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Epigenetic Alterations in Mouse Muscle Cells After In Vitro Treatments with COVID-19 and Influenza Vaccines

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Abstract

The importance of vaccination has come up again with a new form of coronavirus disease, COVID-19, which appeared in late 2019. This virus spread very fast around the globe, and it has numerous variants determined so far. Many studies focus on the effects of COVID-19 in humans and clinical-follow up after vaccination for the understanding whether the disease has been taken under control. Other studies mostly focus on omics analyses and molecular characteristics of COVID-19 itself. However, this is not clear whether COVID-19 vaccines induce epigenetic differences in the host tissues. This study aimed to reveal whether *in vitro* treatment of muscle cells with mRNA-based vaccine for COVID-19 and/or attenuated vaccines (whole virus attenuated for COVID-19 or split virion for quadrivalent influenza) can result in the changes in the global levels of DNA methylation (5meC) and/or DNA hydroxymethylation (5hmC). DNA methylation and DNA hydroxymethylation were individually detected by immunofluorescence and global patterns of epigenetic marks were analysed by fluorescence microscopy in mouse muscle cells after the incubation with vaccines for 24h or 48h. Results showed that each type of attenuated vaccine induced epigenetic changes by different patterns, but the mRNA-based vaccine affected both global levels of 5meC and 5hmC in a similar manner. Findings indicate that vaccines can affect epigenome. These preliminary results suggest that epigenetic profiles of specific genes across different human tissues after vaccination may add further information, therefore, reveal biological significance in detail.

Keywords: COVID-19, coronavirus, influenza, vaccine, epigenetics, DNA methylation, DNA hydroxymethylation

1. Introduction

The COVID-19 pandemic has been an important situation around the globe since late 2019. This is caused by SARS-CoV2 (**S**evere **A**cute **R**espiratory **S**yndrome **Co**rona**V**irus-**2**) that has several mutant variants (1,2). Although there are other members of *Coronaviruses* that share similarity with COVID-19 in terms of genomic pattern and symptomatic effects, COVID-19 is the variant that spread rapidly around the world. Today, all countries suffer from clinical, social, and economic outcomes of the pandemic. Dealing with the spread, treatment and protection has been of the main interest in governments and scientific institutions. There are different vaccine strategies to protect people from the mortal effects of COVID-19 as inducing the immune recognition and response in advance. Vaccination rate around the world has been increasing by time. Some groups of vaccines are produced by classical attenuation methods, but other types are produced using new approaches of biotechnology, such mRNA technology, adenovirus-based methods, and virus-like particles, or combined of classical and new methods.

Use of vaccines has been an old but still useful approach to fight with infections. The classical vaccines derived by Pasteur's method are attenuated whole viruses that induce immune response against the pathogens before possible infection in the future. mRNA-based vaccine technology is a rather new and biotechnologically produced vaccine. These are not routinely used in the clinic but for COVID-19 infection F.D.A. has urgently approved mRNA vaccine. This technology has been broadly examined for cancer prevention (3,4) as well as

infectious diseases including viral (5) and bacterial antigens (6). In the clinic, the immunity of vaccines is primarily studied by comparing unvaccinated and vaccinated people (7,8). There are also some studies using drug repurposing strategies (9,10) or newly synthesized compounds for COVID-19 therapy (11,12). However, the effect of vaccines at molecular level has remained unclear. Epigenetic regulations are the machineries in the cells that manage differential gene expression for a range of various extra-, intra-, and intercellular stimuli. The vaccines can regulate the epigenome of the host organism by the changes in the genes/proteins involved in the innate and adaptive system (13,14) as well as the infections themselves (15). The epigenetic response in the cells after COVID-19 vaccination is one of the interests, and this study aims to elucidate global methylation and hydroxymethylation patterns after vaccine treatments *in vitro*. To the best of knowledge, there is no understanding of epigenetic (including 5meC and 5hmC) patterns in the genome of host muscle cells right after vaccination for COVID-19.

