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ORIGINAL ARTICLE

Semen Analysis

Comparison of sperm parameters and DNA fragmentation index between infertile men with infection and vaccines of COVID-19

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Several preventive measures, including vaccination, have been implemented owing to the severe global effect of coronavirus disease 2019 (COVID-19), but there is still limited evidence in the effect of this disease and vaccination against it on male fertility. Therefore, this study is to compare sperm parameters of infertile patients with or without COVID-19 infection and the effect of COVID-19 vaccine types on them. Semen samples of infertile patients were collected consecutively at Universitas Indonesia - Cipto Mangunkusumo Hospital (Jakarta, Indonesia). COVID-19 was diagnosed by rapid antigen or polymerase chain reaction (PCR) tests. Vaccination was performed with three types of vaccine, namely inactivated viral vaccine, messenger RNA (mRNA) vaccine, and viral vector vaccine. Spermatozoa were then analyzed on the World Health Organization recommendations, and DNA fragmentation was assayed with the sperm chromatin dispersion kit. The results showed that the COVID-19 group experienced a significant decrease in sperm concentration and progressive motility (both $P < 0.05$), but there was no significant change in morphology or sperm DNA fragmentation index (DFI; both $P > 0.05$). The viral vector vaccine caused a decrease in morphology as well as an increase in DFI compared with the control (both $P < 0.05$), meanwhile results for those who were vaccinated with the inactivated and mRNA types were not significant compared with the control (both $P > 0.05$). We conclude that COVID-19 has negative effects on sperm parametes and sperm DNA fragmentation, and we found that the viral vector vaccines affect sperm parameter values and DNA fragmentation negatively. Further studies with a larger population and longer follow-up are needed to confirm the results.

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Keywords: COVID-19; semen parameter; sperm DNA fragmentation; sperm quality; vaccines

INTRODUCTION

Human coronavirus, which was initially known as a minor human pathogen, was later discovered to cause severe respiratory tract infection, which was known as severe acute respiratory syndrome (SARS). Furthermore, it was then declared as a pandemic in 2002, and its name was changed to SARS-coronavirus (CoV). At present, there is a coronavirus pandemic, caused by another variant of coronavirus, namely SARS-CoV-2. The disease is named coronavirus disease (COVID), and as the year it was found as the culprit was 2019, the disease was renamed COVID-19. The World Health Organization (WHO) predicts the result of this new variant infections is more than 14.83 million deaths globally.[1] Several studies also revealed that the infection rate is higher among men than women.[2,3] In this case, endocrine profile differences between men and women play a significant role. It is known that a higher serum total testosterone level is associated with a low immune cell-mediated response due to the immunosuppressive effect of testosterone.[3]

Clinical characteristics of COVID-19 are similar to those of SARS, both being often expressed in the respiratory tract. Furthermore, several new studies have also reported symptoms of infection in other organ systems, such as the cardiovascular and gastrointestinal.[4,5] Furthermore, previous studies also revealed its negative effects on the reproductive system, although some of them stated it had no effect. Other types of virus, such as mumps, Zika virus, hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), herpes, and Ebola virus have also been reported to be detrimental to male reproductive health.

Vaccination is one of the preventive measures implemented to reduce the prevalence of the disease. Several types of vaccine have also been developed owing to the recent discovery of SARS-CoV-2, ranging from virus particles to inactivated whole virus, and their therapeutic effects are inseparable from the adverse effects. COVID-19 vaccines also cause similar short- and long-term symptoms of the COVID-19 disease itself, such as it is expressed in the male reproductive system.[6]

The impact of COVID-19 and anti-COVID-19 vaccines on sperm quality needs to be assessed because it is a new disease to health practitioners, especially within the reproductive health services unit, including obstetrics-gynecology and andrology. Therefore, this study is aimed at determining the effect of COVID-19 and its vaccines on sperm quality by assessing semen analysis, including

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sperm concentration, motility and morphology, as well as sperm DNA fragmentation index (DFI).

