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Bioinformatics-based therapeutic RNA Hepatitis C Virus genotype 4 vaccine production and immunogenicity

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ABSTRACT

Background: *Hepatitis C* is a serious health challenge. Infection is often limited to the hepatocytes. Because of the Hepatitis C virus's high mutation rate, immunity lasts a long time and reinfection occurs (HCV). HCV infection causes chronic hepatitis, cirrhosis, and liver cancer. HCV affects around 2% of the world's population. It is a sexually transmitted and blood-borne infection. HCV vaccinations are being utilized as a preventive vaccination to prevent infection as well as therapeutic immunization to cure and prevent disease progression to cirrhosis and liver cancer. Because current HCV medications are limited, it is necessary to create preventative and therapeutic vaccinations to address this issue. Thus, bioinformatics is being employed in the development of a therapeutic mRNA vaccine against HCV by screening experimental testing. **Methodology**: A lipid nanoparticles mRNA vaccine including nonstructural NS2 transmembrane protein, highly conserved nonstructural NS3 and NS4A proteins was created using bionformactics. With a particle size of roughly 65 nm, the lipid nanoparticles vaccine delivery system was developed. Immunogenicity tests were performed on animals, followed by phases 1/2 of clinical trials. The lipid nanoparticles were synthesized by microemulsion technique.**Results**: In preclinical research, the therapeutic mRNA vaccine achieved 65% efficacy and 60% efficacy in human clinical trials stages 1 and 2. It demonstrated more biological activity and fewer side effects than other conventional immunizations now being studied in clinical trials. Long-term efficacy was achieved by eliciting powerful neutralizing antibodies to the NS2 nonstructural transmembrane protein and modest cell-mediated immunity against the NS3 and NS4A proteins.**Conclusion**: The therapeutic mRNA HCV genotype 4 immunization was both effective and long-lasting in the current study. It must be updated frequently to meet the issue of HCV's high mutation rate.

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Introduction

Hepatitis C virus[HCV] impacts roughly 170 million people on a chronic basis, and 3 to 4 million people become infected each year. Egypt has the greatest frequency (up to 15-20%). Chronic infection affects around 80% of newly infected patients. Cirrhosis of the liver, which affects 10% to 20% of persistently infected people, has been associated with an elevated risk of liver cancer (1% to 5% of chronically infected people during a 20- to 30-year period)[1]. Hepatitis C virus (HCV) is a positive-polarity enclosed single-stranded RNA virus that is the sole member of the genus Hepacivirus within the family Flaviviridae. Many modelling studies demonstrate that direct acting antiviral medicines (DAAs) will not be enough to eradicate HCV. Vaccines are essential for preventing viral infection because few medications are effective against HCV viral infections. Preventing viral HCV illnesses can be accomplished by the delivery of premade antibodies that give passive immunity or through the use of vaccinations that create active immunity[2]. Hepatitis C is a disease that is linked to hepatocellular cancer. In Egypt, HCV is the most common blood-borne pathogen. Yellowing of the skin (jaundice), dark urine, white-colored faeces, nausea, and pain in the upper right portion of the abdomen are all signs of acute hepatitis C infection. These indications and symptoms last between two and twelve weeks[3]. Characteristics: Virus enveloped with a single strand of positive-polarity RNA. There is no polymerase in the virion. HCV has many serotypes. HCV attaches to the surface of host liver cells via glycosaminoglycans (GAGs) and low density lipoprotein receptors (LDLR)[4]. Replicative Cycle Synopsis: It replicates in the cytoplasm and translates its genomic RNA into large polyproteins from which functional viral proteins are cleaved by a protease encoded by the virion. Strong anti-HCV medications target this protease. Moreover, HCV genome RNA encodes a protein known as NS5A, which collaborates with the virus's RNA polymerase to generate offspring genome RNAs. Effective anti-HCV medication also targets the NS5A protein. MiR-122, a liver-specific microRNA, promotes HCV replication in the liver. This micro-RNA operates by increasing HCV mRNA synthesis. It has been demonstrated that MicroRNAs boost cellular mRNA synthesis in a number of tissues. In 2013, researchers discovered that an anti-sense nucleotide that bonded to and hindered the action of miR-122 lowered HCV RNA levels in infected people for a prolonged length of time[5]. The bulk of transmission takes place via blood. Both sexual transmission and transmission from mother to kid are possible[6]. Pathogenesis: Cytotoxic T cells cause hepatocellular injury. Since HCV replication does not kill cells, it has no cytotoxic effect. More than half of all infections occur in the chronic carrier state. Chronic carriers are more likely to develop chronic hepatitis and hepatocellular carcinoma[7]. Serologic testing finds HCV antibodies in the blood. To assess if there is a present infection, a PCR-based test for "viral load" might be utilized[8].

