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The effect of ChAdOx1 nCov-19 vaccine on arterial thrombosis development and platelet aggregation in female rats

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ABSTRACT

ChAdOx1 nCoV-19 adenoviral vector vaccine (ChAd) against coronavirus disease 2019 has been associated with vaccine-induced thrombosis and thrombocytopenia (VITT), especially in young women who have presented with unusual localized thrombosis after receiving the vaccine. The pathogenesis of VITT remains incompletely understood. We tried to provide new insights into mechanisms underlying this phenomenon in the model of arterial thrombosis electrically induced in the carotid artery of female rats. At 28 days post-vaccination, ChAd induced SARS-CoV-2-specific neutralizing antibody responses in all animals. The analysis of the blood vessel/thrombus area showed slight luminal narrowing of the carotid artery with extravasation of blood in vaccinated rats. These small changes were not accompanied by differences in thrombus weight and composition. The vaccinated animals presented a slight increase (by around 14–24%) in platelet aggregation. ChAd did not significantly affect blood coagulation, platelet counts, and their activation markers. Unaffected thrombus formation, the lack of thrombocytopenia and all the measured blood and hemostasis parameters that predominantly stayed unchanged, indicate that the ChAd does not increase the risk of arterial thrombosis development in female rats.

1. Introduction

By November 2021, almost 250 million cases and over 5 million deaths of coronavirus disease 2019 (COVID-19) were reported worldwide [1]. The European Medical Agency (EMA) has authorized 4 vaccines for the prevention of symptomatic COVID-19 in the European Union: Spikevax (Moderna Biotech), Comirnaty (BioNTech Manufacturing GmbH), COVID-19 Vaccine Janssen (Janssen-Cilag International), and Vaxzevria (ChAdOx1 nCoV-19, ChAd, AstraZeneca) [2]. The efficacy and safety of the COVID-19 vaccine were proven in the registration phases and recent clinical trials [3,4].

ChAd vaccine, a recombinant chimpanzee adenoviral vector encoding the spike protein of SARS-CoV-2, has been reported to be associated with vaccine-induced thrombosis and thrombocytopenia (VITT) [5,6]. VITT is still an extremely rare adverse event, and the overall benefits of the ChAd outweigh the thromboembolic risk. The presence of unusual localized venous or arterial thrombosis, thrombocytopenia, high levels of D-dimer, low platelet counts, and low fibrinogen have been described at diagnosis. Estimated clinical manifestations of symptoms present 5 to 30 days following the vaccination [7]. The evidence came from case studies of young women with atypical thrombosis [6]. To date, the pathogenesis of VITT remains incompletely understood. VITT has been associated with high-titer immunoglobulin G (IgG) class antibodies directed against the cationic platelet factor 4 (PF4). Anti-PF4 antibodies potently activate platelets with platelet activation greatly enhanced by PF4. The immune complexes promote cross-linking of FcγRIIa receptors on the platelet surface, which is then followed by platelet activation and aggregation. The underlying pathophysiology is associated with IgG antibodies that recognize PF4 and activate platelets through their Fcγ receptors [5,8].

In the absence of strong human data, studies in rodents may provide new insights into the pathogenesis of VITT thanks to strain homogeneity and shorter life-cycle with preserved high

Abbreviations: aPTT, activated partial thromboplastin time; COVID-19, coronavirus disease 2019; EMA, European Medical Agency; IgG, immunoglobulin G; PF4, platelet factor 4; PT, prothrombin time; TPO, thrombopoietin; VITT, vaccine-induced thrombosis and thrombocytopenia; βTG, β-thromboglobulin.

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interspecies similarity. Thus, we aimed to study the possible mechanisms underlying this phenomenon in the model of arterial thrombosis electrically induced in the carotid artery of female rats.

2. Materials and methods

2.1. Animals

The rats were obtained from the Centre of Experimental Medicine in the Medical University of Bialystok. The animals were bred in a 12-hour light/dark cycle, in a temperature- and humidity-controlled room, and were allowed to have *ad libitum* access to sterilized water and standard chow in specific pathogen-free conditions. After the end of the experiment, all animals were euthanized by cutting the heart muscle and exsanguination. All the procedures were approved by the Local Ethical Committee for Animal Testing (permit number: 63/2021) and conducted under ARRIVE guidelines [9].