PATIENTS AND METHODS

Patients

A total of 70 infertile men aged between 18 years and 49 years at Universitas Indonesia - Cipto Mangunkusumo Hospital (Jakarta, Indonesia) were included in the study from December 2020 to June 2022 after written informed consent was obtained. The study was approved by the Ethics Committee of Faculty of Medicine of Universitas Indonesia (FMUI; Jakarta, Indonesia; Approval No. KET-1044/UN2.F1/ETIK/PPM.00.02/2021). These patients were divided into three groups: 20 infertile patients without COVID-19 infection and vaccination were enrolled as the control group, 20 infertile patients with COVID-19 infection but without vaccination were enrolled as the COVID-19 group, and 30 infertile patients without COVID-19 infection but with vaccination were enrolled as the vaccine group. The vaccine group was then divided into three subgroups, each of 10 subjects, who were selected randomly to receive vaccination with the prepared COVID-19 vaccine (inactivated viral vaccine group, viral vector vaccine group, or mRNA vaccine group, respectively) using the protocol given by the government. The inclusion criteria in these groups were as follows: no history of previous COVID-19 infection for vaccinated group, no previous history of COVID-19 vaccination for COVID-19 group, and no history of previous COVID-19 infection and vaccination for control group. Semen analyses were done in 2–4 weeks after the second dose of vaccine, in order to study the effect of vaccine on the current spermatogenic cycle.

For the COVID-19 group, the inclusion criteria consisted of previous infection proven by PCR test or rapid antigen test, no sequelae, negative PCR result for COVID-19 (2–4 weeks) before semen collection, and still eligible to receive COVID-19 vaccination. As for the control group, the inclusion criteria were as follows: no history of previous COVID-19 infection, no history of COVID-19 vaccination, and still waiting for their schedule to be vaccinated.

The exclusion criteria for all study groups included those who did not agree to the semen analysis or had aspermia characterized by the absence of seminal fluid. The consecutive sampling technique was used by taking all infertile men at Ciptomangunkusumo Hospital who were registered and met the inclusion criteria. Protocols of rapid antigen tests, PCR tests, semen analyses, and sperm DNA fragmentation were carried out based on the protocol of the hospital.

Semen analyses

All infertile men were asked to collect their semen samples into sterile plastic containers by masturbating. Semen analyses were then performed on the basis of the WHO 2010 criteria.[7] Subsequently, the samples were left for liquefaction, and the process was continued with an examination of sperm concentration and motility using a Makler® counting chamber (Sefi Medical Instrument Ltd., Haifa, Israel). Sperm morphology was performed after Diff-Quick staining (Ankebio, Chongqing, China). The samples were then forwarded for further processing of DNA fragmentation.[7]

Sperm DNA fragmentation test

The DNA fragmentation test was assessed via sperm chromatin dispersion with the Spermfunc® DNA kit (BRED Life Science Technology Inc., Shenzhen, China). After the samples were melted by placing them at 90°C–100°C for 5 min, the agarose was transferred, heated at 37°C for 5 min, and mixed thoroughly with the samples. A total of 25 μl of the suspension was poured on agarose-coated slides and covered with a 20 mm × 20 mm cover glass. The slides were then cooled at 4°C for 5 min and incubated in a denaturing solution containing 0.08 mol l^{-1} HCl at 22°C for 7 min. Subsequently, the slides were incubated with lysing solution at room temperature for 25 min and washed with ddH_2O for 5 min. They were then dehydrated with graded ethanol of 70%, 90%, and 100% for 2 min in each. The slides were stained with Wright's solution for 25 min, followed by drying and observation in a light microscope (Nikon, Yokohama, Japan). There are five categories of sperm halo images, namely large, medium, small, no halo, and degraded. The big and medium halos were classified as unfragmented DNA, while the small, no halo, and degraded sperm cells were considered fragmented.[8] The appearance of fragmentation in 500 spermatozoa was defined as the DFI.

Hormone assay

Peripheral blood samples were taken to obtain sera, and the levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were assessed with chemiluminescent immunoassays commercial kits (Shenzhen Yahuilong Biotechnology, Shenzhen, China).

COVID-19 testing

Mucus specimens were collected from the respondents' nasopharynx or saliva using a nasopharyngeal swab. They were then extracted from the nasal swab in a buffer and transferred to the sample well of the coronavirus antigen test cassette. The result was read and interpreted within 15–20 min from the buffer-specimen dispense. A positive sample was indicated by the presence of one colored band on the color (C) line (COVID-19 antigen rapid test kit, Joysbio, Dongguan, China).