2. Materials and Methods

2.1. Cell culture and vaccine treatments

Sol8 (ATCC, CRL-2174™) mouse muscle cells were cultured in DMEM (Wisent Inc., Cat No 319-005-CL, Quebec, Canada) media including 10% fetal bovine serum (Capricorn Scientific GmbH, Cat No FBS11-A, Ebsdorfergrund, Germany) and 1% penicillin-streptomycin (Wisent, Cat No 450-201-EL) antibiotics at 37°C humidified with 5% CO_2 until reach to 80% confluency. Cells were seeded as 25000 cells per well in a 96-well plate. After they reached to confluency, they were treated with vaccines individually for 24h or 48h.
COVID-19 vaccines used were 1) inactive whole virus attenuated (Sinovac/CoronaVac, Sinovac Biontech Ltd.; Beijing, P.R. China) and 2) mRNA-based vaccine, BNT162b2 (Biopharmaceutical New Technologies, BioNTech; Meinz, Germany). Both COVID-19 vaccines have been developed using wild type COVID-19 which is the first variant raised in China resulting in the pandemic. Adjuvant compounds in inactive attenuated COVID-19 vaccine are aluminium hydroxide, sodium chloride, sodium hydroxide and monosodium and disodium hydrogen sulphate, dissolved in water (Sinovac Research & Development Co., Ltd, China). Ingredients in mRNA COVID-19 vaccine includes mRNA (for spike protein of the virus) and lipids ((4-hydroxybutyl) azanediyl)bis(hexane-6,1-diyl)bis(2hexyldecanoate), 2 [(polyethylene glycol)-2000]-N,N-ditetradecylacetamide, 1,2 Distearoyl-sn-glycero-3-phosphocholine, and cholesterol). Adjuvant compounds in mRNA based COVID-19 vaccine include potassium chloride, monobasic potassium phosphate, sodium chloride, dibasic sodium phosphate dihydrate, and sucrose or tromethamine, tromethamine hydrochloride, and sucrose (16) **(Table 1)**.
Influenza vaccine (VAXIGRIP TETRA; manufactured by Sanofi Pasteur, France to TURKEY) used were inactive split virion. Influenza vaccine includes 1) 15 micrograms of A/Victoria/2570/2019 (H1N1) pdm09-(A/Victoria/2570/2019, IVR-215) like variant, 2) 15 micrograms of A/Cambodia/e0826360/2020 (H3N2) –(A/Tasmania/503/2020, IVR-221) like variant, 3) 15 micrograms of B/Washington/02/2019-(B/Washington/02/2019, wild type) like variant and 4) 15 micrograms of B/Phuket/3073/2013, wild type) like variant. Adjuvants in influenza vaccine are sodium chloride, potassium chloride, disodium phosphate dihydrate, potassium dihydrogen phosphate, dissolved in water and traces of ovalbumin (egg protein), neomycin, octoxinol-9, and formaldehyde (Sanofi Pasteur, Turkey) **(Table 1)**. But prescriptions do not indicate final concentrations of adjuvants in the vaccines used.

Estimation of the dose /concentration for application to cells was carried out as follows: 1) In clinical use, 500 microliters of vaccine (for inactive COVID-19 and inactive influenza vaccines) and 300 microliters of vaccine (for mRNA-based COVID-19) have been applied to individuals intramuscularly. An average number of total human cells in a body is around 30 trillion cells ($30x10^{12}$) (17). 2) 500 µl of vaccine is applied for $30x10^{12}$ cells so that 1 µl of vaccine is applicable for $6x10^{10}$ cells, only 1 µl of vaccine is taken from leftovers of vaccines that were used in the clinic. 3) Main stocks of the vaccines were prepared as 1 µl of vaccine in 1000 µl of culture media (for $6x10^{7}$ cells). 4) Main stock was diluted depending on the cell number subjected to be treated with vaccine and the total volume of media needed for the cells cultured in a number of wells in the 96 well plate. *i.e.,* in a design that 400.000 cells seeded into 16 wells, the main stock vaccine was diluted 1:150 in total media (ml) needed (200 µl per well) and split into the wells. Control wells were left untreated, but media without vaccines was renewed. For mRNA-based vaccine used as 300 µl, a similar calculation was performed.

2.2. Immunofluorescence

After treatment, cells were stained by immunofluorescence method as previously described (18). In this protocol, media was removed from the vessels and cells were then washed with 1xPBS (phosphate buffered saline) (with calcium and magnesium ions that facilitate adhesion of cells onto culture vessel) (Wisent, Cat No 311-011-CL) for 3 times. After washing, cells were fixed with 4% paraformaldehyde (w/v) (ChemSolute, Th. Geyer GmbH & Co., Cat No 8416-0500, Germany) for 15 minutes at 37°C followed by 1xPBS wash for 3 times.

Fixed cells were then permeabilized with 1xPBS including 0.75% (v/v) Triton-X (Biomatik, Cat No A4025) and 0.75% (v/v) Tween-20 (Sigma Aldrich Co., Cat No P1379, St. Louis) for 1 hr at RT. After permeabilization, cells were blocked in 50% (v/v) goat serum (Capricorn, Cat No GOA-1B) in 1XPBS at 4°C overnight. Some cells were incubated with mouse anti-5meC-antibody (1:400) (Active Motif, Carlsbad, CA, US, Cat No 39649) for 1h at room temperature (RT), others with rabbit anti-5hmC antibody (1:500) (Active Motif, Cat No 39791) for 2.5h at RT. After primary antibody incubation, secondary antibodies including either anti-mouse Alexa-488 (Abcam, Cat No 150113) for 5meC (1:400) or 2) anti-rabbit Alexa-488 (Abcam, Cat No 150077) for 5hmC (1:500) were treated for 1h and 2h at RT in the dark, respectively. Secondary antibodies were then removed and washed with 1xPBS for 3 times. Staining patterns were visualized under the fluorescence microscope (AxioVert, Zeiss, Germany). Microscopy images were captured with an integrated camera using a set of gamma and exposure values.