Antibodies detected in donated blood can help prevent post-transfusion hepatitis. There is no vaccine, and there are no hyperimmune globulins available[9]. Therapy: The use of pegylated interferon alpha significantly reduces the number of people who become chronic carriers of hepatitis C. A combination of three medications is used to treat chronic hepatitis C: an RNA polymerase inhibitor (sofosbuvir), an NS5A inhibitor (ledipasvir), and a protease inhibitor (paritaprevir)[10]. Testing for immunogenicity of therapeutic mRNA Vaccination against HCV: Antigen and antibody reactions are extremely selective[11]. An antigen will only elicit antibodies in response to itself or a closely comparable antigen[12]. Because of their high specificity, interactions between antigens and antibodies can be used to identify one using the other. Several immunologic tests provide a titer, which is defined as the maximum dilution of the material, such as serum, that produces a positive reaction in the test[13]. It is important to keep in mind that a patient's serum with, say, an antibody titer of 1/64 contains more antibodies and has a higher titer than one with, say, a titer of 1/4. Evaluation of Enzyme-Linked Immunosorbent (ELISA)[14]. Antigens or antibodies in subject specimens can be quantified using this method. It operates by covalently bonding an enzyme to a recognized antigen or antibody, reacting the enzyme-linked substance with the patient's specimen, and then determining the enzyme activity by including the enzyme's substrate. The process is nearly as sensitive as radioimmunoassay (RIA), but it does not need specialized gear or radioactive labelling[15]. Flow Cytometry (Fluorescence-Activated Cell Sorting) is a test that is frequently used to count the various types of immunologic-ally activated blood cells. In this test, the patient's cells

are labelled with a monoclonal antibody unique to the target cell, such as the CD4 protein if the goal is to gauge the patient's helper T cell count. The monoclonal antibody has a luminous dye added to it, such fluorescein or rhodamine. A device known as a fluorescence-activated cell sorter passes single cells through a laser light beam to count the number of fluorescing cells (FACS)[16]. In order to combat the potentially deadly HCV viral disease, the current effort aims to produce a therapeutic lipid nanoparticles RNA HCV immunization. The objective of the study was the development of a therapeutic HCV RNA vaccine based on the co-expression of NS2, NS3, and NS4A proteins in host cells.

Material and methods

Material:

Algomhoria Pharmaceutical Company, Cairo, Egypt, and Alnasr Pharmaceutical Company, Abo zabal Alkhanka, Qalyobia

The pharmacology and toxicology division of the college of pharmacy at Cairo University in Egypt gathered them and determined their legitimacy.

Animal model inclusion standards:

Mature animals, such as transgenic mice weighing 40-50 gm, can be rendered by Hepatitis C virus infection.

Exclusion criteria:

Female pregnant mice; young mice.

Place and date of the study:

This research was conducted at Cairo University's Faculty of Pharmacy from 2020 to 2022.

Type of study:

Experimental testing investigation.

METHODS

Bioinformatics found possible open reading frames for highly conserved and coherent nonstructural NS2, NS3, and NS4A proteins.

The mRNA of the nonstructural conserved proteins NS2, NS3, and NS4A of the HCV genotype 4 serotype 4a that predominates in Egypt was produced and purified using the RNA organic extraction technique.

After utilizing PCR to clone the desired genes (cDNA), the plasmid is digested with Bam HI and Sphl restriction endonucleases II, which is followed by ligase enzyme ligation. The pDNA template was linearized by the restriction enzymes EcoRl, Eam11041, and Lugul (obtained from ThermoFisher scientific company, USA). The linearized pDNA template is in vitro transcribed into mRNA using a mixture of T7 recombinant RNA polymerase, nucleoside triphosphates, RNAse inhibitor, and 5X transcription buffer (obtained from ThermoFisher scientific company, USA). By including a cap analogue, such as the dinucleotide m7G(5,)-ppp-(5,)G, transcription caps mRNA transcripts (called regular cap analogue obtained from ThermoFisher scientific company,USA) Together with mRNA tailing, poly(A) polymerase changes mRNA transcripts (obtained from ThermoFisher scientific company,USA).

Afterwards, for post-in vitro transcription cleaning, DNase I and protein kinase K were added (both purchased from ThermoFisher Scientific Corporation in the United States). The purification process uses the mRNA extraction method. A technique known as acid guanidinium thiocyanate-phenol-chloroform extraction was used to isolate mRNA. It is a technique for liquid extraction-based RNA isolation.