2.2. Vaccine

ChAd was collected from the remaining Vaxzevria's multidose vial (Lot number 210071) after patient vaccination. One dose contains chimpanzee adenovirus encoding the SARS-CoV-2 spike glycoprotein produced in genetically modified human embryonic kidney 293 cells and by recombinant DNA technology, ethanol, L-histidine, L-histidine hydrochloride monohydrate, magnesium chloride hexahydrate, polysorbate 80, sucrose, sodium chloride, disodium edetate and water for injections.

2.3. Study design

Twenty-six female Wistar rats were randomly assigned to 2 equal groups. ChAd was administered by deep intramuscular injection in the femoral muscle at a single dose of $5x10^6$ IU (final volume of 0.1 mL) on the same day. 0.9% sodium chloride solution was used as a vehicle/placebo. Rats were weighed once every three days and observed regularly for 28 days. Then, animals were anesthetized by an intraperitoneal injection of pentobarbital (45 mg/kg) and arterial thrombosis was induced by electrical stimulation (1 mA/10 min) of the common carotid artery as described previously [10] with our minor modifications [11]. During isolation of the artery, 1 rat from the group receiving ChAd died due to operator error. Four rats from each group were selected for histological analysis of any resulting thrombus. The remainder thrombi were removed, air-dried at 37 °C, and weighed 24 h after the end of the experiment. The thrombi, blood samples from the heart, bone marrow from the femur were collected 45 min after the end of electrical stimulation for further measurements. A graphical scheme of the experiment is presented in Fig. 1.

2.4. Antibodies measurement

Blood samples were put into tubes without anticoagulants and allowed to coagulate, then centrifuged at 10,000 × g at 20 °C for 5 min. The sera were collected and deep-frozen (−80 °C) for subsequent measurement of rat anti-SARS-CoV-2 IgG antibody (Krishgen Biosystems, India) by the ELISA technique in a microplate reader (BioTek, USA), according to the kit manufacturer instructions.

2.5. Thrombus and bone marrow analysis

The occluded fragments of the common carotid artery were collected and fixed in 10% buffered formalin. Paraffin blocks were sectioned by a rotating microtome (Leica Microsystems, Germany). Sections were stained by hematoxylin-eosin. The bone marrow smears were stained by the May-Grünwald-Giemsa method. Evaluation of the slides and bone marrow morphology were performed using a light microscope Olympus BX41 with Olympus DP12 camera magnification of 100 (10 the lens and 10 the eyepiece). Histopathological evaluations were performed by two independent blinded veterinary pathologists. The criteria for histopathological evaluation were based on the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice

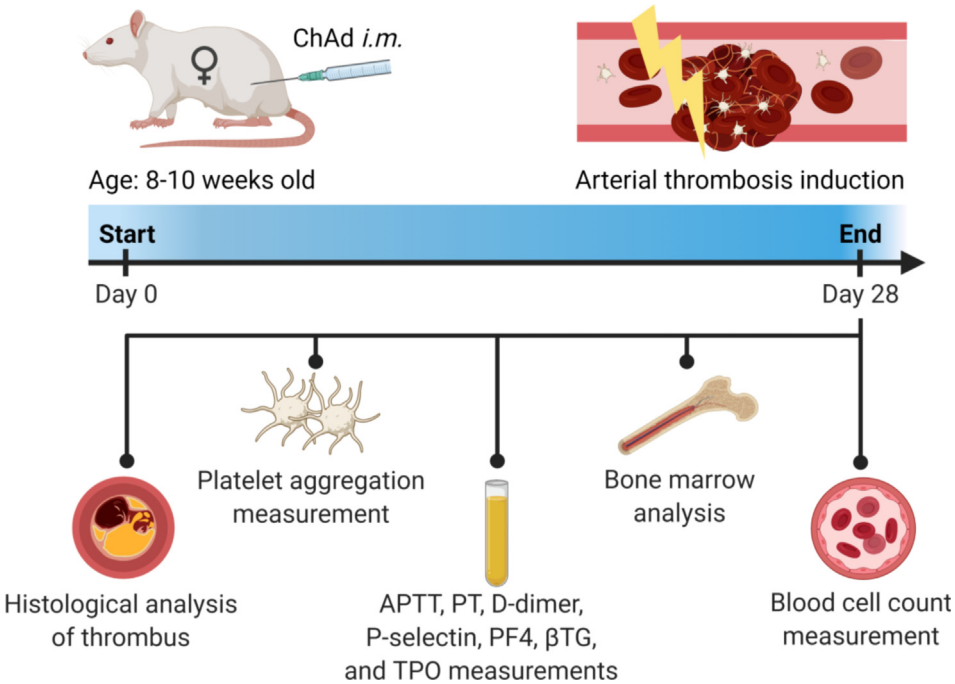

Fig. 1. A graphical scheme with a timeline of the experiment (created with BioRender.com). Abbreviations: APTT, activated partial thromboplastin time; PF4, platelet factor 4; PT, prothrombin time; TPO, thrombopoietin; βTG, β-thromboglobulin.

for the cardiovascular system [12]. Cytological assessments of the bone marrow were performed based on the recommendations of the American Society for Veterinary Clinical Pathology and the Society for Toxicologic Pathology working group [13].

2.6. Coagulation parameters

Sodium citrate-anticoagulated blood samples were centrifuged at 3500 × g at 4 °C for 10 min, and the plasma was deep-frozen (−80 °C) until further assays could be performed. The activated partial thromboplastin time (aPTT) and prothrombin time (PT) values were automatically determined by an optical method (Coag-Chrom 3003; Bio-ksel, Poland) adding routine laboratory reagents to collected plasma. The plasma concentration of D-dimer was measured by the ELISA technique, using a microplate reader (Bio-Tek, USA) to monitor the changes in absorbance according to the kit manufacturer directions (Cloud-Clone Corp., USA).

2.7. Platelet aggregation

Platelet aggregation was assessed in sodium citrate-anticoagulated blood directly after the experiment by measuring electrical impedance with an aggregometer (Chrono-log Corp., USA). The whole blood (500 μL) and 0.9% NaCl solution (500 μL) were incubated for 20 min at 25 °C, and then for 15 min at 37 °C. The changes in impedance were registered for 6 min after the collagen addition (7.5 μg/mL). The aggregation curve was described by the maximal extension, the slope of the platelet aggregation, lag phase, and the area under the curve.

2.8. Platelet activation markers and thrombopoietin measurement

Blood samples were put into tubes without anticoagulants and allowed to coagulate, then centrifuged at 10,000 × g at 20 °C for 5 min. The sera were collected and deep-frozen (−80 °C) for subsequent measurement of P-selectin, PF4, β-thromboglobulin (βTG), and thrombopoietin (TPO) concentrations (Cloud-clone Corp., USA) by the ELISA technique in a microplate reader (BioTek, USA), according to the kit manufacturer instructions.

2.9. Blood cell count

The blood samples were drawn into 3.13% trisodium citrate in a volume ratio of 9:1. Blood cell count was assessed with an Animal Blood Counter (Horiba ABX, France) according to the manufacturer's directions.

2.10. Statistical analysis

Shapiro-Wilk's test was used for data distribution analysis. Data are shown as individual points with mean ± S.D. or median with interquartile range and analyzed using the unpaired Student's *t*-test or Mann-Whitney test using Prism 8 (Graphpad, USA). When *P* was smaller than 0.05, differences between the two groups was considered to be of statistical significance.

3. Results

3.1. Clinical observations and SARS-CoV-2 IgG antibody responses

No deaths, vaccine-related clinical signs of toxicity, effects on food consumption, or visual changes were reported during the 28-day observation. Body weight gain and final body weight at the end of the experiment were not significantly different among control and vaccinated rats (Fig. 2A). At 28 days post-vaccination, ChAd induced SARS-CoV-2-specific neutralizing antibody responses in all animals. High antibody levels (>10 μg/mL) were detected in 75% of rats (Fig. 2B). A positive result of the anti-SARS-CoV-2 IgG antibody confirmed the dose was correctly chosen.

