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RESEARCH ARTICLE

THE EFFECT OF A MULTI-EPITOPE COVID-19 VACCINE WITH CHITOSAN ON THE CARDIAC HISTOPATHOLOGY OF BALB/C MICE

(PENGARUH VAKSIN COVID-19 MULTIEPITOP DENGAN KITOSAN TERHADAP HISTOPATOLOGI JANTUNG MENCIT BALB/C)

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ABSTRACT

The multi-epitope COVID-19 vaccine contains epitopes designed to induce an immune response, using SARS-CoV-2 as the model antigen. This study examined its effects on the cardiac histopathology of male BALB/c mice. A total of 25 mice were divided into five groups: a negative control that received no treatment (K), a 500 µg multi-epitope COVID-19 vaccine evaluated on day 7 after the second dose (P1), a 500 µg multi-epitope COVID-19 vaccine evaluated on day 14 after the second dose (P2), a 500 µg multi-epitope COVID-19 vaccine formulated with chitosan and evaluated on day 7 after the second dose (P3), and a 500 µg multi-epitope COVID-19 vaccine formulated with chitosan and evaluated on day 14 after the second dose (P4). Myocardial tissue was examined microscopically for inflammatory cell infiltration, degeneration, and necrosis. The study used a completely randomized design with a post-test-only control group. Based on the Dallas criteria, no myocarditis was identified, with or without necrosis, as all groups showed no inflammatory cell infiltration. However, vacuolar and fatty degeneration were observed in both the negative control and the treatment groups (P1, P2, P3, P4), which were likely unrelated to the administration of the multi-epitope COVID-19 vaccine, with or without chitosan adjuvant. These findings indicate that the vaccine induces an immune response without causing hyperactivation or hypersensitivity, so the response remains limited and controlled.

Keywords: cardiac histopathology, cardiomyocyte degeneration, chitosan adjuvant, COVID-19, multi-epitope vaccine

ABSTRAK
Vaksin COVID-19 multiepitop adalah jenis vaksin yang terdiri dari epitop-epitop sebagai unit dasar yang menghasilkan respons imun dengan SARS-CoV-2 sebagai model pembuatannya. Penelitian ini bertujuan untuk mengetahui pengaruh pemberian vaksin COVID-19 multiepitop terhadap gambaran histopatologi jantung mencit jantan galur BALB/c. Penelitian dilakukan pada 25 ekor mencit jantan Galur BALB/c dengan lima kelompok, yaitu kontrol negatif yang tidak diberikan perlakuan (K), vaksin COVID-19 multiepitop dosis 500 µg yang dinilai pada hari ke-7 pasca second dose (P1), vaksin COVID-19 multiepitop dosis 500 µg yang dinilai pada hari ke-14 pasca second dose (P2), vaksin COVID-19 multiepitop dosis 500 µg dengan tambahan adjuvan kitosan yang dinilai pada hari ke-7 pasca second dose (P3), vaksin COVID-19 multiepitop dosis 500 µg dengan tambahan adjuvan kitosan yang dinilai pada hari ke-14 pasca second dose (P4). Penilaian dilakukan secara mikroskopis dari infiltrasi sel-sel inflamasi, degenerasi, dan nekrosis pada miokardium jantung mencit. Penelitian menggunakan rancangan acak lengkap dengan rancangan penelitian post-test only control group design. Hasil penelitian berdasarkan kriteria Dallas menunjukkan tidak adanya tanda-tanda miokarditis dengan atau tanpa adanya nekrosis, karena semua kelompok tidak mengalami infiltrasi sel inflamasi. Namun, degenerasi vakuolar dan degenerasi lemak ditemukan pada kelompok kontrol negatif serta kelompok perlakuan (P1, P2, P3, P4), yang kemungkinan tidak terkait dengan pemberian vaksin COVID-19 multiepitop, baik dengan maupun tanpa adjuvan kitosan. Temuan ini menunjukkan bahwa vaksin mampu memicu respons imun tanpa menyebabkan hiperaktivasi atau hipersensitivitas, sehingga respons imun yang dihasilkan tetap terbatas dan terkontrol.