It exhibits high RNA recovery and purity. Chloroform solutions were composed of a 25:24:1 solution of phenol, 96% chloroform, and 4% isoamyl alcohol (which ensures RNase inactivation and inhibits foaming).

The PH was tuned at 5 for mRNA purification, which favoured keeping mRNA in the aqueous phase. This method involved centrifuging an aqueous sample with a solution of phenol and chloroform that was water-saturated to create an upper aqueous phase and a lower organic phase (mainly phenol). A chaotropic substance called guanidinium thiocyanate was added to the organic phase to aid in the denaturation of RNases that break down mRNA. Protein partitioned in the organic phase whereas RNA did so in the aqueous phase. DNA separates into the organic phase in an acidic environment (PH 5), whilst mRNA remains in the aqueous phase. The aqueous phase was used to extract the RNA, which was then precipitated with 2-propanol, rinsed with ethanol, immediately dried by air drying, and dissolved in Ribonuclease-free water. The absorbance of mRNA transcripts was calculated at 260 and 280 nanometers.

The efficiency of mRNA transcripts was assessed using the absorbance ratio at 260 and 280 nm. mRNA transcripts were separated by agarose gel electrophoresis according to size, followed by denaturation and transfer to nitrocellulose

membrane, where they were hybridized to radio-labeled probes, in order to further analyse the quantities and sizes of recombinant mRNA transcripts. Dimethyl dioctadecyl ammonium bromide (DDAB), a quaternary ammonium lipid that forms compounds with mRNA and activates innate immunity, was used to make lipid nano-particles that formed bubbles around the recombinant mRNA transcripts in the vaccine delivery system. The lipid nanoparticles vaccine delivery system used 65nm-sized particles. The lipid nanoparticles were synthesized by microemulsion technique.

Formulation:

The intramuscular injection of the sterile HCV vaccine with lipid nanoparticles and mRNA is recommended. Each 1 mL dose comprises 0.5 mg of aluminium hydroxide, 50 mcg of dimethyl dioctadecyl ammonium bromide lipid (DDAB), and 10 mcg of HCV genotype 4 proteins NS2, NS3, and NS4A. Each dose also contains 0.619 mg of sodium dihydrogen phosphate dihydrate and 4.5 mg of sodium chloride.

100 transgenic mice were intraperitoneally injected with pure RNA to investigate the immunogenicity in animal models.

In vitro evaluation of vaccine on transgenic mice:

Transgenic mice are those whose genes have been altered using recombinant DNA and tissue culture techniques. An animal that has had a gene or DNA sequence (a trans-gene) artificially inserted into its genome is said to be transgenic. 100 transgenic mice were given the vaccine. They got two doses, separated by 21 days. Half of the second booster dose was given in the first dose. Not less than 100 RNA virions were required to cause substantial viremia and cellular immune responses from HCV.

The pathogens were genotype 4a serotype RNA HCV virions, which are widespread in Northern Africa, particularly Egypt. In order to boost the production of HCV viral proteins and effectively elicit humoral and cell-mediated immunity, human hepatocytes were injected into transgenic mice[17].

Bio-assay and testing of the natural activeness and toxicologic consequences of mRNA vaccine:

Vaccine potency was determined using protection testing.

Active: After being immunized with the vaccine under study, groups of transgenic mice were challenged with increasing numbers of microorganisms(ranged from 100 RNA virions to 1000 RNA virions). In order to assess the vaccine's protective potential, the lowest number of germs fatal for 50% of animals (i.e., LD50) is determined and compared to LD 50 in non-vaccinated animals.

Passive: Serum from vaccinated persons was delivered in graded quantities to normal mice, which were subsequently challenged with the infectious agent(ranged from 100 RNA virions to 1000 RNA virions). The highest serum dilution effective at protecting 50% of animals (i.e. ED50%) was established as a measure of vaccine effectiveness.

Human evaluation of mRNA HCV vaccines via randomized human clinical trials phases 1/2:

The present explore includes three groups of human participants. Each group had 100 people in it: The placebo was administered intramuscularly to Group(1) (the negative control group). The conventional HCV-VLPs vaccination was administered intramuscularly to group (2) (positive control group). The test therapeutic mRNA HCV vaccine was administered intramuscularly to group (3) (test group).

