Letter to Editor

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Comments on "Comparative Immunogenicity and Neutralization Potency of Four Approved COVID-19 Vaccines in BALB/c Mice"

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Dear Editor

We read the following article in your valuable journal:

"*Comparative Immunogenicity and Neutralization Potency of Four Approved COVID-19 Vaccines in BALB/c Mice*"; Iranian Journal of Immunology; March 2024; 21(1):1-14

I acknowledge that the topic of study is interesting and aligns with some of the current needs for developing vaccines. Also, the study design and techniques used to assess the effectiveness of vaccines in a mouse model are interesting and informative. However, the presence of ambiguities and unanswered questions in the text that come to the reader's mind hinder the formation of a clear and integrated picture of the methodology, data analysis, conclusions, and the ability to reference the study's results.

Below, I draw your attention to the following points.

1. Introduction Section

On page 2 of the article, the following information is included:

"*Different effects of various COVID-19 vaccines in preventing infection, hospital admission, and death have been reported in the Iranian population (15). Addressing these questions is crucial for public health officials, healthcare providers, and policymakers to have a thorough understanding of the strengths and weaknesses of the different COVID-19 vaccines currently used in each country (16).*

Accordingly, we conducted a comparative study on immunogenicity and neutralization efficacy of the four approved COVID-19 vaccines in Iran, including the PastoCovac Plus, Sinopharm, SpikoGen, and Noora. Since the majority of individuals received heterologous prime-boost vaccine regimens, conducting a comparative study using human serum samples is difficult. Consequently, to examine the immunogenicity and neutralization efficacy of these vaccines, multiple groups of female BALB/c mice were vaccinated with three doses of each vaccine."

The above text has been managed in a way that suggests this animal study could serve as a guide for public health officials, healthcare providers, and policymakers. Furthermore, it has been mentioned that since vaccinated individuals have received heterologous prime-boost vaccine regimens, this study was conducted on laboratory animals. Given that the study on vaccines in humans and animals is not equivalent, it was necessary to address and emphasize this issue both in this section and also in the "discussion" section of the article.

Now the question arises: If the effectiveness of these four types of vaccines in humans was the question, can investigating the effectiveness of each of these four vaccines on a number of mice adequately answer this question and essentially replace a study on humans?

Some parts of the article implicitly suggest to the reader that the animal study is assumed to be equivalent to a study on humans. Certainly, the esteemed authors of this article also confirm that an animal study cannot be considered equivalent to a human study; otherwise, experimental studies would suffice with animal models, and the studies would not progress to clinical trials.

On the other hand, these vaccines have also received clinical trial approval and have been administered to thousands of people, and undoubtedly their effectiveness has been investigated by the relevant research teams. Therefore, what added value does returning to preclinical studies have? The reason mentioned for conducting this study on mice does not seem logical.

2. Materials and Methods Section

In the stages of conducting research, all ethical principles must be fully and accurately observed, and should be mentioned in a way that builds trust among readers to study. Additionally, the methodology section should be logically designed and executed, and written in a way that enables other researchers to replicate the study. In the present study, there are many ambiguities that prevent the fulfillment of the above conditions. For example:

2.1. In the article, an ethical code for this study has been announced as follows:

"*Research Ethics Committee of Tehran University of Medical Sciences approved this study (IR.TUMS.SPH.REC.1400.334).***"**

However, when searching for the above code on the website of the "Iran National Committee for Ethics in Biomedical Research (URL: https://ethics.research.ac.ir/IndexEn.php)", it becomes apparent that the aforementioned code has been issued for a study with the following title:

"*Production of recombinant fusion protein containing receptor-binding domain (RBD) of SARS-CoV-2 virus with human-Fc (RBD-Fc) and investigation of its immunogenicity and toxicity as a candidate vaccine against SARS-CoV-2***"**

Since there is no clear evidence of the results of the study mentioned above in this article, it can be concluded that this ethical code is not related to the present study (1).

However, on page 4 of the article, the RBD synthesized by the researchers of this study has been used to make an ELISA kit, which suggests that the ethics code listed in the article may be for a study related to the synthesis of this specific piece.

2.2. If the above-mentioned ethical code is not related to this study, it can be inferred that this study is not related to the following sentence mentioned in the "Acknowledgment" section of the article:

"*Partial financial support for this study was provided by a grant from Tehran University of Medical Sciences (Grant No. 1401-1-99-57094).*"

Unless the intention is to cover the expenses of producing the RBD vaccine, which was used in a small part of this study to prepare the ELISA kit. In any case, the source of financial support for this study (which seems to be a high-cost study) has not been clearly and explicitly stated.