3.2. Arterial thrombosis development and blood coagulation

The analysis of the blood vessel/thrombus area showed slight luminal narrowing of the carotid artery with extravasation of blood in vaccinated rats. There were no differences in the thinning of the artery walls, segmental widening of the artery lumen, vascular smooth muscle cell degeneration or necrosis, endothelial damage, and artery edema between control and vaccinated rats. Thrombi were found to be similar in red blood cell and fibrin composition (Fig. 3A and Table 1). These slight carotid artery wall

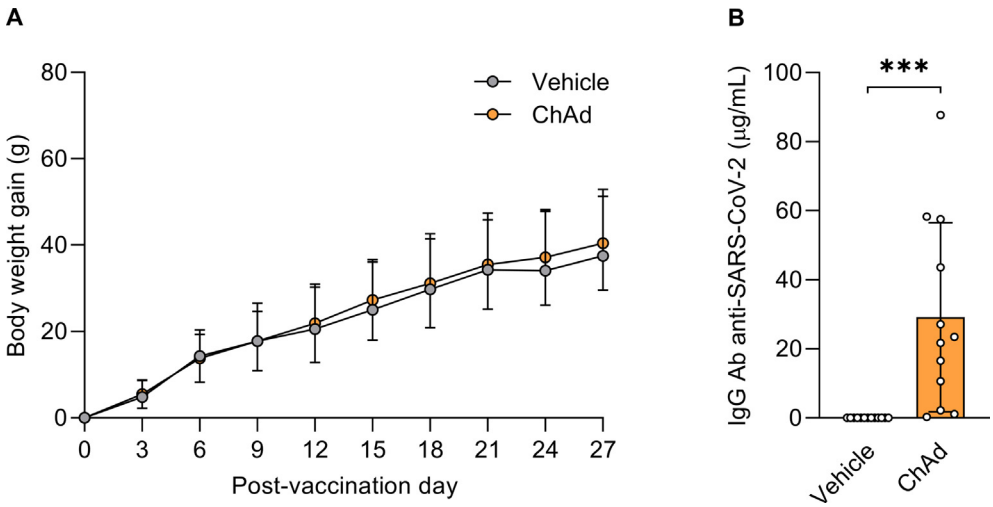

Fig. 2. ChAd does not affect body weight and induces SARS-CoV-2-specific neutralizing antibody responses in female rats. (A) Three-day average cumulative body weight gain in rats, and (B) the anti-SARS-CoV-2 IgG antibody concentration in rat serum measured by ELISA technique according to the kit manufacturer instructions. ***$P < .001$, unpaired Student's *t*-test. Results are shown as individual points with mean ± S.D. and analyzed with Prism 8.