Mucus specimens were collected from the respondents' nasopharynx or saliva using nasopharyngeal and oropharyngeal swabs. Subsequently, they were treated to extract the RNA. The extracted RNA was reverse transcribed to DNA using a specific enzyme and the addition of short DNA fragments that are complementary to specific parts of the transcribed sample. The mixture of extracted RNA and additional DNA short fragments was then placed in a reverse transcription (RT)-PCR machine (Bioneer, St. Ingbert, Germany). It was cycled 35 times through different temperatures to trigger specific chemical reactions that can create new identical copies of the target sections of the viral DNA. A fluorescent dye was released as the amount of viral DNA increased, and when a certain level of fluorescence is surpassed, it can be concluded that the virus is present (BioRad SARS-C-V-2 RT-PCR Assay Kit, Singapore, Singapore).

COVID-19 vaccines

The sample population in this study consists of semen donors who had completed both vaccine doses, which were given 28 days apart. Three types of vaccine were included, which were inactivated viral vaccine (Sinovac, Beijing, China), viral vector vaccine (AstraZeneca/AZ, Cambridge, UK), and mRNA vaccine (Moderna, Cambridge, MA, USA).

Statistical analyses

All statistics were analyzed with SPSS software version 22 (IBM, Armonk, NY, USA). The data of each study parameter were analyzed between the COVID-19 and control groups, as well as among the three different vaccine subgroups and control group. Kruskal–Wallis test and analysis of variance (ANOVA) were used to analyze sperm quality parameters between the COVID-19 group and the control group and among the inactivated viral vaccine, viral vector vaccine,

and mRNA vaccine groups, respectively. The significance level was set at 5% with $P < 0.05$.

RESULTS

Patient characteristics

A total of 70 patients met the criteria for inclusion in this study, of which 20, 20, and 30 infertile patients were recruited in the control group, COVID-19 group, and vaccine group, respectively. Within the vaccine group, the inactivated viral vaccine, viral vector vaccine, and mRNA vaccine were each received by 10 patients. Furthermore, there was no significant difference in the demographic and clinical characteristics among these three groups, except for age and infertility period (**Table 1**).

Semen analyses

Comparisons were made between the COVID-19 group and the control group for sperm parameters and DFI, and in the vaccine group to determine the effect of each vaccine on the sperm parameters and DFI. The statistical average of the vaccine group was then compared with the result of the COVID-19 group and the control group.

The COVID-19 group experienced a significant decrease in sperm concentration and progressive motility (both $P < 0.05$), but there was no such decrease in morphology or increase of DFI (both $P > 0.05$) compared with the control group (**Table 2**). Moreover, a comparison of DNA fragmentation between the two groups showed that there were large and medium halos of spermatozoa in the control group than in the COVID-19 group, which had smaller and no halo of spermatozoa cells (**Figure 1**).

In the vaccine group, the sperm quality and DFI varied among the different vaccine types. The lowest sperm concentration and progressive motility were observed in samples from men vaccinated with the viral vector vaccine, but they were insignificantly different from the control group (**Table 3**). Sperm concentration and progressive motility in those who received the inactivated virus and mRNA vaccines were decreased insignificantly different from the control group. There was a significant decrease in nonprogressive motility in the viral vector vaccine group compared with that in the control group ($P < 0.05$). They also experienced a significant reduction in normal sperm morphology, while the inactivated virus and mRNA vaccine groups showed insignificant differences compared with those in the control group (both $P > 0.05$). There was thus a significant increase in DFI among men vaccinated with viral vector vaccine compared with that in the control group ($P < 0.05$), while the inactivated virus and mRNA vaccine groups showed insignificant decreases (both $P > 0.05$), as shown in **Table 3**.

There were variations in the results of sperm DNA fragmentation in infertile men after COVID-19 vaccination. Spermatozoa from men who received the inactive vaccine mostly had big and medium halos (**Figure 2a**). Those who were vaccinated with mRNA had more medium sperm halos (**Figure 2b**). Men who received the viral vector vaccine had medium-to-small sperm halos or no sperm halo (**Figure 2c**).