2.3. There is no mention of the conditions for the care and work on animals and how ethical principles and animal protection from pain and suffering arising from research work

(such as injections, blood sampling, humane killing) were observed. The exact number of animals studied is not disclosed. The control groups have not been well introduced.

In this regard, researchers are recommended to adhere to "ARRIVE guidelines" (URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7393194).

2.4. The structure of Table 1 with the title "*Table 1. Approved SARS-CoV-2 vaccines employed in this study*" has not been drawn correctly, making it somewhat time-consuming to understand. It would have been better if the names of the vaccines (Sinopharm, PastoCovac Plus, SpikoGene, and Noora) were placed in the first column from the left under the "Vaccine" title, and the words "Platform", "Expression System", "Adjuvant", and "Injected dose (μg)" were placed in the header of the second to fifth columns.

2.5. The first paragraph under the title Animal Vaccination states:

"*Based on previously published preclinical studies (17-20), the doses of Sinopharm, PastoCovac Plus, SpikoGen, and Noora vaccines were determined to be 2, 10, 5, and 40 μg per injection, respectively (Table 1).*"

However, in references 18 and 20 of this article, the effective doses of the aforementioned vaccines are different (2, 3). For example, in reference number 20, it is clearly stated that a dose of 40 μg of the vaccine was not effective, and a dose of 80 μg of the vaccine was declared as the effective dose. However, in the present study, a dose of 40 μg was used, and naturally, you have reached the same conclusion as previously reached in reference number 20.

Another noteworthy point is that instead of using the declared effective doses in the preclinical reports of these two vaccines, the researchers have repeated the study on these two vaccines using a "different batch".

2.6. ELISA kits for measuring antibodies have been developed by the researchers of this study; however, information such as their sensitivity and specificity, and the controls used, has not been provided.

2.7. It is not specified what serial dilutions were used in the tests.

2.8. In some of the tests, the response to the Wuhan strain was examined, while in others, the response to the Delta strain was investigated. Not only has the reason for this not been explained, but also these responses have also been the basis for judging the effectiveness of the vaccines in the analyses.

This is important because all four vaccines discussed in the study are made against the Wuhan strain, and during the COVID pandemic, it was revealed that the Delta strain is capable of evading existing vaccines.

2.9. Additionally, in this study, some test results using the Delta strain have been reported as positive and valuable for some vaccines. It would have been better if this was also mentioned as a valuable difference in the "Discussion" section of the article.

2.10. It is not clear why both complete Freund's adjuvant and incomplete Freund's adjuvant were used for one mouse simultaneously.

2.11. For some of the tests, strains of the COVID-19 virus have been used. However, it is not clear where the Wuhan and Delta strains of this virus have been obtained from and under what conditions they have been stored. Additionally, it has not been disclosed what level of biosafety laboratory has been used and whether high biosafety levels are even available at the site where this study is being conducted. There is no mention of these equipment and how safety principles are observed.

2.12. It is not clear how much blood was taken from the tail vein of the mice each time. The maximum volume of blood that can be taken from a mouse's tail vein is 200 microliters, and the serum will be much less. Considering the number of tests performed using mouse serum, each run in triplicate with different dilutions of serum, it appears that either the total number of mice used in this study is much higher than implied in this article, or the volume of blood taken from the

animals was very high, which could have led to serious harm or death of the animals, making it practically impossible to continue the work. Both approaches are considered unethical.

2.13. The protocol for hyperimmunization of a mouse using RBD has not been mentioned.

3. Discussion Section

3.1. In the discussion section, not only are the results of vaccine efficacy not compared with the results of corresponding preclinical studies, but also the various differences in the methodology of this study compared to preclinical studies of the four vaccines (such as the type of adjuvants used, vaccine dosages, vaccine injection sites, injection frequencies, intervals between injections, intervals between the last injections and second blood sampling, and the tests used to evaluate vaccine efficacy) are not mentioned. These differences, although they can certainly lead to different responses, have not been discussed, examined, or analyzed.

If it was not intended to exactly replicate the methodology of the preclinical studies of the 4 vaccines under investigation, at the very least, it should have been mentioned in the discussion section.