2.3. ImageJ Analysis

Microscope images were analysed using ImageJ (NIH, US) software. For this, the colour threshold was set first for each image. After individual nuclei were automatically selected in each image, mean fluorescence intensity and area were measured for each nucleus. Total staining of 5meC or 5hmC (arbitrary units, a.u) were then calculated by mean fluorescence intensity × nucleus area. Total staining values (Sum values, arbitrary units, a.u) were represented by bar graphs using SPSS software (Version 23) and shown with +/- standard error of the mean from three repeats.

2.4. Statistics

The comparison of sum values was performed by univariate analysis of variance (UNIANOVA) of SPSS software. Pair-wise analyses were performed using post-Hoc analysis. Significance levels used were $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****).

Table 1. Comparison of adjuvants in each vaccine.

Adjuvant Compounds	Attenuated inactive COVID-19	Split virion inactive Influenza	mRNA based COVID-19
Sodium chloride	Yes	Yes	Yes
Aluminium hydroxide	Yes	No	No
Potassium chloride	No	Yes	Yes
Sodium hydroxide	Yes	No	No
Monosodium hydrogen sulphate	Yes	No	No
Disodium hydrogen sulphate	Yes	No	No
Monobasic potassium phosphate	No	No	Yes
Dibasic sodium phosphate dihydrate	No	No	Yes
Sucrose	No	No	Yes
Tromethamine	No	No	Occasional
Tromethamine hydrochloride	No	No	Yes
Disodium phosphate dihydrate	No	Yes	No
Potassium dihydrogen phosphate	No	Yes	No
Ovalbumin (egg protein)	No	Yes	No
Neomycin	No	Yes	No
Octoxinol-9	No	Yes	No
Formaldehyde	No	Yes	No
Water	Yes	Yes	No

3. Results and Discussion

After the treatment with whole virus attenuated, global level of DNA methylation (5meC) significantly increased at 24h ($p = 0.000$) but decreased for additional 24h ($p \leq 0.025$) **(Figure 1)**. After mRNA-based vaccine, its level did not change at 24h ($p>0.05$), whereas this amount decreased at 48h ($p = 0.000$) compared to untreated cells **(Figure 2)**. After the inactive split virion vaccine, DNA methylation pattern was completely opposite compared to treatment with whole virus attenuated as it decreased at 24h ($p \leq 0.013$) but increased at 48h ($p = 0.000$) **(Figure 3)**.

In contrast to DNA methylation, the level of DNA hydroxymethylation (5hmC) decreased at 24h ($p = 0.000$) but increased at 48h ($p = 0.000$) after whole virus attenuated **(Figure 4)**. mRNA-based vaccine induced similar alterations in DNA hydroxymethylation as in DNA methylation with a significant decrease at 48h only **(Figure 5)**. After the inactive split virion vaccine, 5hmC level was only changed at 48h with an increase as well as 5meC. But differentially this was not affected at short-

term (p>0.05) **(Figure 6)**. **Table 2** shows comparisons for each vaccine treatment with significance (p values) and level trends (increase or decrease) for each epigenetic mark. Preliminary results indicate that 5meC and 5hmC patterns have a tendency towards similar reprogramming after mRNA-based and split virion vaccines, but whole virus inactivated vaccine induced an opposite effect for both.

This study aimed to understand whether two major epigenetic modifications occurring on DNA (5meC and 5meC) are significantly affected after vaccine treatments of cells in the culture. These results suggest that vaccines are able to induce epigenetic reprogramming in the cells (muscle cells in the present study) by different patterns. However, this study does not explore or declare any biological significance of these patterns at gene or cell level. Nevertheless, the results create awareness about the potential of vaccines in terms of epigenetic regulations.