After two weeks, the three groups were challenged with varying doses of the pathogenic bacteria to see if protective neutralizing antibodies appeared. Following 21 days, the three groups were given intradermal booster dosages. The test vaccine's protective power was assessed during a two-year period. The protective antibodies were detected using an enzyme linked immunosorbent test (ELISA), whereas protective cell mediated immunity was assessed using a flow cytometry technique.

ELISA technique for therapeutic mRNA HCV vaccination neutralizing antibody detection:

An 9 µl Antigen suspension/ well was affixed to the well's bottom. Antibodies bound to antigen in serum samples from patients. The antibody to human IgG was joined to the patient's IgG and enzyme-linked for 15 minutes (100µl Horseradish peroxidase enzyme/well). For 15 minutes, a substrate of 70 µl/well was introduced for the enzyme, which changes colour when acted upon by the enzyme. Enzyme activity is evaluated in a UV spectrophotometer at 450nm by applying the enzyme substrate and measuring the colour response. Throughout this procedure, an ELISA

MPR-A9600/MPR-A9600T microplate reader was used. Its wavelength varied from 340 to 750 nm, with an absorbance range of 0.000 to 4.000 Abs and an incubation temperature of +4 to 50 degrees Celsius[18].

Flow cytometry for detection and counting CD+4 and CD+8 T lymphocytes specific to therapeutic mRNA HCV vaccine:

Equipment: Invitrogen Attune Cytpix flow cytometer, USA.

A cell interacted with a fluorescent Rhodamine dye-labeled monoclonal antibody. When the cell moved down the tube, ultraviolet light caused the dye to glow, and a sensor counted how many cells were in the tube[19].

Statistical analysis

Triplets were used for every culture. Means and standard deviation served as the framework for their presentation. A one-way analysis of variance (p value 0.05) and statistical analysis using Excel spreadsheet software were both used to complete the statistical analysis. F test was utilized all through this investigation.

RESULTS

In preclinical (animal) studies, the non-adjuvanted lipid nanoparticle RNA HCV therapeutic vaccine achieved 65% effectiveness, but only 60% efficacy in human clinical trials. 90 participants in the negative control group, 41 in the positive control group (standard), and 40 in the test group got infected during human clinical trials. The UV spectrophotometer absorbance ratio of recombinant mRNA transcripts at 260 and 280 nm was nearly 2, suggesting that the organic extraction technique produced exceptionally pure recombinant mRNA transcripts. The HCV virus's LD50 was discovered to be greater than 60 mcg/mL. With the RNA-HCV vaccine, the ED50% was 16 mcg/ml.

The RNA-HCV vaccine formulation contained 30 mcg/ml of HCV genotype 4 proteins (NS2, 10 mcg/ml; NS3, 10 mcg/ml; NS4A, 10 mcg/ml HCV genotype 4 proteins). Immunogenicity rose from 60% to 68% in the presence of 0.5 mg of aluminium hydroxide as an adjuvant. HCV-infected convalescent human sera identified purified RNA transcripts as heterogeneous vesicular structures. Small animal models and human vaccination attempts have resulted in the production of neutralizing antibodies, the induction of cell-mediated immunity, and the protection against sickness produced by a subsequent live viral challenge.

Table 2 depicts the efficacy of a therapeutic RNA HCV vaccine throughout phase 1/2 clinical trials. Table 5 shows the ELISA absorbance of various serum neutralizing antibodies to RNA HCV vaccination. Graph 5 depicts an increase in the number of cytotoxic CD+8 T cells following immunization with a therapeutic RNA HCV vaccine. The immunization of transgenic mice against Hepatitis C virus genotype 4 serotype 4a is shown in Table 4. Table 3 shows the T lymphocyte count after immunization against Hepatitis C virus genotype 4. Graph 4 depicts an increase in the number of helper CD+4 T cells following immunization with an RNA HCV therapeutic vaccine. Graph 2 demonstrates that the mRNA HCV vaccine provided 60% protection throughout human clinical trials stages 1-2. Graph 3 depicts the ELISA absorbance of various serum concentrations of neutralizing antibodies to therapeutic RNA HCV vaccination. Graph 1 indicates that the mRNA HCV vaccine provided 65% protection throughout preclinical animal testing. Figure 1 depicts the various quantities and sizes of mRNA transcripts of NS2, NS3, and NS4A proteins determined using the Northern method. The purity of mRNA transcripts was estimated to be around 85%.