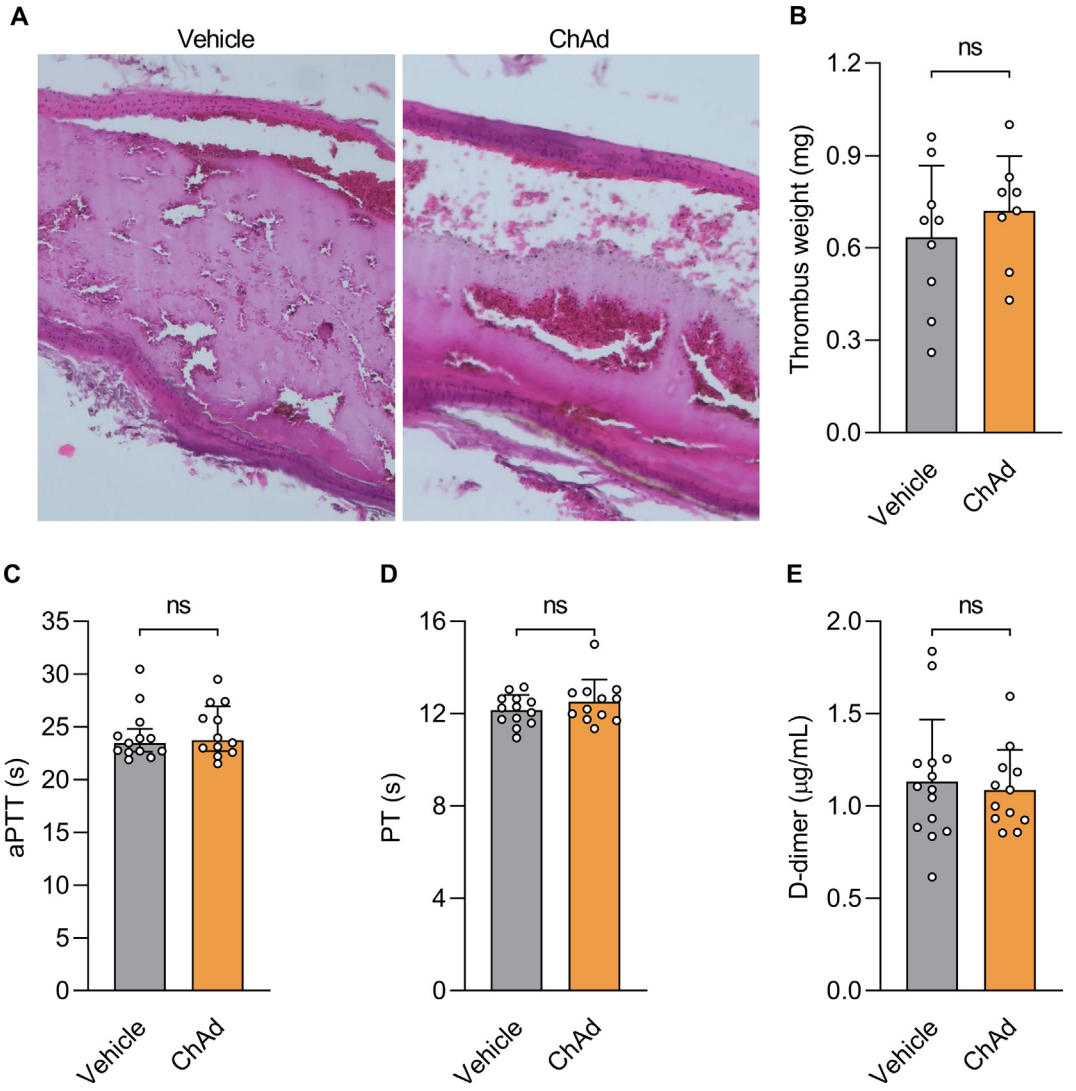

Fig. 3. ChAd does not promote electrically induced arterial thrombosis in female rats. (A) Representative micrographs of thrombus formed in the carotid artery of vaccinated rats. Paraffin blocks were sectioned by a rotating microtome (Leica Microsystems, Germany). Sections were stained by hematoxylin-eosin (H&E). Evaluation of the slides was performed using a light microscope Olympus BX41 with Olympus DP12 camera magnification of 100 (10 the lens and 10 the eyepiece). (B) Thrombus weight 24 h after the end of the procedure using an analytical scale. (C) Activated partial thromboplastin time (aPTT) and (D) prothrombin time (PT) measured by an optical method in sodium citrate-anticoagulated plasma collected from rats. (E) D-dimer concentration in sodium citrate-anticoagulated rat plasma measured by ELISA technique according to the kit manufacturer instructions. ns = not significant, unpaired Student's *t*-test or Mann-Whitney test. Results are shown as individual points with mean ± S.D. or median with interquartile range and analyzed with Prism 8.

Table 1
Summary of notable histological abnormalities in arterial thrombus of rats vaccinated with a single dose of ChAd.