Kata kunci: adjuvan kitosan, COVID-19, degenerasi kardiomiosit, histopatologi jantung, vaksin multiepitop

INTRODUCTION

Coronavirus Disease 2019 (COVID-19) is an infectious disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2).[1] COVID-19 can be prevented through vaccination. COVID-19 vaccines are classified into several platforms, including inactivated vaccines, viral vector vaccines, nucleic acid approaches that utilize DNA or mRNA, and subunit vaccines, each of which induces an immune response through different mechanisms.[2,3] All of these platforms have been shown to be safe in clinical trials. However, various complications and adverse effects continue to be reported. One example is cardiac complications such as myocarditis, particularly associated with mRNA vaccine platforms.[4–6] Histopathological examinations have demonstrated focal and interstitial inflammatory cell infiltration in the myocardium, along with cardiomyocyte degeneration and necrosis.[7,8]

Multi-epitope vaccines containing both T-cell and B-cell epitopes are currently being developed. These vaccines are capable of inducing cellular and humoral immune responses against specific pathogens. They also carry a lower biohazard risk compared to other vaccine types because they are designed to

minimize components that may trigger pathological immune responses or undesirable side effects.[9,10] Multi-epitope vaccines can be combined with adjuvants to enhance immunogenicity.[9,11–13] One example is chitosan, a nano-adjuvant typically within the size range of 1–1000 µm.[12,13] In this study, a multi-epitope vaccine using SARS-CoV-2 as the model for epitope development and chitosan as an adjuvant was used to evaluate its effects on the cardiac histopathology of BALB/c mice. The results were compared based on the presence or absence of chitosan as an adjuvant in the multi-epitope COVID-19 vaccine.[13,14]

MATERIALS AND METHODS

This study was an experimental laboratory investigation using BALB/c mice as experimental animals. The research design used was a post-test-only control group design. One control group and four treatment groups were included according to the scheme in Figure 1, with a Completely Randomized Design (CRD).

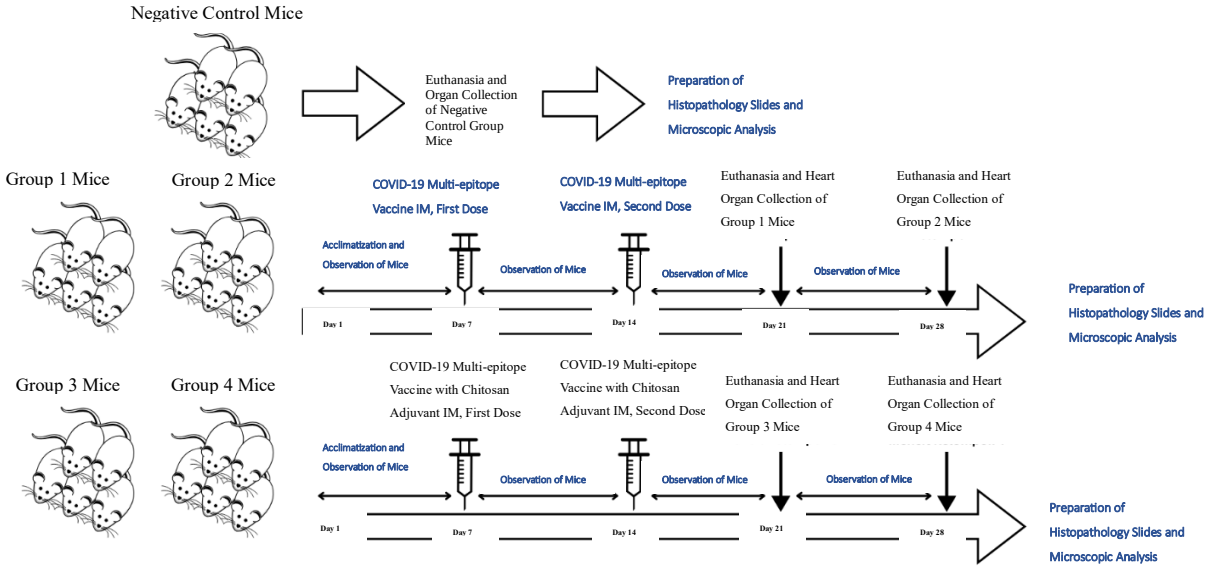

Figure 1 Research scheme for each group.

The preparation of the chitosan adjuvant and vaccine formulation was conducted at the Biochemistry Laboratory of Universitas Padjadjaran and the Microbiology Laboratory of the Faculty of Medicine, Jenderal Achmad Yani University, in September 2024. The multi-epitope COVID-19 vaccine was obtained from purified cultures of Escherichia coli (E. coli). The chitosan used in this study was commercial chitosan processed from shrimp shells, in powder form, white to orange in color, with a molecular weight ranging from 3,800 to 20,000 Daltons. Its technical specifications included a solubility of 10 mg in 1 mL of dilute acetic

acid, a moisture content (based on the Karl Fischer method) not exceeding 10.00%, an ash content not exceeding 2.0%, and a degree of deacetylation of ≥75.00%.