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 -80 ^{0}C	Artiko
Refrigerator 5	whirlpool
PH meter electrode	Mettler-Toledo
Deep freezer -20 ^{0}C	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 protection power of therapeutic RNA HCV vaccine during clinical trials phases1/2:

	vaccinated		**nonvaccinated**
Description	test	standard	control
% infected	40	41	90
%noninfected	60	59	10

Table 3. It represents the count of T. lymphocytes following vaccination against Hepatitis C virus genotype 4:

Description	**Vaccinated**	**Pre-vaccinated**
CD+4 COUNT[Ku/l]	1160	1000
CD+8 COUNT[Ku/l]	740	600
Total	**1900**	**1600**

Table 4. Vaccination of transgenic mice against Hepatitis C virus genotype 4:

Description	**Vaccinated**	**Non-vaccinated**
Alive	65	21
Dead	33	79
Total	**100**	**100**

Table 5. It represents the absorbance of different serum neutralizing antibodies to RNA HCV vaccine via ELISA:

Concentration(ng/ml)	Absorbance
1.55	0.18
3.07	0.265
6.19	0.331
12.6	0.558
25	1.029
50	1.902
100	3.048

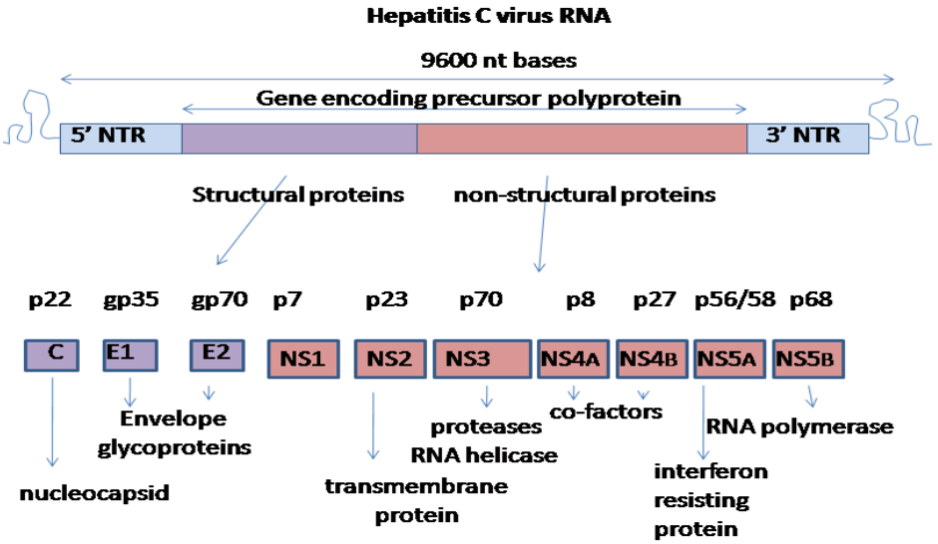
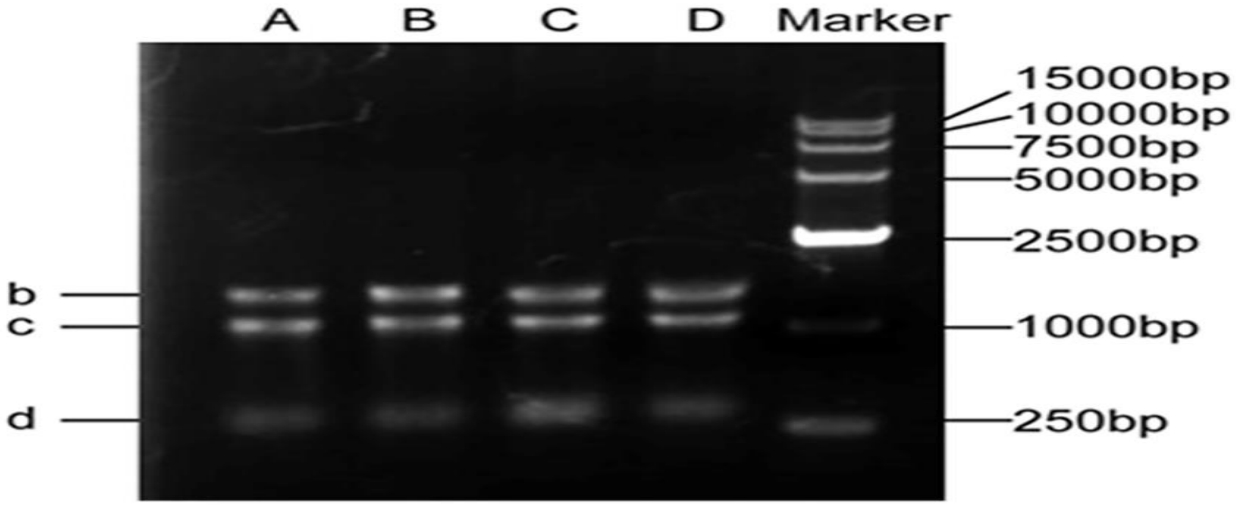
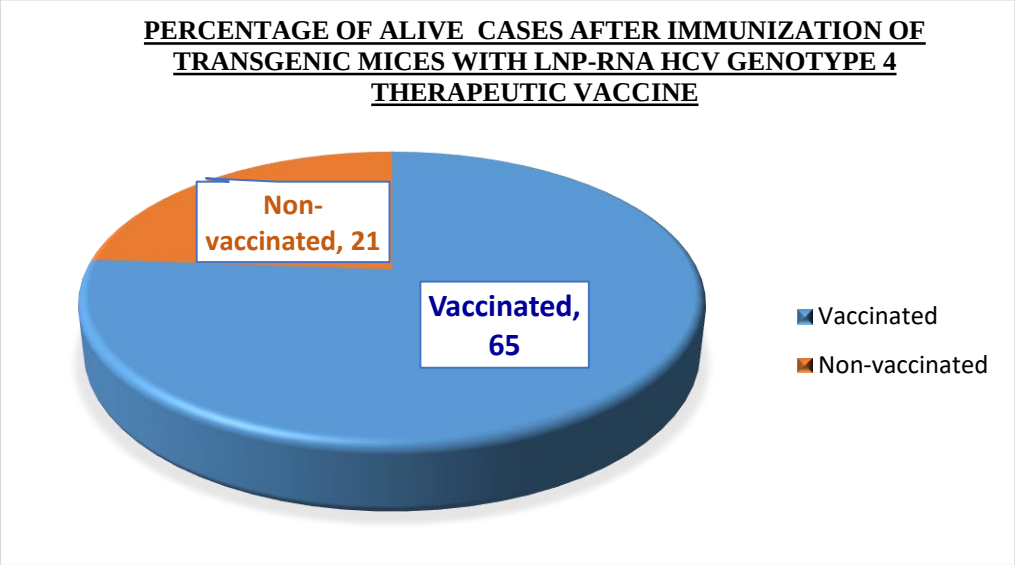