Vaccines are the savers of human life by assisting the immune system to prepare itself before infections and even cancer. As all the compounds including numerous drugs, vaccines are supposed to change epigenetic mechanisms in the cells. This is not surprising that the epigenome, a dynamic representative of the genome, is subject to alter after internal and external stimuli. Although the genome is more robust and rigid in terms of changes in DNA sequence, the epigenome is a kind of dynamic response of cells to any conditions so that it regulates gene expressions to manage the current situation. The vaccination can regulate the host epigenome in particular genes involved in adaptive and innate immunity. For instance, BCG vaccine, (Bacille Calmette-Guerin, a vaccine for tuberculosis) induced NOD2 receptor pathway which is involved in innate immunity, compared to unvaccinated counterparts (19,20). BCG vaccine also stimulated the changes in TNF-α and IL-6 promoters which are related to inflammation (19). A study with healthy people (50–74-year-olds) showed that the changes in the genome wide DNA methylation patterns were at low level whereas the levels of methylation in a specific group of CpG sites were decreased, and these changes were associated with lower humoral immune response to influenza vaccination (13). Correspondingly, decrease in the level of *RNF39* gene which encodes a transcription factor in the major histocompatibility complex (MHC) class I region, was found to be related with a weak response against HBV vaccine in newborns (14). The response of vaccines in terms of epigenetics has been found to be associated with age. The study showed a high level of epigenetic reprogramming in older people (>50 years-old) after influenza vaccination (21). These suggest that vaccination can reprogram the host's epigenome during the response of immunization. This should be also noted that infections can also induce epigenetic changes in the infected organism. The patterns of DNA methylation in more than 500 genes were found to be different in newborns exposed to active HBV infection in utero (15). But, to the best of knowledge there has been no study that tried to experience global epigenetic changes in the host genome after COVID-19 vaccination. This study presents the first preliminary findings for possible changes in epigenetic patterns after *in vitro* treatment of mouse cells with two different types of COVID-19 vaccines and an influenza viral vaccine, and the findings point out that each type of viral vaccine significantly affected the host epigenome but through different ways. This is also the first study to examine the second-common DNA modification, 5hmC-DNA hydroxymethylation, after both COVID-19 and influenza vaccine treatments *in vitro*. However, this study does not provide any information at the gene level and is limited to only one cell line from rats. Human cells should be included, and even *in vivo* studies can give more comprehensive outcomes, *i.e.*, blood cells can be obtained from vaccinated people. This will lead to understanding the direct effect of vaccines on immune system cells. The vaccines may regulate the epigenome of the immune system cells in the process of pathogen recognition for the future. This is also possible that infection, *i.e.*, COVID-19, can affect the host's epigenome to trigger immunizations (22). Previous works identified detailed epigenetic changes in the host genome after trivalent influenza vaccination containing the influenza A/California/7/2009 H1N1-like, A/Perth/16/2009 H3N2-like and B/Brisbane/60/2008-like viral strains. (23). These changes include a range of pathways regulating immune response such as T cell receptor pathway, T cell activation and golgi to plasma membrane protein transport for short term (3 days) and long term (28 days). The presented study, to the best of the knowledge, is the first to evaluate epigenetic response in the muscle cells after COVID-19 vaccination. However, by this study, it is hard to conclude the biological significance of the observed changes, but statistical significance exists. Revealing gene and/or tissue specific changes provides a broader perspective for the effects of vaccines in detail. Upregulation of genes by changes in epigenetic markers on DNA may be relevant to immunization of cells against the pathogen. Immunization is not only for immune system cells but also fibroblast cells were shown to have immunization activity as well (24–26). Muscle has been known to play a mediator role for immune system training (27) so that it supports the epigenomic changes in muscle cells after vaccination. Vaccines are applied intramuscularly; thereby muscle cells are the first responsive cells in the body.

Another limitation of this study is not to include a group of control cells treated with adjuvant compounds only. This is because lists of adjuvants are given in each vaccine **(Table 1),** but the concentrations of adjuvants are not provided. This limits the mimicking of the precise content within the vaccines and reliability of the

experiments. Control cells were therefore designed with only canonical cell culture media environment. One or more adjuvants possibly can affect the host epigenome itself. For instance long term exposure of low dose formaldehyde (exist in only influenza vaccine used in the present study) induced decrease in the global level of DNA methylation in human bronchial epithelial cell line (28). A kind of detergent, Octoxinol-9, another adjuvant in influenza vaccine, is declared as safe compared to other octoxinols (octoxynols) which are shorter chain than 8 (29), but there is no study showing its effect on epigenetic profile. A part of the epigenome is open to change after intra- and extracellular inducements, but these do not always mean a pathological response.

The attenuated vaccines are a kind of whole virus inactivated vaccine, such one produced by Sinovac. The split-virion vaccines are also inactivated but these are produced by disruption of virus envelope and releasing the virion particles (30), such as the quadrivalent influenza vaccine used in the study. mRNA based vaccines are totally different from other types as mRNA of target protein within the virus genome is packaged within a liposomal structure. The different designs of three vaccines examined suggest the different epigenetic response in the cells. The changes in the levels of DNA methylation and DNA hydroxymethylation were found to differ from treatments of each vaccine in this study. The variation may be derived from the differential structures of vaccines suggesting regulation of different cellular responses. To reveal the biological action within the cells, detailed investigations based on whole genome sequencing are required.