	Vehicle	ChAd
Thinning of the artery walls	3 (3–4)	3.5 (2–4)
Segmental widening of the artery lumen	3 (2–4)	3.5 (2–4)
Narrowing of the artery lumen	0.5 (0–2)	2 (0–3)
Extravasation of blood	1 (0–3)	2 (1–2)
Vascular smooth muscle cell degeneration	3 (2–4)	3 (3–4)
Vascular smooth muscle cell necrosis	1 (1–2)	1 (1–3)
Endothelial damage	3 (3–3)	3 (3–4)
Artery edema	2 (1–3)	1.5 (1–2)
Red blood cell-rich thrombus	2 (1–4)	2 (1–4)
Fibrin-rich thrombus	2 (1–3)	2 (2–3)

The severity was rated on a 5-point scale; with 0 for no effect, 1 for minimal, 2 for mild, 3 for moderate, through 4 for severe effect. Results are shown as median with range.

deformations were not accompanied by the increased weight of the thrombus (Fig. 3B). ChAd did not affect aPTT, PT, and D-dimers (Fig. 3C-E).

3.3. Platelet aggregation, platelet activation markers, and thrombopoietin measurement

Vaccinated rats presented a slight increase (by around 14–24%) in the maximal extension, slope of platelet aggregation, and area under the curve (Fig. 4A-D). The increase in these parameters indicates that platelets are slightly activated by ChAd. No significant differences in P-selectin, βTG, PF4, and TPO concentrations (Fig. 4-E-H), complete blood count (Table 2), and bone marrow morphology (Table 3) were observed in the rats vaccinated with ChAd when compared to the control group.

