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ORIGINAL ARTICLE

Effects of a multiepitope covid-19 vaccine with chitosan nano-adjuvant on colon histopathology in BALB/c mice

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ABSTRACT

The COVID-19 pandemic caused by SARS-CoV-2 has posed major global challenges in infectious disease control. Vaccination remains the primary strategy to reduce morbidity and mortality; however, concerns persist regarding vaccine safety, including gastrointestinal adverse events. Multiepitope vaccines represent a promising approach to induce broader immune responses, while chitosan nanoparticles are widely studied as biocompatible adjuvants to enhance vaccine safety and efficacy. This study aimed to evaluate the histopathological effects of a multiepitope COVID-19 vaccine, with and without chitosan nanoparticles, on the colon of male BALB/c mice. A post-test-only control group design was employed using 25 male BALB/c mice, divided into five groups (n = 5 per group). Colon tissues were examined histopathologically using Hematoxylin–Eosin (H&E) staining and assessed according to the Harpaz Histological Scoring System (HSS). The results demonstrated that 100% of samples across all treatment groups exhibited an HSS score of 1, indicating mild inflammatory cell infiltration involving less than 50% of crypts without erosion or ulceration. No samples showed HSS scores of 0, 2, or 3. Due to the homogeneous distribution of histopathological scores across all groups, inferential statistical analysis could not be performed. In conclusion, administration of the multiepitope COVID-19 vaccine, either alone or combined with chitosan nanoparticles, did not induce pathological changes in the colon of BALB/c mice. These findings support the gastrointestinal safety of the vaccine formulation and its potential for further development as a safe and effective COVID-19 vaccine candidate.

Keyword: COVID-19, multiepitope vaccine, chitosan nanoparticles, histopathology, colon, BALB/c, adjuvant

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INTRODUCTION

COVID-19 is an infectious disease that poses significant challenges to public health control due to factors such as mobility, population density, and compliance with health protocols.[1] The disease is caused by SARS-CoV-2, first identified in Wuhan, China, in late 2019 and rapidly spread worldwide.[2] To reduce morbidity and mortality, the Indonesian government implemented a national COVID-19 vaccination program.[3] Despite its effectiveness, vaccine safety remains a critical concern, particularly in relation to Adverse Events Following Immunization (AEFI). Variations in post-vaccination side effects, including gastrointestinal symptoms, have been reported in Indonesia and other countries.[4–7] Case reports have documented prolonged diarrhea, ulcerative colitis, and severe colitis following COVID-19 vaccination, while cohort studies suggest potential effects of vaccination on inflammatory bowel disease activity.[8–10] Early identification of SARS-CoV-2 as the cause of severe pneumonia provided the scientific foundation for vaccine development.[11]

Evaluation of vaccine safety has been conducted across various populations, including healthcare workers who are at high risk of exposure. Reported AEFI in this group are generally mild to moderate, highlighting the need for ongoing and systematic safety assessment.[12] To enhance immunogenicity and broaden protection against viral variants, multiepitope vaccines have been developed by combining multiple viral protein epitopes to induce comprehensive humoral and cellular immune responses. Initial vaccine design strategies focused on surface glycoproteins due to their role in viral entry, while recent advances in bioinformatics and deep learning–based *in silico* methods have facilitated epitope prediction and multiepitope SARS-CoV-2 vaccine design.[13,14]

Effective vaccination relies on coordinated innate and adaptive immune responses that lead to the formation of immunological memory.[15] The use of adjuvants is a key strategy to enhance vaccine-induced immunity, with evidence showing improved immunogenicity of COVID-19 vaccines following adjuvant incorporation.[16] Chitosan is a widely studied adjuvant with biocompatible, biodegradable, and immunostimulatory properties, making it suitable for vaccine formulations.[17] Preclinical evaluation using animal models, particularly mice, is essential to assess both vaccine efficacy and safety prior to human application.[18] Although SARS-CoV-2 transmission pathways are well characterized, data on the histopathological safety of the colon following administration of a multiepitope COVID-19 vaccine combined with chitosan nanoparticles remain limited. Therefore, this study aims to evaluate the effects of a multiepitope COVID-19 vaccine, with and without chitosan nanoparticles, on the colon histopathology of male BALB/c mice.[19]

METHODS AND SUBJECT

Research Design

This study employed a laboratory experimental design using a post-test-only control group design. This design was selected because it allows direct evaluation of intervention effects by comparing the control and treatment groups without requiring baseline measurements. All samples consisted of stored biological materials in the form of colon tissues from male BALB/c mice that had previously been fixed in 10% formalin. Thus, this study is retrospective, as it utilized pre-existing tissues obtained from prior experimental procedures.