DISCUSSION

COVID-19 is an emerging disease that has affected many people as well as various aspects of life. This study shows that it has a negative impact on sperm quality in infertile men, especially sperm concentration and motility. This finding is consistent with Tiwari *et al*.,[9] and Erbay *et al*.,[10] who reported that men infected with COVID-19 experienced a progressive decrease in semen volume, concentration, and motility. Decreased sperm quality can be caused by injury due to COVID-19 infection, which triggers an immune response in the seminiferous tubules.[11] Another study reported that the reduction was due to an inflammatory process enhanced by fever, which damages the testicular germ cells through infiltration caused by the destruction of the blood–testicular barrier.[12] Furthermore, Li *et al*.[13] revealed that there was a decrease in the cellular layer as well as an extensive germ cell destruction in the seminiferous tubules of the testes of COVID-19 patients. Wang and Xu[14] stated that SARS-CoV-2 requires the angiotensin-converting enzyme (ACE) receptor and spike protein to enter the host, while the S protein is filled by transmembrane serine

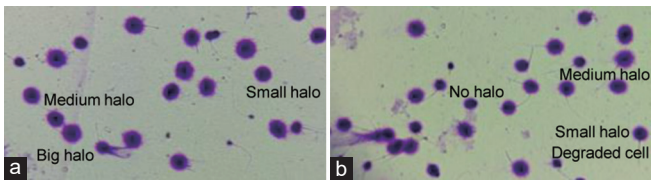

Figure 1: The appearance of sperm DNA fragmentation in infertile men of (**a**) the control group and (**b**) the COVID-19 group. COVID-19: coronavirus disease 2019.

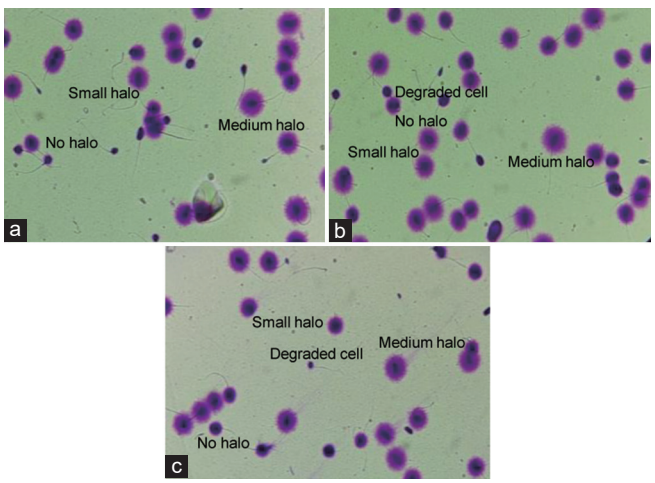

Figure 2: The appearance of sperm DNA fragmentation in infertile men with different COVID-19 vaccinations: (**a**) inactivated viral vaccine group, (**b**) mRNA vaccine group, and (**c**) viral vector vaccine group. COVID-19: coronavirus disease 2019; mRNA: messenger RNA.

Table 1: Characteristics of patients in coronavirus disease 2019, vaccine, and control groups

Characteristic	*Control group, mean±s.d.*	*COVID-19 group, mean±s.d.*	*Vaccine group, mean±s.d.*	*P*
Age (year)	29.3±4.8	29.8±3.5	28.6±3.8	0.032*
Infertility period (year)	3.6±2.4	4.2±2.6	3.9±2.9	0.026*
BMI (kg m^{-2})	25.9±4.1	27.3±3.3	26.5±4.4	0.297
Basal serum FSH (IU l^{-1})	0.7±3.8	0.2±0.8	0.8±4.1	0.528
Basal serum LH (IU l^{-1})	2.4±4.1	0.8±2.0	1.5±0.7	0.124
Basal serum testosterone (IU l^{-1})	150.0±230.2	62.4±154.2	328.0±10.4	0.126

*Significant level: $P<0.05$. COVID-19: coronavirus disease 2019; BMI: body mass index; FSH: follicle-stimulating hormone; LH: luteinizing hormone; s.d.: standard deviation

protease 2 (TMPRSS2). Sertoli, Leydig, and spermatogenic cells express TMPRSS2 as well as the ACE receptor, possibly explaining the causes of various effects of COVID-19 on the male reproductive system.[14,15] All these mechanisms impair spermatogenesis and decrease sperm quality, leading to oligozoospermia and asthenozoospermia.