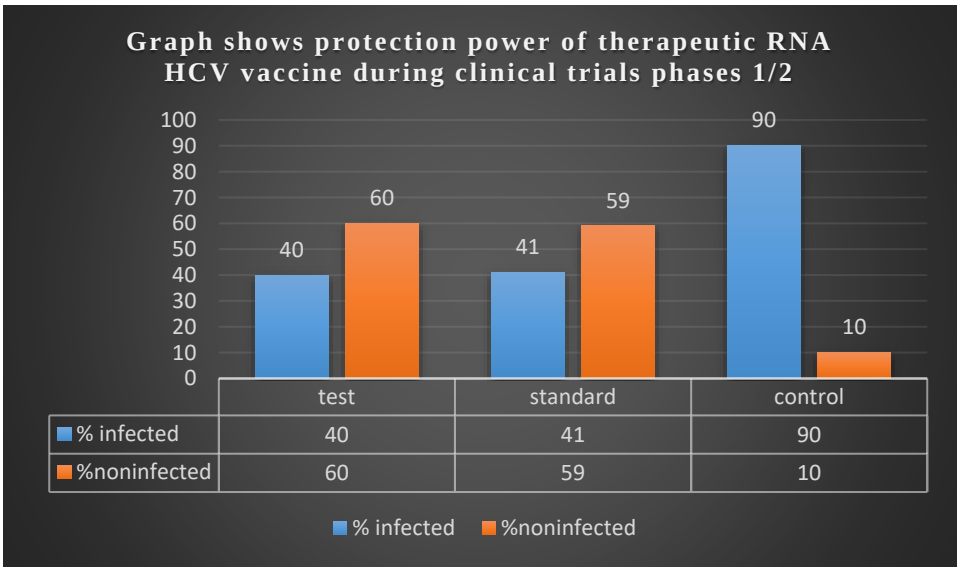
Figure 1. It displays various Northern technique-measured mRNA transcript quantities and sizes for the NS2, NS3, and NS4A proteins. There were around 85% of mRNA transcripts that were pure.