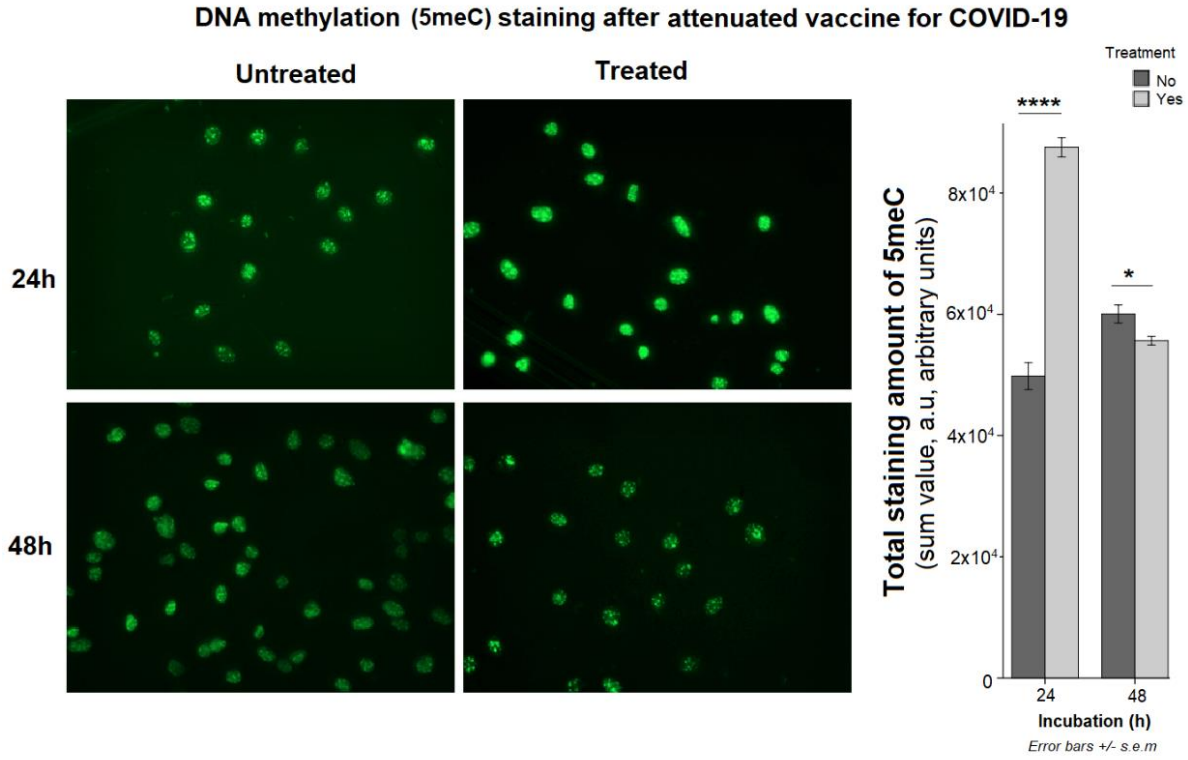

Figure 1. DNA methylation profile after attenuated vaccine for COVID-19.

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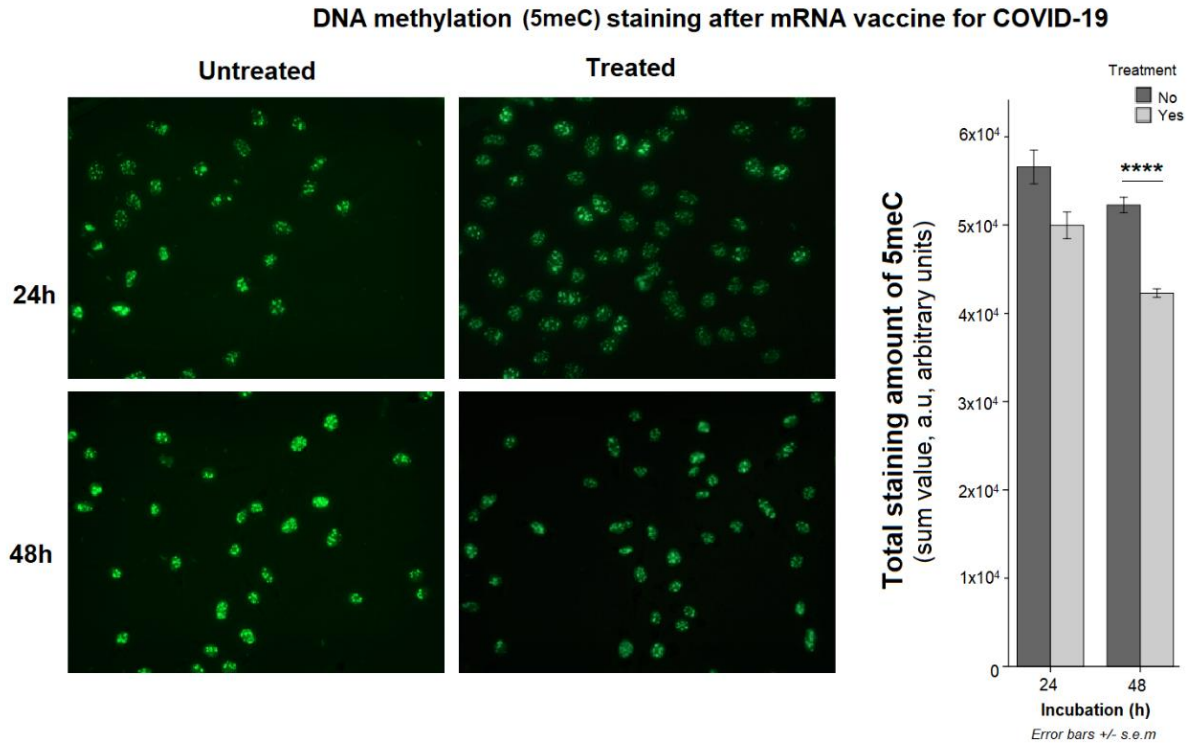