Preparation of nanochitosan was performed using a titration method that required two solutions. Solution one consisted of 2% acetic acid, into which chitosan was added in a beaker. Solution two consisted of sodium tripolyphosphate dissolved in distilled water and stirred until fully dissolved. Solution two was then placed in a burette, and solution one was placed on a magnetic stirrer beneath the burette. Solution two was added dropwise into solution one while stirring until a cloudy appearance developed, resulting in a chitosan-to-sodium tripolyphosphate ratio of 3:1. The interaction between the two solutions caused the chitosan to crosslink with the tripolyphosphate, forming nanoparticles.[15]

The maintenance, treatment, termination, and cardiac organ collection were conducted at the Animal Laboratory of the Faculty of Medicine, Unjani, from September to October 2024. Maintenance was carried out concurrently with acclimatization and observation. The mice used in this study were male BALB/c mice aged six to eight weeks, weighing 20 to 40 grams, healthy, active, free of wounds or deformities, and had never received prior drug treatment.[16,17] A total of twenty-five mice were used, with five mice per group, based on the Federer formula. The mice were weighed and assessed for homogeneity using an animal scale with a target body mass of 20 to 40 grams. If a mouse experienced more than a 20% reduction from its initial body weight, it was excluded from the study. The same exclusion applied to any mouse that died during the study. Mice were removed from the cage carefully using proper handling techniques.[16]

Three types of tools and materials were used during maintenance. First, adult mouse feed was provided at three to five grams per day. Second, drinking water was supplied *ad libitum* through a sterilized bottle, with a daily intake ranging from three to six milliliters, and the bottle was sterilized once every two weeks. Third, cages measuring 30 × 27 × 20 centimeters, made of materials resistant to gnawing, were prepared with approximately three centimeters of wood shavings as bedding. The bedding was replaced every three days.[16,18]

The treatment was administered by intramuscular injection of the multi-epitope COVID-19 vaccine. A one-milliliter syringe fitted with a 25-gauge needle was used, and injections were given into the thigh muscle.[16] Mice in groups 1 and 2 received 400 micrograms of the multi-epitope COVID-19 vaccine per mouse. Groups 3

and 4 received 500 micrograms of the vaccine combined with chitosan adjuvant per mouse.

The tools used during treatment included a one-milliliter syringe, a centrifuge tube, and gloves. The materials consisted of the multi-epitope vaccine, chitosan adjuvant, distilled water, and tissues.

Euthanasia was performed using a physical technique, namely cervical dislocation. Before euthanasia, mice were anesthetized by inhalation in a CO_2 chamber. After anesthesia, the mice were euthanized using the physical cervical dislocation method.[16,19]

Cardiac organ collection was performed after euthanasia. The mouse was placed in a supine position on a surgical board. The skin of the abdomen along the midsagittal line, the rectus abdominis muscle, and the peritoneal layer were lifted using forceps. The incision was extended cranially with surgical scissors in a vertical direction and then continued laterally to the right and left anterior regions. The incision was further extended dorsally until reaching the area beneath both forelimbs. The heart was then carefully removed by gently separating any remaining lung or tracheal tissue surrounding the organ, and care was taken to avoid separating the thymus from the heart.[19,20] Before preparing the histological specimen, the heart was sectioned beginning from the apex. The cut was angled to divide the four cardiac chambers into two halves. If space allowed, both halves of the heart were used for observation.[20]

The tools and materials used for euthanasia and dissection included containers, scalpels, tissue scissors, and gloves. The material used was the treated male BALB/c mice.

Specimen preparation was carried out in the Anatomical Pathology Laboratory in October 2024. The process involved fixation, dehydration, infiltration, embedding, clearing, sectioning, staining, and mounting. After these steps were completed, the slides were ready for microscopic examination. The tools and materials required included a microtome, paraffin containers, cutting instruments, glass slides, cover slips, a hot plate, an incubator, a slide holder, and forceps.[20,21]

Observation of the study results was conducted at the Anatomical Pathology Laboratory of the Faculty of Medicine, Unjani, in October 2024. Microscopic examination was performed using a microscope at 400x magnification across five fields of view of the myocardium. The assessment included the presence of inflammatory cell infiltration, degeneration, and necrosis. A total of five fields of view were examined microscopically. The criteria used in this study were based on the

Dallas Criteria for grading and diagnosing myocarditis, as shown in Table 1.[22–25]

Table 1 Microscopic grades according to the Dallas Criteria

Grade	**Description**
Grade 0 (No Myocarditis)	No inflammatory cell infiltration, degeneration, or necrosis in the myocardium.
Grade 1 (Borderline Myocarditis)	Presence of inflammatory cell infiltration in the myocardium, without degeneration or necrosis.
Grade 2 (Active Myocarditis)	Presence of inflammatory cell infiltration in the myocardium, accompanied by degeneration or necrosis.

