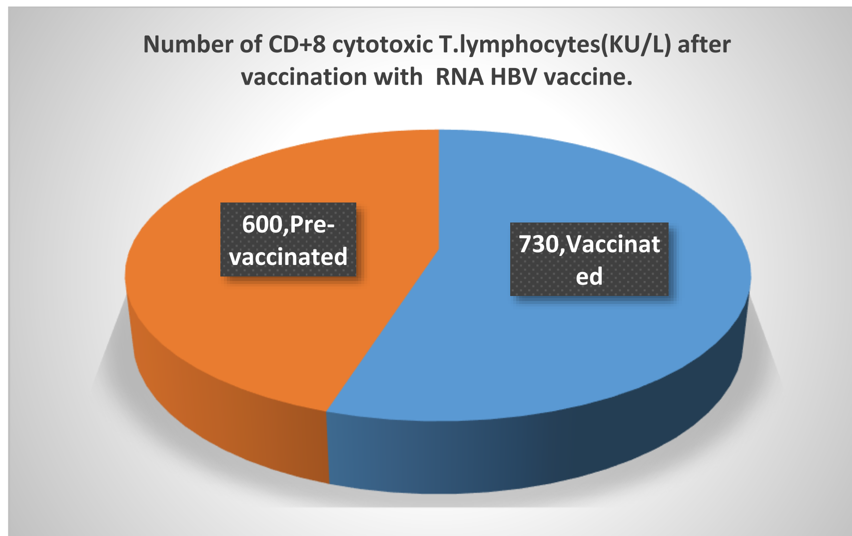


	test	standard	control
% infected	21	10	90
%noninfected	79	90	10

Graph 3. It shows that the protection power of test RNA HBV was 79% during clinical evaluation phases 1/2.



Graph 4. It represents moderate increase in the count of CD+4 helper T lymphocytes after vaccination with mRNA-HBV vaccine. This was based on the count of T.lymphocytes before and after vaccination.



Graph 5. It represents mild increase in the count of CD+8 cytotoxic T lymphocytes after vaccination with RNA HBV vaccine. This was based on the count of T.lymphocytes before and after vaccination.

Discussion

The goal of the current work was to create an HBV vaccination using lipid nanoparticles. In clinical testing stages 1/2, the test mRNA-HBV vaccine's protection efficacy was 79%. The hepatitis B surface antigen mRNA was successfully used to create a lipid nanoparticles vaccination in the current work. The existing HBV subunit vaccinations showed protective power below 90% in prior research by Luisa Romano et al, 2015.19 The present mRNA-HBV vaccine in this study showed 81% efficacy in preclinical studies (animal studies). In contrast, other standard subunit vaccines for hepatitis B surface antigen have shown efficacy of approximately 90%. Based on the protection power, the present trial mRNA-HBV vaccine showed moderate biological activity and fewer side effects than other standard vaccines. The effect was long lasting. In a human clinical trial, 90 people in the negative control group, 10 people in the positive control group (standard group), and 21 people in the test group were infected. Recombinant mRNA HBV transcripts can self-assemble and mimic the shape and size of HBV particles; but are unable to infect the host. The size of HBV virus-like particles (HBV-VLPs) generated from RNA transcripts introduced into host cells by injection was approximately 45 nm and formed solely a monolayer incorporating

coherent HBV structural surface antigen[that envelops the viral nucleocapside]. LD50% of Hepatitis B virus was found to contain more than 60 mcg/ml of virus. The ED50% of mRNA-HBV vaccine was ascertained to be 13 mcg/ml. The mRNA-HBV vaccine product concentration was 20 mcg/ml. The absorbance ratio of mRNA transcripts at 260 and 280 nm utilizing UV spectrophotometer was nearly 2, indicating high purity of recombinant mRNA transcripts refined by organic extraction method. The present mRNA-HBV vaccine is recommended for all age groups, especially young children and people in countries where HBV infection is prevalent. Test vaccines are contraindicated in people who were allergic to their ingredients. Observed side effects were mild pain at the site of intramuscular and intradermal injection and low-grade fever for several days, relieved by simple analgesics such as paracetamol and ibuprofen. The present vaccine contained RNA of HBV surface antigens which were the main mode of prevention. Storing the mRNA-HBV vaccine at -70°C was recommended. The vaccine strongly stimulated humoral immunity and moderately stimulated cell-mediated immunity. Humoral immunity is the body's primary defense mechanism against infection. The main neutralizing antibodies in blood were IgM, IgG1 and IgG2. The test vaccine moderately activated cell-mediated immunity against HBV infection. During the ELISA, high concentrations of neutralizing antibodies to the monolithic surfactant antigen protein were accompanied by high absorbance (optical density). In immunocompetent individuals, the mean number of normal CD+4 helper T lymphocytes was approximately 1000 ku/L, whereas after vaccination it exceeded 1000 ku/L. Moreover, the average number of CD+8 cytotoxic T lymphocytes increased from 600 KU/L to nearly 730 KU/L after the vaccination procedure. advantage: No reversion to virulence is possible. Cons: It was impossible for the vaccine viral particles to shed in feces and infect unimmunized contacts, so it did not contribute to the development of herd immunity to this serious infection worldwide; has a shorter duration of action than live attenuated vaccine; and must be refrigerated at -70°C to avoid spoilage and contamination.

Overview of bioinformatic analysis of hepatitis B surface antigen demonstrated that Hepatitis B surface antigen was composed of three structurally essential envelope glycoproteins. The large pre-S1 protein demonstrated an important role in HBV infectivity, the moderate pre-S2 protein displayed a minor role in infectivity; while the small S protein, was involved in viral morphogenesis and HBV entry into liver cells. All three proteins induced neutralizing antibodies that obstructed overwhelming fatal HBV infection. According to Hong Z, 2020 study, the recombinant subunit HBV vaccination offered defence against all HBV genotypes (from A to H).20 Additionally, the current mRNA-HBV vaccination in this trial showed encouragingly comparable outcomes to Hong Z et al., 2020 study.

Conflict of interest: There is no conflict of interest.

Funding: This study was done in a research project number 46361/2021 funded by STDF.

Conclusion

This study was a promising due to evolution of mRNA vaccine of hepatitis B surface antigens which demonstrated efficacy as prophylaxis against viral infection with hepatitis B virus. It can be used for all ages.

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