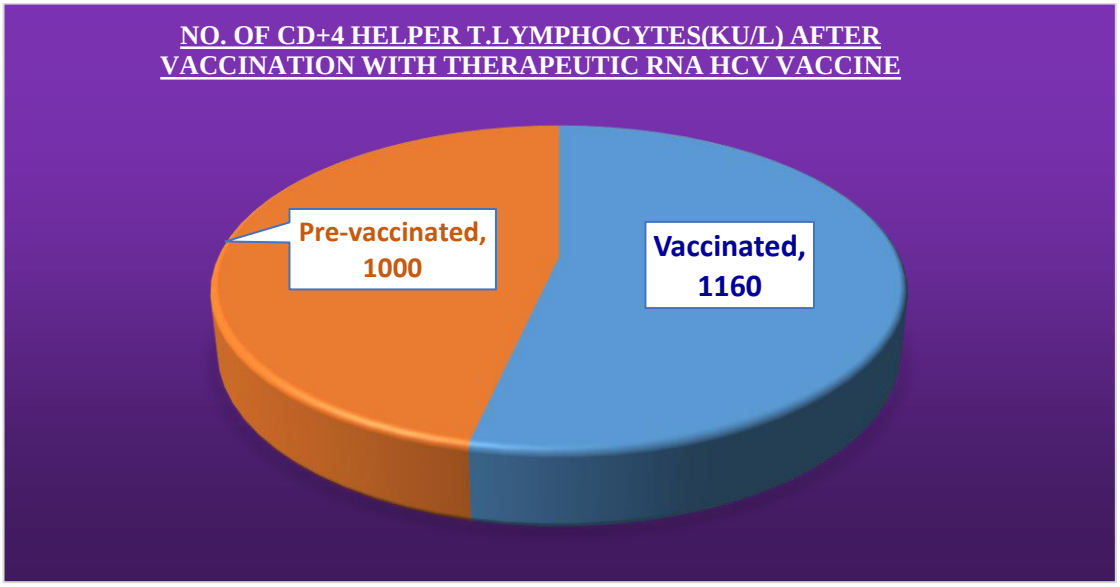
Graph 1. It demonstrates that 65% of animals were protected by the mRNA HCV vaccination throughout preclinical testing.



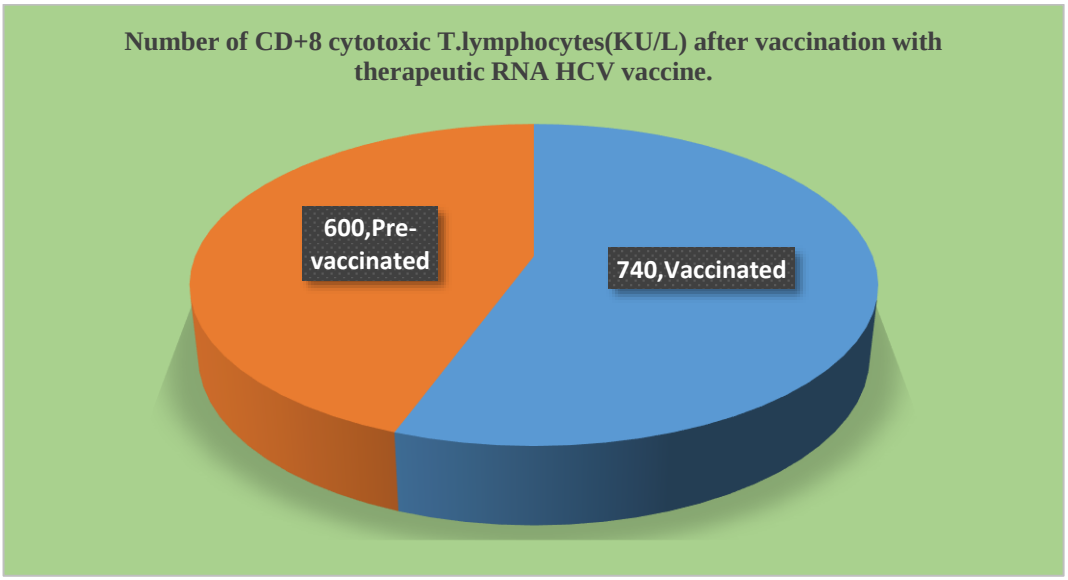
Graph 2. It demonstrates that throughout stages 1-2 of human clinical trials, the mRNA HCV vaccine's protective efficacy was 60%.



Graph 3. It depicts the absorbance of various serum concentrations of therapeutic RNA HCV vaccine-neutralizing antibodies using ELISA.



Graph 5. It denotes a rise in the number of cytotoxic CD+8 T cells following the administration of the therapeutic RNA HCV vaccine.