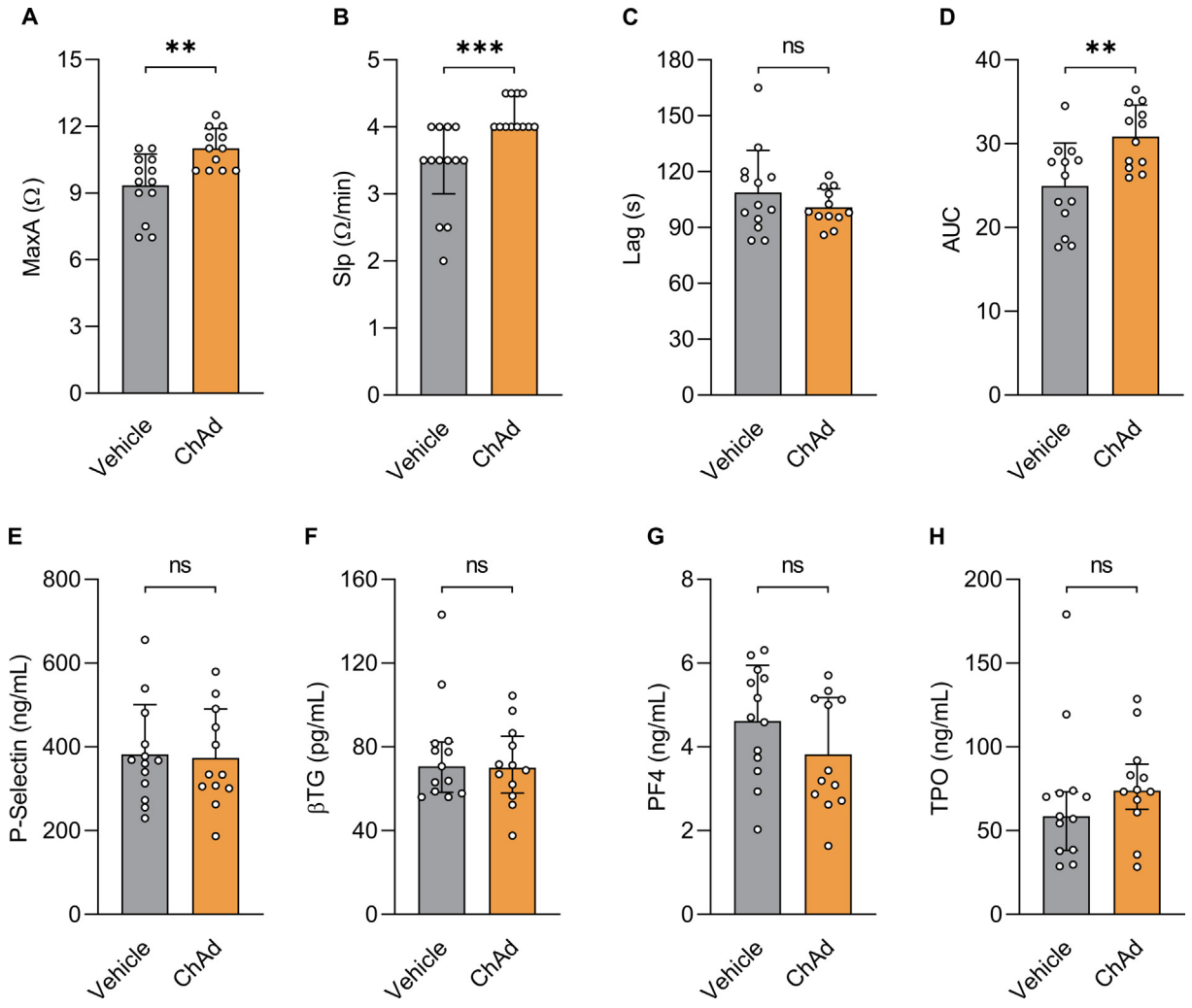

Fig. 4. ChAd enhances platelet aggregation in female rats with electrically induced arterial thrombosis. (A) The maximal extension (MaxA), (B) slope of platelet aggregation (Slp), (C) lag time (Lag), and area under the curve (AUC) in whole blood after the collagen addition (7.5 μg/mL) measured by impedance technique in sodium citrate-anticoagulated blood with 0.9% saline solution (volume ratio 1:1). Before the measurement, the mixture was incubated for 20 min at 25 °C and then for 15 min at 37 °C. The changes in impedance registered were recorded as aggregation curves. (E) P-selectin, (F) β-thromboglobulin (βTG), (G) platelet factor 4 (PF4), and (H) thrombopoietin (TPO) concentration in rat serum measured by ELISA technique according to the kit manufacturer instructions. $^{**}P < .01$, $^{***}P < .001$, ns = not significant, unpaired Student's *t*-test or Mann-Whitney test. Results are shown as individual points with mean ± S.D. or median with interquartile range and analyzed with Prism 8.

Table 2
Complete blood count results after arterial thrombosis induction in rats vaccinated with a single dose of ChAd.

	Vehicle	ChAd
White blood cells, $10^3/mm^3$	1.3 ± 0.3	1.4 ± 0.4
Red blood cells, $10^6/mm^3$	7.56 ± 0.46	7.49 ± 0.26
Hemoglobin, g/dL	14.3 ± 0.9	14.2 ± 0.5
Hematocrit, %	41.7 ± 2.6	41.2 ± 1.5
Mean corpuscular volume, μm^3	55.2 ± 0.9	55.1 ± 0.8
Mean corpuscular hemoglobin, pg	18.9 ± 0.4	18.9 ± 0.3
Mean corpuscular hemoglobin concentration, g/dL	34.3 ± 0.4	34.4 ± 0.4
Blood platelets, $10^3/mm^3$	510 ± 33	521 ± 51

Results are shown as mean ± S.D.

Table 3
Bone marrow results after arterial thrombosis induction in rats vaccinated with a single dose of ChAd.

	Vehicle	ChAd
Myeloblasts, %	2.32 ± 0.67	2.08 ± 0.90
Promyelocytes, %	5.20 ± 0.98	4.66 ± 0.69
Myelocytes, %	9.17 ± 2.81	9.29 ± 3.61
Metamyelocytes, %	11.21 ± 2.36	10.97 ± 0.92
Band neutrophils, %	9.72 ± 1.91	9.69 ± 2.02
Segmented neutrophils, %	13.81 ± 4.33	12.65 ± 4.34
Eosinophilic cell line, %	5.43 ± 1.01	6.31 ± 2.30
Basophilic cell line, %	0.10 ± 0.13	0.14 ± 0.19
Monocytic cell line, %	2.67 ± 1.21	2.80 ± 0.63
Proerythroblast, %	1.41 ± 0.56	1.43 ± 0.68
Basophilic erythroblast, %	9.76 ± 2.33	9.49 ± 2.30
Polychromatophilic erythroblast, %	14.28 ± 2.60	14.85 ± 3.11
Acidophilic erythroblasts, %	4.47 ± 1.92	6.05 ± 2.82
Lymphoid cell line, %	9.22 ± 2.20	8.84 ± 2.04
Plasmocytes, %	0.28 ± 0.39	0.28 ± 0.21
Megakaryocytes, %	0.75 ± 0.37	0.71 ± 0.23

Results are shown as mean ± S.D.