The result also revealed that infertile men who were infected with the virus also experienced an increase in LH and prolactin (PRL), while the uninfected group had an increase in FSH and testosterone, though not statistically significant. This was due to the direct effect of the virus on the testis and several indirect effects, including inflammation, fever, and dysregulation of the hypothalamic–pituitary–gonadal (HPG) axis. The impairment induced by modeling the androgen, in relation to the hormonal axis, has been established as a proposed therapy in infertility.[15] On the basis of post-COVID-19 recovery, Guo *et al.*[16] concluded that there was an increased sperm concentration and progressive motility by 56 days after COVID-19 infection. Furthermore, the long-term of COVID-19 infection symptom has been observed on the male reproductive system, which can last for 74 days.[17]

This study also revealed that COVID-19 can negatively affect DFI. Apart from the inflammatory phenomena, which contribute to the enhancement of the apoptotic process leading to sperm DNA fragmentation, round cells in semen, such as immature germ cells and leukocytes, are known to have a role in this process by producing reactive oxygen species (ROS) in ejaculation.[18] Li *et al.*[12] revealed that apoptosis also plays a role in the COVID-19 mechanism, thereby increasing sperm DFI. The increase in the number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive apoptotic cells in the testes of men who died from the infection was caused by oxidative stress due to increased circulating ROS as well as suppression of glutathione peroxidase activity.[18] The DFI was examined slightly after the vaccination (in the vaccination group), and after previous COVID-19 infection (in the COVID-19 group) in order to assess the damage to sperm DNA rapidly, since by waiting for another spermatogenic cycle, the result of DFI might not be as accurate, as the fragmentation of sperm DNA can be caused by multiple factors.

Vaccination is an important primary prevention for COVID-19. Several vaccine manufacturers are involved in these preventive measures. At present, there are three main types of COVID-19 vaccine, namely whole, nucleic acid, and subunit viruses. They can also be divided into active and inactive viruses based on their components. "Sinovac" (Bio-Institute Biological, Beijing, China) and "Sinopharm" (Bio-Institute Biological) are inactivated virus vaccines, while those "AstraZeneca/AZ" (AstraZeneca, Cambridge, UK) and "Johnson & Johnson/Janssen" (Johnson & Johnson, Leiden, The Netherlands) are viral vector vaccines. "Moderna" and "Pfizer" (BioNTech, Mainz, Germany) vaccines contain viral nucleic acids, which are made from COVID-19 RNA. COVID-19 subunit vaccines, including "Novavax" (Novavax, Gaithersburg, MD, USA), have not yet been produced in Indonesia. Although the part and activation status of the COVID-19 virus used is different, several studies have reported different side effects on the male reproductive system.[3–6]

Of the three types of vaccine used in this study, the viral vector (AZ) induced a low sperm concentration and progressive motility compared with the inactivated and mRNA groups. Similar results were also obtained by Kumar and Kaur[19] and Zhu *et al.*[20] who reported that inactivated vaccines had no effect on sperm parameter values. In addition, Lifshitz *et al.*[21] strengthened the notion that mRNA vaccines do not influence sperm quality. Nevertheless, a different methodology was used in the previous study.

Table 2: Comparison of sperm quality and sperm DNA fragmentation index in infertile men, in the coronavirus disease 2019 and control groups

Sperm characteristic	*Control group, mean±s.d.*	*COVID-19 group, mean±s.d.*	*P*
Sperm concentration ($\times10^6$ ml^{-1})	36.1±27.1	20.1±24.6	0.035*
Motility (%)			
Progressive	43.9±18.7	32.9±19.7	0.049*
Nonprogressive	11.5±3.6	15.4±9.1	0.076
Immotile	45.2±17.4	49.6±20.4	0.432
Normal morphology (%)	2.4±1.3	2.2±1.4	0.664
DFI (%)	19.4±21.4	21.1±16.6	0.76