3.2. Sinopharm vaccine, due to its possession of multiple antigens, can generate a greater range of antibodies; therefore, it is not comparable to the other vaccines studied in this research. It would have been better to mention this difference in the discussion section.

3.3. Why is it claimed that the present study can be used for vaccine selection and non-selection strategies by working on a number of mice? In this regard, please pay attention to the following (page 11):

"*The observed differences in immunogenicity and neutralization potency among the four vaccines emphasize the significance of comparative studies for currently used vaccines in Iran to address their relative advantages and disadvantages. Our findings have important implications for vaccine selection strategies.*"

4. Conflicts of Interest Section

Considering the analytical approach taken to evaluate the efficacy of the vaccines under study, the generalizations made, the study limitations, differences in the study compared to the referenced articles No. 17 to 20, and the way the discussions and analyses were conducted, it appears that there are indeed conflicts of interest with at least two of the author teams of references No. 19 and 20.

CONCLUSION

Given the numerous ambiguities in research ethics and methodology, as well as the lack of thoroughness, and weakness in the discussion section of the article, it seems that the peer-review conducted for this article was not strong and thorough. Furthermore, there has been negligence in examining the adherence to ethical principles in this study.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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AUTHOR'S RESPONSE

Dear Professor Abbas Ghaderi,
Editor-in-Chief, Iranian Journal of Immunology

Our point by point response to the comments and suggestions raised by the authors of the Letter to Editor with regards to our article recently published in IJI (Comparative immunogenicity and neutralization potency of four approved COVID-19 vaccines in BALB/c mice; doi:10.22034/iji.2024.101060.2728) is given below. We are grateful for the interest of the critics in our paper and also for their constructive comments and hope that our response will be useful and helps to clarify the questions and ambiguities raised by the authors.

1. Introduction section

R1) We agree with the respected critics' opinion that the results of animal studies are not necessarily the same as the results of human studies. For this reason, we have clearly highlighted this issue in several important parts of the article, for example in the conclusion statement of the Abstract (page 1):

"This suggests the need for additional comparative assessment of the potency and efficacy of these four vaccines in vaccinated subjects."

We have also reiterated this point in the discussion section (page 11, right column, lines 4-10):

"It is important to note that the findings of our study were obtained in the BALB/c mice model and may not be fully applicable to human responses. Animal models are valuable tools for the initial evaluation of vaccines, but further research in human populations is needed to confirm and extend these findings."

Further emphasis was given by highlighting this issue in the conclusion section (page 12, lines 9-11).

The above mentioned descriptive statements clearly indicate that our study cannot replace studies comparing the effectiveness of these vaccines in human. We clearly pointed out that these four vaccines have been studied in human, and the human study of the Noora vaccine was cited in our article (page 10, left column, lines 35-38 and ref 38). Also, the SpikoGen vaccine, has undergone more extensive human trials (1-4), and both vaccines were licensed for public use in Iran by the Ministry of Health of Iran.

Actually, the main reason for conducting this study was to use these four vaccines as a control for our RBD-Fc candidate vaccine and to compare the neutralizing potency of these vaccines with our own candidate vaccine (page 9, left column, lines 33-40). We intentionally did not include the data of our own candidate vaccine in this article to prohibit any assumption of conflicts of interest. The results of our candidate vaccine has recently been published in the European Journal of Microbiology and Immunology (doi: 10.1556/1886.2024.00045).

2. Materials and methods section

R2.1) The results presented in this article are part of an approved research project of Tehran University of Medical Sciences (TUMS). The main goal of this project is to produce a candidate vaccine for COVID-19 and to evaluate its neutralizing potency in mice and rabbits. Although the original proposal did not specifically mention evaluating and comparing our candidate vaccine against others, it is reasonable to compare the effectiveness and immunogenicity of the new vaccine with similar approved vaccine platforms once they are available. Despite not being explicitly stated in the initial plan, conducting this study aligns with the core objectives of the approved research plan. As a matter of fact, all four studied vaccines were prepared and obtained from the vaccination centers affiliated to TUMS through official correspondence with the university, stating our intention to use these vaccines for this approved project. Therefore, inclusion of

these vaccines and preclinical evaluation of their immunogenicity and neutralization potency, despite not being included in the initial proposal, is not necessarily considered a major misconduct or a violation from an ethical point of view.

This issue was later discussed by TUMS ethics committee. Based on the committee's decision, although analysis of these four vaccines should has already been amended and included in the approved proposal in advance, but this is not a major misconduct and would not compromise the validity of the results.