The research subjects were male BALB/c mice randomly assigned into five groups, consisting of one negative control group, two groups receiving the multiepitope COVID-19 vaccine, and two groups receiving the combination of the multiepitope COVID-19 vaccine and chitosan nanoparticles. Observations were performed on days 7 and 14 after the administration of the second dose. Through this approach, the study is expected to provide valid insights into the effects of the vaccine on histopathological changes in the mouse colon.

Research Location and Time

This study was conducted at two primary locations. The maintenance of BALB/c mice and the collection of colon organs were carried out at the Animal Laboratory of the Faculty of Medicine, Universitas Jenderal Achmad Yani, Cimahi. Subsequently, tissue preparation and histopathological examination were performed at the Anatomical Pathology Laboratory of the same faculty. All stages of the research were conducted in July 2025 according to the predetermined schedule.

Population and Research Sample

a. Population

The study population consisted of stored biological materials in the form of colon organs from male BALB/c mice aged 6–8 weeks and weighing 20–30 grams, maintained at the Animal Laboratory of the Faculty of Medicine, Universitas Jenderal Achmad Yani, Cimahi. The BALB/c strain was selected because it exhibits consistent immune responses and is widely used as an animal model in immunology and vaccine development research.

b. Inclusion and Exclusion Criteria

The inclusion criteria in this study consisted of colon organs from mice that had been fixed in 10% formalin, were well preserved, and showed no signs of tissue degradation. Samples that met the quality standards for histopathological preparation, were free from contamination, and originated from previous research procedures conducted according to laboratory protocols were included in the study. Conversely, the exclusion criteria consisted of organ samples that were damaged, contaminated, or exhibited structural deterioration, making them unsuitable for analysis.

c. Sample Size and Calculation

The sample size was determined using the Federer formula $[(n–1)(t–1) \geq 15]$, where n represents the number of animals per group and t represents the number of treatment groups. With five treatment groups, the calculation yielded $(n–1)(5–1) \geq 15$, resulting in a minimum n value of 4.75 animals per group. Based on this calculation, five mice were included in each group. Thus, a total of 25 stored biological samples consisting of colon organs from male BALB/c mice were analyzed.

d. Microscopic Examination and Data Recording

Histopathological examination was performed using a light microscope at 100× and 400× magnification across five randomly selected fields of view per slide. Assessment focused on inflammatory cell infiltration, erosion, and ulceration based on the Harpaz Histological Scoring System (HSS). All observations were recorded using a standardized histopathological observation sheet, which included sample codes, treatment group codes, microscopic findings, and HSS scores. Each slide was assigned a unique anonymized code to ensure objective evaluation. Histopathological assessment was conducted by two independent assessors with expertise in anatomical pathology, who evaluated the slides independently.

e. Blinding Procedure

To minimize observer bias, blinding was applied during histopathological assessment. The assessors were blinded to group allocation and treatment assignment at the time of slide evaluation. Slide coding and decoding were performed by a separate researcher who was not involved in microscopic assessment. In cases of discrepancy between the two assessors, the final score was determined through joint re-evaluation and consensus.

Research Materials and Equipment

a. Material

The primary materials used in this study were the multiepitope COVID-19 vaccine and chitosan nanoparticles, which were combined according to the designated research doses. The research subjects consisted of colon organs from male BALB/c mice aged 6–8 weeks and weighing 20–30 grams, which had been previously fixed in 10% formalin as stored biological specimens. In addition, histotechnical supporting materials were used, including graded alcohols for dehydration, xylene for tissue clearing, liquid paraffin for infiltration and embedding, and hematoxylin–eosin (H&E) staining solutions for the preparation of histopathological slides.

b. Equipment

The equipment used included a microtome for cutting tissue sections at a thickness of 4–5 µm, paraffin containers and molds for eembedding, as well as knives, forceps, and tissue scissors for preparation procedures. Tissue sections were mounted on glass slides and covered with coverslips using a mounting medium. A hot plate and incubator were used to facilitate slide adhesion and drying. Histological examination was performed using an Olympus DP74 light microscope equipped with ImageJ software for documentation and histopathological image analysis.