*Significant level: $P<0.05$. COVID-19: coronavirus disease 2019; DFI: DNA fragmentation index; s.d.: standard deviation

Table 3: Comparison of semen analysis and sperm DNA fragmentation index in infertile men who received coronavirus disease 2019 vaccination

Sperm characteristic	*Control group, mean±s.d.*	*Inactivated viral vaccine group, mean±s.d.*	*Viral vector vaccine group, mean±s.d.*	*mRNA vaccine group, mean±s.d.*	*P*
Sperm concentration ($\times10^6$ ml^{-1})	36.1±27.1	20.3±30.2	14.6±2.8	27.0±27.9	0.801[a] 0.648[b] 0.933[c]
Motility (%)					
Progressive	43.9±18.7	27.5±20.2	25.0±7.1	53.0±19.5	0.685[a] 0.510[b] 0.868[c]
Nonprogressive	11.5±3.6	16.4±9.2	27.0±5.7	4.0±9.6	0.203[a] 0.043[b] 0.696[c]
Immotile	45.2±17.4	52.9±23.8	48.0±12.7	43.0±22.3	0.391[a] 0.179[b] 0.868[c]
Normal morphology (%)	2.4±1.3	1.8±1.4	3.5±0.7	1.0±1.4	0.258[a] 0.032[b] 0.958[c]
DFI (%)	19.4±21.4	16.1±24.6	26.0±8.8	17.1±23.3	0.141[a] 0.013[b] 1.000[c]

[a]Inactivated viral vaccine group *vs* control group; [b]viral vector vaccine group *vs* control group; [c]mRNA vaccine group *vs* control group. COVID-19: coronavirus disease 2019; s.d.: standard deviation; DFI: DNA fragmentation index; mRNA: messenger RNA

However, Gonzales *et al.*[22] reported that mRNA vaccine decreased sperm parameter values, but not significantly. The results of this study showed that the vector virus group had higher DFI than the inactivated and mRNA groups. This condition was caused by the vaccination mechanism, which involves the transmission of COVID-19 genetic material using live viruses. This study used genetic material to produce COVID-19-specific proteins, which are recognized by the immune system to trigger a response. Other types of vaccine, such as inactivated variant, contain the COVID-19 virus, which can initiate a response without causing disease. Furthermore, mRNA vaccines contain segments of the virus that code for a specific protein (mRNA). The side effects of vaccination on spermatozoa are similar to the impact of COVID-19. The incidence of adverse events, such as palpitations, irregular heartbeat, tongue edema, abnormal blood pressure, chest discomfort, angioedema, body contusion, urticaria, and anaphylactic reaction, is higher with the viral vectors than the inactivated virus and mRNA vaccines.[20] Chen *et al.*[23] revealed that the viral vector vaccines are live viruses as vectors to induce a strong immune response. Inactivated vaccine is a mature development with good safety levels, but it is less immunogenic compared to viral vector vaccine, while mRNA is a new and safe type of vaccine. All these explained mechanisms show that viral vector vaccine has the highest detrimental effect on spermatozoa.

This study compares the effects of three types of COVID-19 vaccine on semen parameters and DNA fragmentation, in infertile men, though it has several limitations, including the small number of samples, lack of healthy fertile men, and short follow-up. The results showed that COVID-19 infection and vaccination negatively affect sperm quality in infertile men, particularly viral vector vaccine. Inactive and mRNA vaccinations are more recommended to men wishing their partners to conceive.

The timeline (2–4 weeks after COVID-19 infection or after vaccination) was chosen in order to investigate the effect of vaccination and COVID-19 infection in the current spermatogenic cycle. However, we propose that future studies with a larger sample size and a longer follow-up period are needed for a better understanding of the effects of vaccination and COVID-19 infection in more spermatogenic cycles, which also could be used to validate these results in this study.

AUTHOR CONTRIBUTIONS

SWL played a major role in the writing of the manuscript, experimental design, semen analysis, data collection, statistical analysis, and supervision of the project. GR contributed to the experimental design, sperm DNA fragmentation test, and data collection. EY and GP contributed to the experimental design and manuscript review. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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