Discussion

To address the global burden of Hepatitis C virus infection, the current research generated a novel therapeutic mRNA-HCV vaccine. In preclinical investigations (animal testing), the non-adjuvant therapeutic lipid nanoparticles RNA HCV vaccine was 65% effective, but only 60% successful in human clinical trials (phases 1/2). They showed significant biological activity and rather mild negative effects. Their effectiveness was long lasting because to the quick pace of HCV virus alterations, although the immunisation required to be refreshed often. In human clinical trials, the test group consisted of 40 participants, 41 participants in the positive control (standard) group, and 90 participants in the negative control group. The recombinant mRNA transcripts purified by organic extraction showed high purity, as evidenced by the

absorbance ratio of mRNA transcripts determined using a UV spectrophotometer at 260 and 280 nm being close to 2. The HCV virus was shown to have an LD50 greater than 60 mcg/ml. The RNA HCV vaccine's ED50% was established to be 16 mcg/ml. The RNA HCV vaccine formulation contained 30 mcg/ml of HCV genotype 4 proteins together with 10 mcg/ml of NS2, 10 mcg/ml of NS3, and 10 mcg/ml of NS4A. Over two-year-olds should receive a therapeutic RNA HCV immunization. The recommended dosage for this vaccination is 30 mcg/ml given intramuscularly, followed 21 days later by a booster shot given intradermally. In the addition of 0.5 mg of aluminium hydroxide as an adjuvant, immunogenicity increased from 60% to 68%.

For those who are allergic to any of the vaccine's components, the test immunization is not advised. The only adverse effects were moderate discomfort at the injection sites in the muscles and skin for a few days and a mild fever, both of which were managed with common analgesics such ibuprofen and paracetamol. The current vaccination, which contains highly conserved and coherent nonstructural NS2, NS3, and NS4A genotype 4 proteins, offers the main means of defence. The recommended storage temperature for therapeutic RNA HCV vaccines is -70 0C[20]. Infection prevention and the function of immunity The humoral immune system was only mildly activated by therapeutic RNA HCV vaccinations, whereas the cell-mediated immune system was highly stimulated. Cell-mediated immunity played a crucial role in the body's ability to defend itself against the virus. The best anti-HCV treatments were cytotoxic CD+8 T cells and helper CD+4 T lymphocytes. These shots only slightly triggered the humoral immune system's defence against viral infection. IgM, IgG1, and IgG3 were the main neutralizing antibodies in blood against the coherent nonstructural proteins of HCV genotype 4 (NS2 proteins). As a result of the immunizations not being given orally or through the respiratory route, little IgA antibody was produced against them. Although no detectable antibodies were generated against the intracellular proteins NS3, NS4A, both proteins were important in eliciting cell mediated immunity. High quantities of neutralizing antibodies to NS2 nonstructural transmembrane proteins were associated with increased absorbance during ELISA (optical density). The typical normal CD+4 helper T cell count in immunocompetent participants was around 1000 Ku/L, but after immunization it exceeded 1000 Ku/L. Also, the average number of CD+8 cytotoxic T cells rose from 600 to approximately 740 Ku/L following immunization. Advantages: No reversion to virulence is conceivable.

Disadvantages: Since vaccine virus excretion and transmission to non-immune contacts are not feasible, it does not contribute to the establishment of herd immunity against HCV infection.

No one vaccine candidate made it through phase II clinical effectiveness testing in humans, per the Alex S et al., 2021 research. This recently concluded experiment, which employed recombinant viral vectors to activate pan-genotype T cell responses against HCV nonstructural proteins, failed to prevent chronic infection in a cohort of high-risk injection drug users, despite strong preclinical data suggesting its potential effectiveness[21]. The present study immunization, on the other hand, exhibited protection against the onset of chronic infection in a group of high-risk injectable drug users. Overview of bio-informatics analysis of HCV genotype 4 nonstructural NS2, NS3 and NS4A proteins: NS3 and NS4A are both highly conserved intracellular nonstructural (functional) proteins. They are involved in HCV replication, and antibodies that inhibit the interaction between these proteins and the receptors on the cell membrane of hepatocytes operate as neutralizing antibodies as well as eliciting robust cell-mediated immunity against them. NS2 is a conserved nonstructural(functional) type 1 transmembrane protein that plays an important role in viral entrance and replication, and antibodies that disrupt the interaction between these proteins and the receptors on the hepatocyte cell membrane are effective neutralizers.

Conclusion:

HCV vaccination candidates with therapeutic mRNA are viewed as promising. They were effective in preventing viral infection with hepatitis C virus genotype 4 and mutant variants. Anyone above the age of two can use them.

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