Research Procedure

a. Sample Preparation

Colon organs of male BALB/c mice previously fixed in 10% formalin were retrieved from storage. The samples were then rinsed under running water to remove residual fixative before further processing.

b. Histotechnical Procedure

The first stage involved dehydration using graded alcohols to remove water from the tissues. This was followed by tissue clearing with xylene and subsequent infiltration with molten paraffin. The tissues were subsequently embedded in paraffin blocks and sectioned using a microtome at a thickness of 4–5 µm.

c. Staining and Slide Preparation

The tissue sections were mounted on glass slides and stained with hematoxylin–eosin (H&E). Hematoxylin stains cell nuclei blue to purple, while eosin stains the cytoplasm pink. After staining, the slides were covered with coverslips using a mounting medium to maintain specimen quality.

d. Microscopic Examination

The completed slides were examined under a light microscope at 100× and 400× magnification across five fields of view. Histopathological features were evaluated using the Harpaz Histological Scoring System (HSS) to assess inflammatory cell infiltration, erosion, and ulceration. Observations were recorded systematically and analyzed statistically to compare differences among treatment groups.

Research Variables

This study employed two main types of variables: independent and dependent variables.The independent variable was the type of vaccine administered, consisting of the multiepitope COVID-19 vaccine alone and the multiepitope COVID-19 vaccine combined with chitosan nanoparticles. The dependent variable was the histopathological changes in the colon of BALB/c mice following vaccination.

Operational Definition

The operational definition of the independent variable was the administration of a multiepitope vaccine containing several SARS-CoV-2 protein epitopes (N, S, E, and M proteins) at a dose of 500 µg/mouse, delivered intramuscularly twice at a seven-day interval, either with or without chitosan nanoparticles as an adjuvant. The dependent variable was defined as microscopic alterations in mouse colon tissues, assessed through hematoxylin-eosin (H&E)–-stained histopathological examination and evaluated using the Harpaz Histological Scoring System (HSS), which categorizes tissue conditions into score 0 (no inflammatory cell infiltration), score 1

(<50% crypt infiltration without ulceration or erosion), score 2 (≥50% crypt infiltration without ulceration or erosion), and score 3 (presence of erosion or ulceration). In this study, the independent variable was categorical (nominal), whereas the dependent variable was measured on an ordinal scale, and the HSS was selected because it is widely used in colitis research, provides clear differentiation of inflammatory infiltration, erosion, and ulceration, and is appropriate for assessing colon tissue safety.

Data Analysis

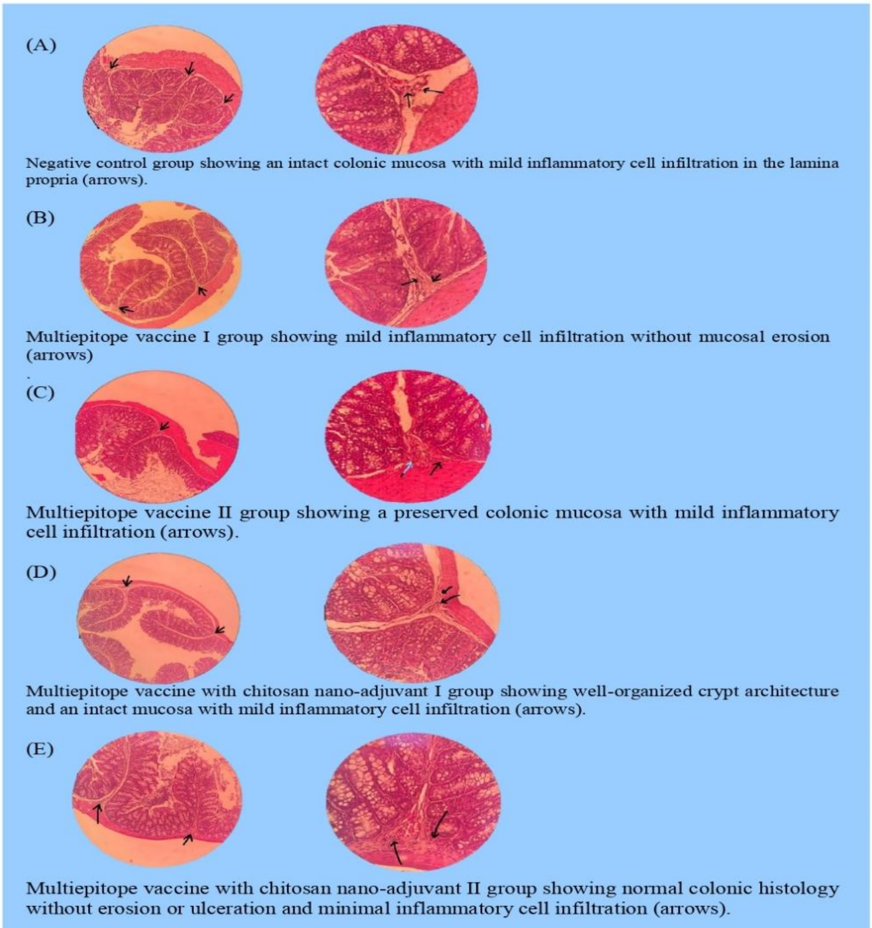
Statistical analysis was performed with a 95% confidence level, and $p < 0.05$ was considered statistically significant. In this study, inferential statistical analysis could not be performed due to the homogeneous distribution of histopathological scores across all groups.