4. Discussion

We report the effect of vaccination with ChAd on blood platelets and coagulation in the model of arterial thrombosis electrically

induced in female rats. We found slight platelet activation and changes in the carotid artery wall without signs of prothrombotic and procoagulant activity of ChAd. The number of platelets, their activation markers and other blood parameters were observed, indicative of the general safety and neutrality of the vaccine to hemostasis in female rats.

According to the American Society of Hematology, VITT is a clinical syndrome characterized by the presence of unusual localized venous or arterial thrombosis, markedly elevated D-dimer and (PF4-dependent) thrombocytopenia. Thrombosis was noticed mostly in women under 50 years [5,14]. In our study, the analysis of the thrombus area showed slight luminal narrowing of the carotid artery with extravasation of blood in vaccinated rats. These small changes were not accompanied by the increased weight of the thrombus. We did not observe any changes in the typical clotting times and D-dimer concentration, although we purposely conducted a study on female rats. The response of the human coagulation system to the vaccine may differ depending on the genetic background, especially if we take into account a very low occurrence of VITT [15,16]. Additionally, the incidence rates could be varied in populations with different genetic risks. There are many doubts about genetic susceptibility that could portend thrombotic risk in VITT [16]. A high homogeneity of rats strain, thus the lack of polymorphisms or species differences in the hemostatic molecular targets of humans and rats could be an explanation of our results.

What we found was a slight increase by around 14–24% in platelet aggregation without any significant change in the number of platelets. However, we did not observe an elevated concentration of soluble P-selectin, PF4 and βTG, which would indicate the activation of platelets *in vivo*. The hyperactive platelets are a component of autoimmune diseases exemplified by lupus erythematosus and immune thrombocytopenic purpura [17]. ChAd could make platelets susceptible to proaggregatory stimuli such as collagen. The vaccine-induced spike protein can stimulate platelets *via* binding to endothelial angiotensin-converting enzyme 2, and turn its antithrombotic properties toward the opposite side [18,19]. A balance of angiotensin (Ang) production may shift from the Ang I/Ang-(1–7) axis, exerting antiplatelet activity *via* release of endothelial prostacyclin and nitric oxide [20], into the Ang-(1–9)/Ang II axis, exerting proaggregatory and prothrombotic activity *via* angiotensin receptor type I, as we had previously shown [21,22]. The derangement of artery wall found in the histopathology could play a role in the activation of platelets observed in our study. On the other hand, spike protein may activate platelets indirectly through inflammation and release of PF4 [15]. It was suggested that the antibodies against complexes of PF4 with heparin seen in heparin-induced thrombocytopenia could mediate VITT, although most cases of VITT do not have prior heparin exposure [15,23]. A cationic PF4 bound to other polyanions could stimulate the anti-PF4 antibody production and promote platelet aggregation. DNA in the virus-vectored vaccine or release from macrophages and neutrophils during exaggerated immune reaction after vaccination is assumed to bind PF4 [24]. During inflammation, injured endothelial glycocalyx can be another source of polyanion, such as heparan sulfate [25]. Although the concentration of PF4 was unchanged, a slight tendency to decrease circulating PF4 could be seen. Increased platelet hyperactivity in COVID-19 patients was linked with virus effect on megakaryocytes [18,26]. However, we did not observe any changes in TPO production and bone marrow morphology.

In conclusion, we would emphasize the role of hyperactive platelets in the mechanism of VITT, thus the therapeutic solutions could potentially interfere with platelets function. However, genetic or other factors seem necessary for VITT development in humans. Within the limitations that the female rat model study presents, we conclude that unaffected thrombus formation, the lack of thrombocytopenia, and all the measured blood and hemostasis parameters that predominantly stayed unchanged, indicate that the ChAd does not increase the risk of arterial thrombosis development.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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