R2.2) As clearly stated in the proposal of our research project approved by TUMS, production of the RBD-Fc fusion protein and its preclinical assessment as a vaccine candidate were the main objectives of this project and these four vaccines were included as control for our own candidate vaccine (page 9, left column, lines 33-40). This study was registered as a PhD thesis with a limited financial support from the university. The remaining budget was provided personally by the research group.

R2.3) All ethical issues with regards to animal experimentations were outlined in the proposal and were reviewed and approved by the Research Ethics Committee of TUMS (IR.TUMS.SPH.REC.1400.334).

On page 3 (Animal Vaccination), the number of animals in each group and the control group are clearly defined.

R2.4) As the respected critics pointed out, the structure of Table 1 could be modified and presented in a better format to make it more easy for the readers to follow. Nevertheless it is not incorrect or so much complicated to describe these modifications.

R2.5) While confirming the point raised by the respected critics regarding the dose used for the immunization of mice with Noora vaccine (40 µg), we clearly stated that the dose of each vaccine was based on previously published preclinical studies (References 17-20 of our published article). In the preclinical studies, different vaccine doses were investigated. For example for the Noora vaccine 40, 80 and 120 µg were tested and 120 µg was selected as the best dose. However, after conducting a human study, a dose of 80 µg was suggested as the human dose. Considering that in most vaccine studies, the dose used in mice is usually several order lower than the human dose (1, 5-24), we used the dose of 40 µg for mice. This rationale aligns with common practices in vaccine studies. All these clarifications have already been given in our published article (Page 10, Left column, Lines 28-38).

As for the PastoCoVac vaccine, contrary to the critics' statement, the selected dose for mice (10 ug) was the same as the selected dose published in the corresponding preclinical study (ref 18).

R2.6) For immunogenicity tests and anti-RBD titer measurements, we used the ELISA test, which has already been employed in many studies. Similar to the preclinical study of the Noora vaccine, the RBD protein was coated to measure the specific antibody level by an indirect antigen specific ELISA.

Although the sensitivity and specificity of this ELISA were tested, but needless to say that we performed a comparative study and all four vaccines were compared with each other simultaneously. Thus the sensitivity and specificity parameters apply to the results obtained for all four vaccines and do not confer limitation on a specific vaccine type.

For the virus neutralization tests (VNTs), we used four different standard tests. To date, the use of all four methods has not been reported in any study. All four VNT tests confirmed the virus neutralization results and were highly significantly correlated, confirming the accuracy of our results.

It should be noted that based on the results of the anti-RBD and neutralization tests, we did not claim that the Noora and SpikoGen vaccines are ineffective. There is no universally accepted sero-protection threshold for COVID-19, and we only conducted a comparative study. It is plausible that the same anti-RBD titer and neutralization

results obtained for the Noora and SpikoGen vaccines may induce protection.

R2.7) Based on the results of the pilot tests, different serum dilutions were used in each test. The important point is that in Figure 2, the Anti-RBD titer was measured based on AU/ml, and for the Anti-spike comparison, the OD of 1:50 serum dilution was used for comparison. As for the neutralization methods, the neutralizing potency was initially determined at three different serum dilutions and the ID50 values were then calculated for different groups.

R2.8) As correctly outlined by the respected critics, the neutralizing potency of the sera was determined in the three sVNT, pVNT and cVNT tests against the Delta variant, but for the inhibitory flow cytometry neutralization test it was tested against the Wuhan variant. The reason is clearly mentioned in the article (page 10, right column, lines 38-46).

Since all the four investigated vaccines were produced based on the Wuhan variant, basically a higher neutralization potency is expected to be induced against the Wuhan as compared to the Delta variant by all four vaccines. Thus, since the study was designed to compare these vaccines with each other, employment of other virus variants, such as the Delta or other variants, is acceptable and does not pose any limitation or problem in the study.

Additionally, most vaccines produced against SARS-CoV-2 were initially based on the Wuhan variant. However, following emergence of different variants, their neutralization potency was investigated against the emerging variants. There are several comparative studies, where the vaccines developed based on the Wuhan variant were later tested against the Delta variant (25-27). Furthermore, in our own article, we outlined that these results pertain to neutralization against the Delta variant, and to generalize the findings, the neutralization potency against other variants should be examined (Page 12, "Conclusion" section).