Research Ethics

All stages of this research were conducted in accordance with ethical principles for animal experimentation. Animal welfare was ensured based on the Five Freedoms, including freedom from hunger and thirst, freedom from discomfort, freedom from pain, injury, and disease, freedom to express normal behavior, and freedom from fear and distress. The study also adhered to the principles of Replacement, Reduction, and Refinement (3R): replacing animal use where possible, minimizing the number of animals used without compromising scientific validity, and refining procedures to reduce suffering. This research protocol underwent ethical review and received approval from the Research Ethics Committee of the Faculty of Medicine, Universitas Jenderal Achmad Yani, Cimahi (Approval Number: 016/UH1.07/2025), confirming that it met formal ethical requirements for implementation.

RESULTS AND DISCUSSION

Results



(A) Negative control group showing an intact colonic mucosa with mild inflammatory cell infiltration in the lamina propria (arrows).

(B) Multiepitope vaccine I group showing mild inflammatory cell infiltration without mucosal erosion (arrows)

(C) Multiepitope vaccine II group showing a preserved colonic mucosa with mild inflammatory cell infiltration (arrows).

(D) Multiepitope vaccine with chitosan nano-adjuvant I group showing well-organized crypt architecture and an intact mucosa with mild inflammatory cell infiltration (arrows).

(E) Multiepitope vaccine with chitosan nano-adjuvant II group showing normal colonic histology without erosion or ulceration and minimal inflammatory cell infiltration (arrows).

Figure 1. Histopathological Features of the Colon in BALB/c Mice Representative hematoxylin–eosin (H&E)–stained sections of colonic tissue from each experimental group

Sections were stained with hematoxylin–eosin (H&E) and examined under a light microscope at ×100 and ×400 magnifications. Scale bar = 50 µm. All groups exhibited mild inflammatory cell infiltration (HSS score = 1) without erosion or ulceration.

Table 1. Distribution of Harpaz Histological Scores (HSS) Assessing Inflammation, Erosion, and Ulceration in the Colon of BALB/c Mice

Treatment Group	n	Score 0	Score 1	Score 2	Score 3
Negative control	5	0 (0%)	5 (100%)	0 (0%)	0 (0%)
Multiepitope vaccine (I)	5	0 (0%)	5 (100%)	0 (0%)	0 (0%)
Multiepitope vaccine (II)	5	0 (0%)	5 (100%)	0 (0%)	0 (0%)
Vaccine + chitosan nanoparticles (I)	5	0 (0%)	5 (100%)	0 (0%)	0 (0%)
Vaccine + chitosan nanoparticles (II)	5	0 (0%)	5 (100%)	0 (0%)	0 (0%)
Total	25	0 (0%)	25 (100%)	0 (0%)	0 (0%)

HSS: Harpaz Histological Scoring System; score 0 = no inflammation; score 1 = mild inflammatory infiltration without erosion or ulceration; score 2 = moderate infiltration; score 3 = erosion or ulceration

The results of the study showed that all mice from the five treatment groups, including both the control group and the groups, receiving the multiepitope vaccine with or without the chitosan nanoparticle adjuvant, had a histopathology score of 1. This score indicates mild inflammatory cell infiltration (<50% of crypts) without the presence of erosion or ulceration. None of the samples exhibited scores of 0, 2, or 3. Thus, no variation in score distribution was observed among the groups.

The absence of score variation rendered statistical analysis using the Chi-Square test impossible. Statistical testing could not be performed due to the homogeneous distribution of scores across all groups (p-value could not be calculated).Descriptively, however, these findings indicate that the multiepitope vaccine and the chitosan nanoparticle adjuvant did not induce colon tissue damage. All groups exhibited the same histological response pattern, allowing the conclusion that the vaccination treatments were safe for the colon of BALB/c mice.