Figure 2. DNA methylation profile after mRNA vaccine for COVID-19.

Table 2. Significant changes in epigenetic markers of DNA (5meC and 5hmC) after vaccines in vitro.

Vaccine Type	Incubation	Comparison	5meC	5hmC
Whole virus attenuated (COVID-19)	24	Untreated vs Treated	$p \leq 0.000$ ↑	$p \leq 0.000$ ↓
	48		$p \leq 0.025$ ↓	$p \leq 0.000$ ↑
mRNA based (COVID-19)	24		n.s ×	n.s ×
	48		$p \leq 0.000$ ↓	$p \leq 0.000$ ↓
Inactive split-virion (Influenza)	24		$p \leq 0.013$ ↓	n.s ×
	48		$p \leq 0.000$ ↑	$p \leq 0.000$ ↑

n.s; not significant ↑ *increase* ↓ *decrease*

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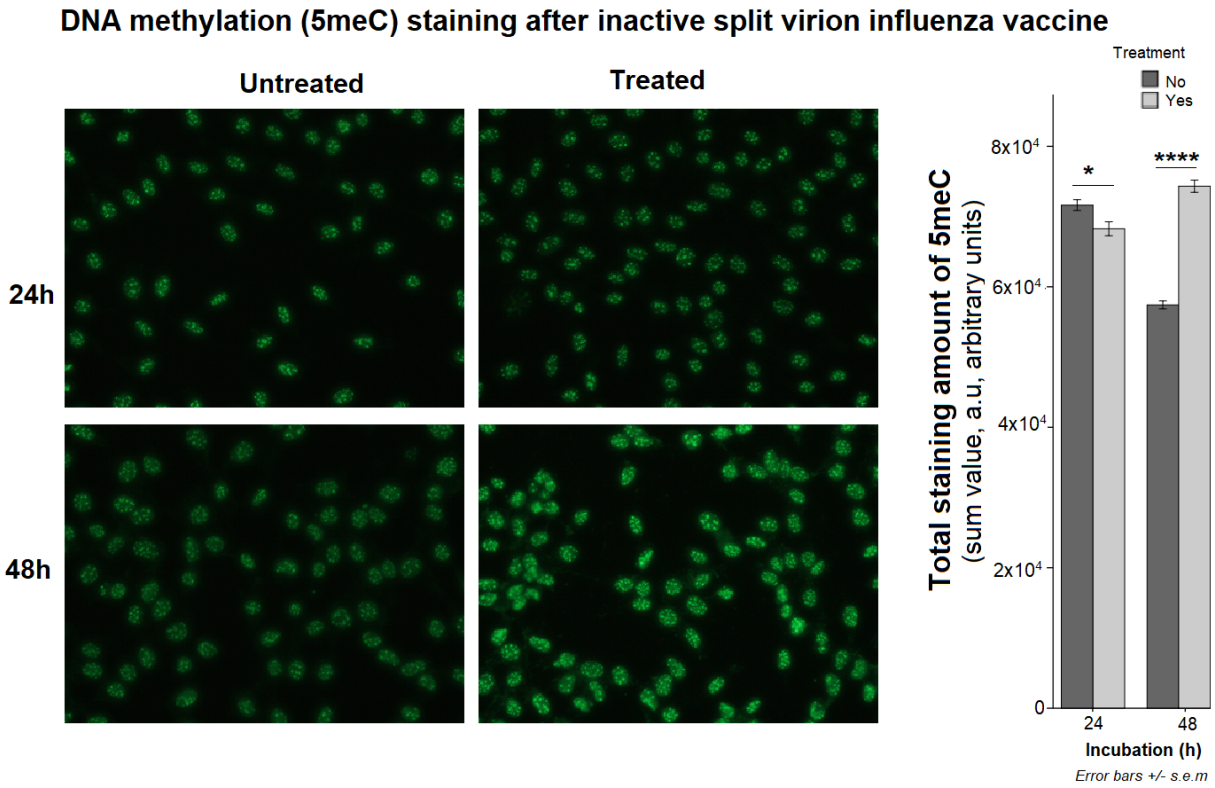

Figure 3. DNA methylation profile after inactive split virion influenza vaccine.