R2.9) Thank you for your comment. In fact, this point has been outlined in our article (page 10, right column, line 3-9):

"Our results showed that the sera from mice vaccinated with Sinopharm and PastoCovac Plus exhibited strong neutralizing activity against the Delta variant of SARS-CoV-2 pseudovirus and live virus, as evidenced by the pVNT and cVNT results, respectively (Figs. 3a, 3b)."

R2.10) We did not state that the two adjuvants were administered simultaneously (page 4, left column, line 31-33). According to the immunization protocol with this adjuvant, the first dose of the antigen is given together with the Freund's complete adjuvant and subsequent doses are administered in combination with Freund's incomplete adjuvant.

R2.11) Live virus was used only for the cVNT test against the Delta variant. Obviously, this test was conducted under BSL-3 conditions and was performed in a specialized virology laboratory that had the necessary safety facilities (Amirabad Pharmed Virology Laboratory). These are straight forward facts and there is no urgent need to mention them in the paper.

R2.12) As correctly outlined by the critics, a large volume of mouse serum is required for the experiments. For this purpose, according to the animal anesthesia protocol, cardiac blood collection was used as a terminal procedure to collect a maximum volume of blood directly from the heart. Based on our knowledge and expertise this detailed information is not stringently necessary to be included in the paper.

R2.13) Hyperimmunized serum is a serum collected from a mouse administered with multiple doses of RBD emulsified in Freund's adjuvant (complete followed by incomplete adjuvant as mentioned above) with a maximum titer of anti-RBD antibody. As mentioned in the article, we used hyperimmunized mouse serum as a standard (page 4, left column, line 30-35).

3. Discussion Section

R3.1) Various methodological parameters may affect vaccine immunogenicity. Some of these parameters such as the antigen expression system (eukaryotic or prokaryotic), type of adjuvant, dose of antigen and number of doses have been discussed and presented in different parts of the paper, such as table 1. Needless to say that all four vaccines were administered similarly and immunogenicity of the vaccines was compared in immunized mice using the same methodology. Meanwhile, in the discussion section of the article, the results of this study have been analyzed and compared with the preclinical study of the corresponding vaccines (page 9, right column, lines 26-40), (page 10, left column, lines 1-6 and 28- 45) and (page 11, left column, lines 5-29).

R3.2) The most important aspect of a COVID-19 vaccine is to induce protection against natural infection, which could be evaluated to some extent by conducting in vitro neutralization tests, especially against the live virus. Therefore, to this extent inclusion of Sinopharm vaccine and its comparison with the other three vaccines, does not seem to be illogic or problematic.

R3.3) Our statement was quoted partially and incompletely by the critics. Our complete statement was: "Our findings have important implications for vaccine selection strategies. Vaccines that are more likely to induce higher neutralizing immune response, such as Sinopharm and PastoCovac Plus are more effective in virus neutralization and achieving optimal protection against SARS-CoV-2. Our findings showed that these two vaccines displayed greater immunogenicity and induced significantly higher virus neutralizing responses compared with SpikoGen and Noora vaccines". It is quite logic and scientifically sound to assume higher effectiveness and protective capacity for vaccines with higher neutralization potency, as indicated above.

Furthermore, we have mentioned in different parts of the article that the results obtained from our mouse model provide only a partial evaluation of the immunogenicity of these vaccines. The findings need to be further investigated in human studies to gain a comprehensive understanding. We have addressed these limitations in our article, specifically on page 10, right column, lines 26-37, and page 11, right column, lines 4-22 and 36-45.

R3.4) It is really surprising how the critics came to the conclusion that there are indeed conflicts of interest with at least two of the author teams of references No. 19 (SpikoGen team) and 20 (Noora team). Conflict of interest is indicated where an individual may preference, or be perceived to preference, their own interests over their duties and responsibilities as a researcher. What are the reasons behind this assumption?!

Finally, it should be noted that, as we mentioned in the article, these vaccines were obtained with the permission of Tehran University of Medical Sciences directly from the vaccination centers affiliated to the university (Page 3, Left column, Lines 12-14 and Page 11, Right column, Lines 23-29), without any manipulation (for example, vortexing), and all storage protocols were strictly followed and the vaccines were administered within the products' expiration dates.

We take this opportunity to thank the critics for their constructive comments and suggestions and hope that our responses would be helpful and could shed more light on the ambiguous issues.

Kind Regards
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