These findings support the initial hypothesis that the combination of a multiepitope vaccine with chitosan nanoparticles is biocompatible and does not increase the risk of inflammatory reactions in the colon. Chitosan nanoparticles, known for their mucoadhesive and biodegradable properties, did not cause gastrointestinal adverse effects. Therefore, the multiepitope vaccine with a chitosan nanoparticle adjuvant can be considered safe for further development, particularly from the perspective of gastrointestinal tissue safety. Overall, the homogeneous score distribution across all groups confirms that the interventions, whether vaccination alone or in combination with chitosan nanoparticles, did not produce significant pathological effects on the colon. This is an important foundation indicating that the multiepitope vaccine candidate with a chitosan nanoparticle adjuvant has strong potential for further development without posing a risk of histopathological disturbances in the colon.

Discussion

The results of this study indicate that administration of the multiepitope COVID-19 vaccine, with or without a chitosan nanoparticle adjuvant, did not induce pathological changes in the colon of BALB/c mice. All samples showed a histopathology score of 1, characterized by mild inflammatory cell infiltration without ulceration or erosion. These findings support the initial hypothesis that the multiepitope vaccine combined with chitosan nanoparticles is safe for colon tissues.[20] BALB/c mice were selected as the animal model because they exhibit consistent immune responses and are widely used in SARS-CoV-2 immunology studies. This model is considered representative for evaluating vaccine safety and assessing histopathological effects in the gastrointestinal tract.[21] Therefore, the absence of abnormalities in the colon indicates that the vaccine does not trigger an excessive inflammatory response in the target organ.

The concept of multiepitope vaccines offers advantages by combining epitopes from SARS-CoV-2 structural proteins such as N, S, E, and M, thereby enabling a broader induction of both humoral and cellular immune responses. This approach is considered more flexible and rapid, as it can be designed using *in silico* methods.[22] In this study, the absence of colon tissue damage indicates that the immune stimulation induced was protective without causing pathological inflammation.[23] The chitosan nanoparticle adjuvant also plays an important role in maintaining the vaccine's safety profile. Chitosan is biocompatible, biodegradable, and mucoadhesive, allowing enhanced antigen penetration and mucosal retention. Other studies have reported that chitosan can stimulate both innate and adaptive immunity without causing gastrointestinal toxicity.[24,25] Thus, the combination of the multiepitope vaccine and chitosan nanoparticles not only enhances immunization effectiveness but also maintains the histopathological safety of the colon.

These findings are also important in the context of reported gastrointestinal side effects associated with first-generation COVID-19 vaccines. Several studies have documented complications such as prolonged diarrhea and ulcerative colitis following vaccination.[26,27] The findings of this study confirm that the multiepitope vaccine candidate formulated with chitosan nanoparticles does not induce histopathological alterations in colon tissues, suggesting that it may be safer than conventional vaccine formulations. Although the results support the hypothesis, several limitations remain. First, observations were limited to the colon, and potential adverse effects on other organs such as the liver, kidneys, or spleen were not evaluated.[28] Second, the sample size was relatively small, which may limitthe generalizability of the findings.[29] Third, the post-vaccination observation period was limited to day 14 after the second dose, preventing assessment of potential long-term effects.[30] Based on these limitations, further research is strongly recommended using a multi-organ assessment, a larger sample size, a longer observation period, and safety testing in non-rodent animal models as a preparatory step before progressing to human clinical trials.

CONCLUSION

This study demonstrates that administration of the multiepitope COVID-19 vaccine, either with or without a chitosan nanoparticle adjuvant, did not produce significant histopathological alterations in the colon of BALB/c mice. All samples exhibited only mild histopathological scores (Harpaz HSS = 1), characterized by inflammatory cell infiltration involving <50% of crypts, without erosion or ulceration. These findings support the hypothesis that the multiepitope vaccine is relatively safe for colon tissue and further strengthen the evidence that the use of chitosan nanoparticles as an adjuvant does not induce gastrointestinal side effects. Thus, the combination of the multiepitope vaccine and chitosan nanoparticles holds strong potential for further development as an effective and histopathologically safe vaccine candidate. However, this study has several limitations, including the focus on colonic tissue only, the small sample size, and the short observation period. Therefore, future studies with larger sample sizes, evaluation of additional organs, and extended follow-up periods are needed to establish a more comprehensive safety profile.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the research, authorship, or publication of this article.

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