Adapted from Caforio, 2012.[25]

Data analysis was performed using Statistical Product and Service Solutions (SPSS). Categorical data were analyzed using the chi-square test to determine correlations and assess the significance of each variable. Logistic regression was applied to evaluate the likelihood between variables. The primary variable assessed was structural changes in the myocardium accompanied by inflammatory cell infiltration.

This study adhered to the principles of the Five Freedoms. These include freedom from hunger, freedom from discomfort, freedom from injury, freedom from fear and distress, and freedom to express normal behavior.[16] In addition, the study followed laboratory animal use guidelines based on the 3Rs, which consist of replacement, reduction, and refinement.[26]

The use of experimental animals in this study received ethical approval from the Animal Ethics Committee of the Faculty of Medicine, Universitas Jenderal Achmad Yani. Ethical approval is documented in the ethical clearance letter No. 026/UH2.11/2024 and protocol No. H2.2410.032, issued on November 4, 2024.

RESULTS AND DISCUSSION

Microscopic examination showed no inflammatory cell infiltration or necrosis. However, vacuolar degeneration and fatty degeneration were observed in the cardiomyocytes of the negative control group, Group 1, Group 2, Group 3, and Group 4, as shown in Figure 2, Figure 3, and Figure 4. In addition, during observation, the mice showed increased body weight, no behavioral changes, and no discoloration of the heart.

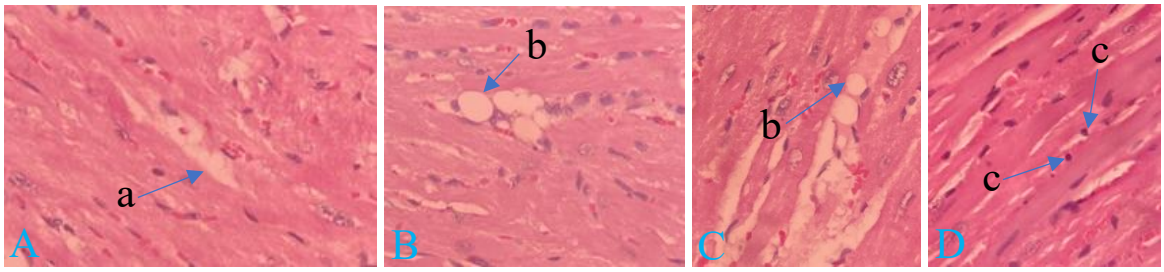

Figure 2 Microscopic appearance of the mouse heart in the negative control group.

Description : A. Mouse 1, negative control group
B. Mouse 2, negative control group
C. Mouse 3, negative control group
D. Mouse 4, negative control group
Arrow a: vacuolar degeneration
Arrow b: fatty degeneration
Arrow c: inflammatory cells

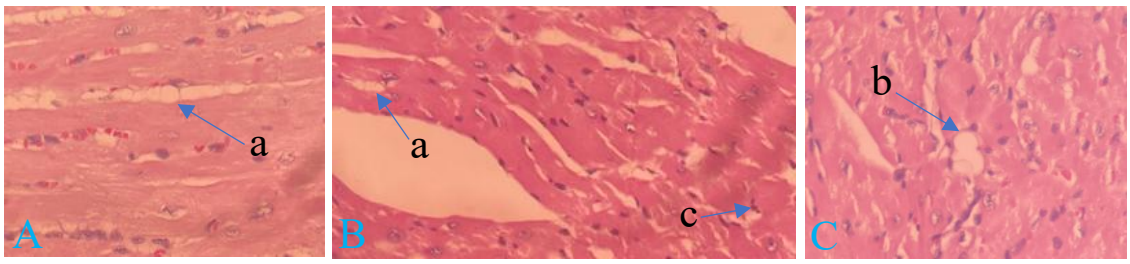

Figure 3 Microscopic appearance of the mouse heart on day seven after the second dose.