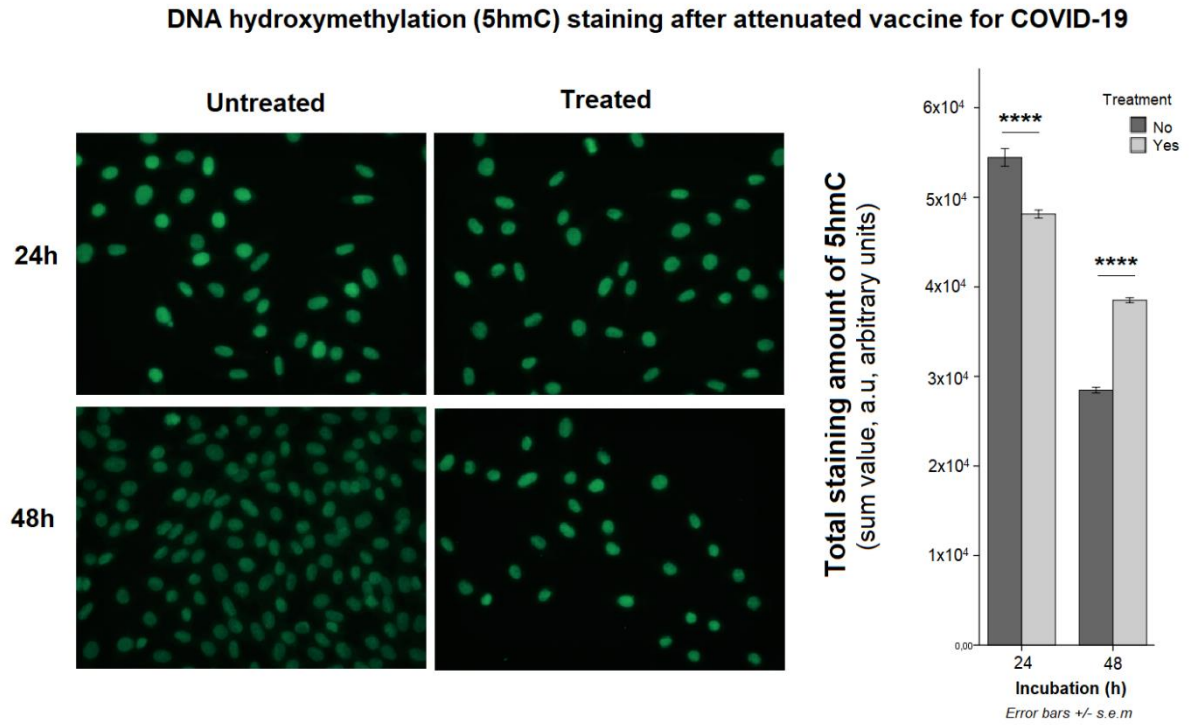

Figure 4. DNA hydroxymethylation profile after attenuated vaccine for COVID-19.

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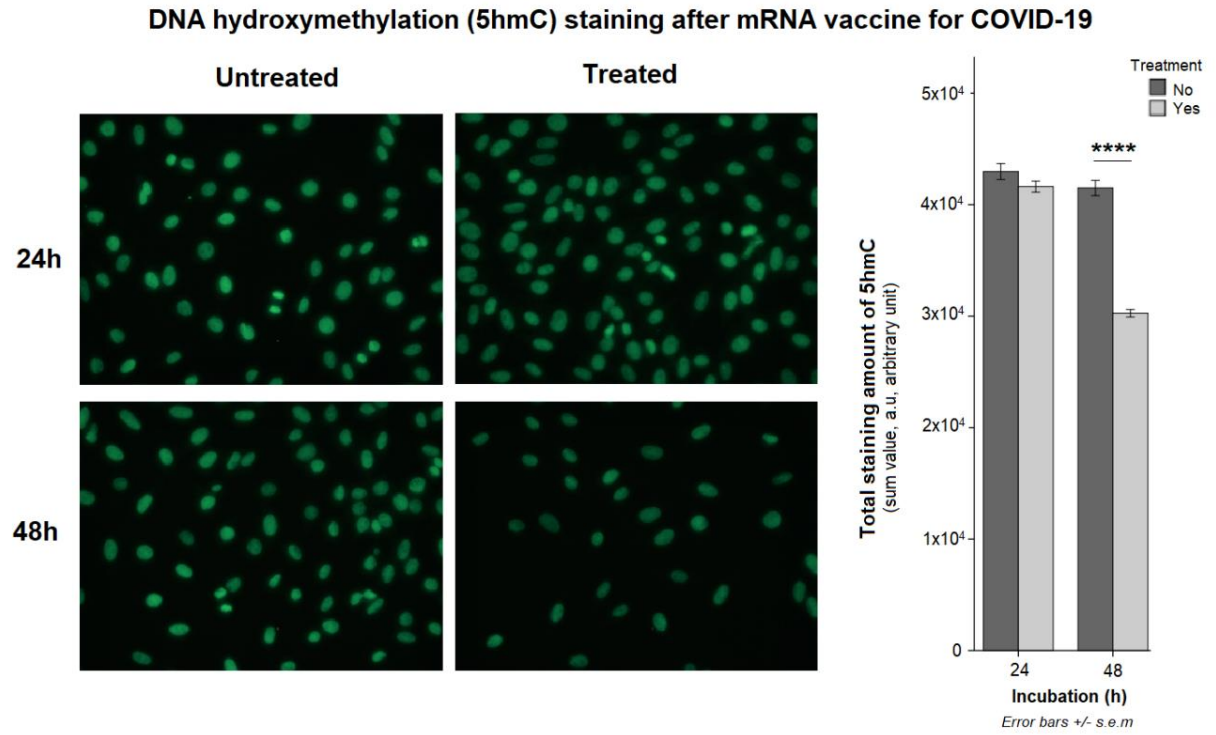