Description: A. Mouse 2, Group 1
B. Mouse 2, Group 2
C. Mouse 5, Group 2
Arrow a: vacuolar degeneration
Arrow b: fatty degeneration
Arrow c: inflammatory cells

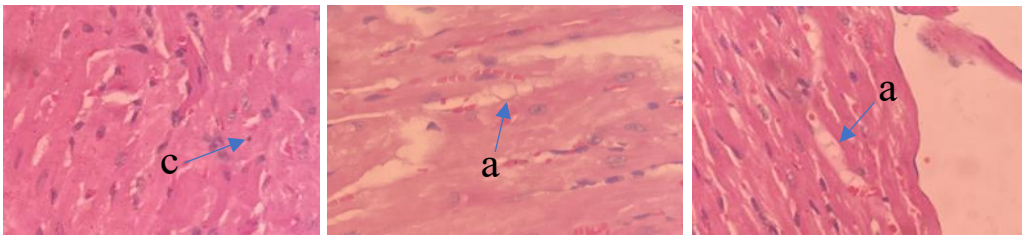

Figure 4 Microscopic appearance of the mouse heart on day fourteen after the second dose.

Description: A. Mouse 5, Group 3
B. Mouse 4, Group 3
C. Mouse 4, Group 4
Arrow a: vacuolar degeneration
Arrow b: fatty degeneration
Arrow c: inflammatory cells

Inflammatory cells that appear only once or twice in a single field of view do not qualify as a basis for diagnosing myocarditis according to the Dallas criteria.[23] The presence of vacuolar degeneration and fatty degeneration is

caused by factors other than vaccination. These factors include hypoxia, environmental toxins, infectious agents, genetic abnormalities, physical injury, and cellular aging. The most likely cause of cellular injury leading to degeneration in this study is hypoxia resulting from CO_2 exposure prior to euthanasia. Hypoxia causes mitochondrial damage and dysfunction. This leads to failure of ATP production. Reduced ATP levels decrease the activity of ATP-dependent sodium pumps in the plasma membrane. As a result, intracellular sodium accumulates, and potassium exits the cell. The increase in intracellular solute concentration, followed by iso-osmotic water influx, causes cellular swelling and dilation of the endoplasmic reticulum. Under microscopic examination, this appears as small, clear cytoplasmic vacuoles. These represent distended or pinched segments of the endoplasmic reticulum, a pattern known as vacuolar degeneration. Mitochondrial injury also disrupts oxidative phosphorylation and promotes the formation of reactive oxygen species (ROS). These molecules damage cellular components. When organelles and lipids are affected, fat can accumulate in injured cells, resulting in fatty degeneration.[27]

The immune response mechanism induced by the multi-epitope COVID-19 vaccine is associated with the epitopes incorporated into the vaccine, which consist of three structural proteins: Membrane (M), Envelope (E), and Spike (S). The receptor-binding domain (RBD) of the spike protein is also included in epitope construction. The antigen combinations used—RBD-E-M and M-RBD-E—are capable of eliciting strong cellular and humoral immune responses.[10,27–29] These epitopes exhibit high antigenicity and immunogenicity, enabling them to be recognized effectively and stimulate an appropriate immune response. Their allergenicity is relatively low, which minimizes the risk of hypersensitivity and excessive immune activation.[27–29]

The characteristics of these epitopes result in limited cytokine release by antigen-presenting cells (APCs). Thus, APCs do not recruit large numbers of leukocytes that would contribute to inflammatory cell infiltration. In contrast, high-allergenicity epitopes would trigger hypersensitivity reactions, leading to excessive cytokine release by APCs, followed by over-recruitment of inflammatory cells capable of releasing lysosomal enzymes and ROS into surrounding tissues, potentially inducing cellular injury such as degeneration or necrosis. In the adaptive immune response, effector cells do not excessively release perforin, granulysin, granzymes, or ROS.

Therefore, uncontrolled cell death leading to necrosis or apoptosis does not occur.[10,27]

Chitosan, used as an adjuvant, enhances vaccine performance through its mechanism of action. Its positive charge binds to negatively charged antigens, allowing the antigen to be retained and released gradually before being presented to APCs. This controlled release helps regulate immune activation, preventing pathological immune responses.[12,13]

CONCLUSION

Administration of the COVID-19 multi-epitope vaccine, both with and without chitosan as an adjuvant, does not affect microscopic changes in the hearts of mice. There is no difference in the microscopic effects between the COVID-19 multi-epitope vaccine alone and the COVID-19 multi-epitope vaccine combined with a chitosan adjuvant on mouse cardiac tissue.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in the preparation of this scientific article.

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