Figure 5. DNA hydroxymethylation profile after mRNA vaccine for COVID-19.

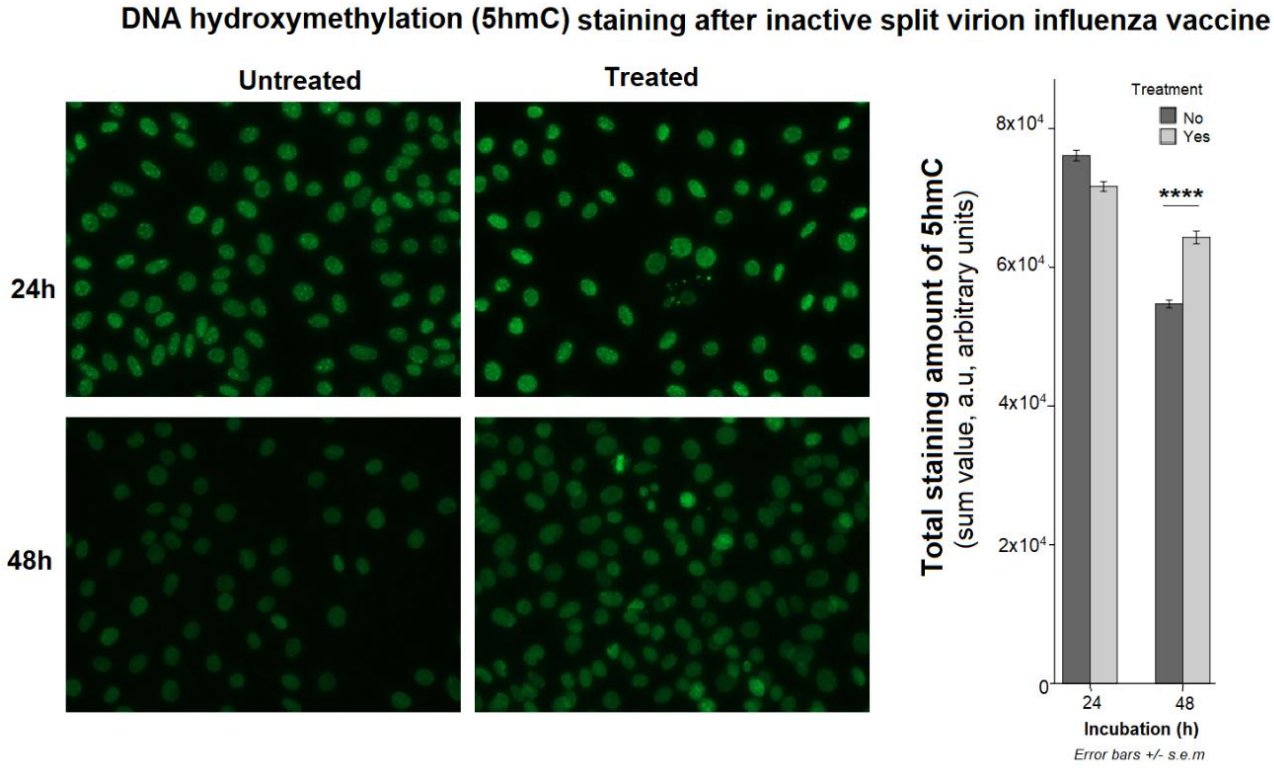

Figure 6. DNA hydroxymethylation profile after inactive split virion influenza vaccine.

4. Conclusion

This study suggests that different type of vaccines can make global epigenetic changes on the individuals who are vaccinated. However, this study only was performed in vitro and using muscle cells. Comprehensive in vitro studies and further in vivo studies are required to make a broader conclusion.

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Author's Contributions

Selcen Çelik Uzuner: Hypothesized the study, performed the experiments, and wrote the manuscript.

Ethics

There are no ethical issues after the publication of this manuscript.

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