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APROBATION OF PLATELET AGGREGATION INHIBITOR FROM *Echis multisquamatis* SNAKE VENOM *in vitro, in vivo* AND *ex vivo*

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Snake venom-derived platelet aggregation inhibitors can be promising antiplatelet medications that can allow to avoid the risk of bleeding and treatment resistance, particularly in aspirin-resistant patients. Our study aimed to assess the effectiveness of a platelet aggregation inhibitor derived from *Echis multisquamatis* snake venom in various settings, including *in vitro, in vivo*, and *ex vivo*.

Methods. We examined a polypeptide from *Echis multisquamatis* venom, purified using a recently developed chromatography protocol, across multiple models. This polypeptide was introduced into platelet-rich blood plasma and administered intravenously to rats. The effects on platelet aggregation were assessed using aggregometry, focusing on ADP-induced aggregation.

Results and Discussion. Our findings revealed that a concentration of 0.040 mg/ml significantly reduced platelet aggregation *in vitro*. Remarkably, this dosage also proved effective when administered intravenously in laboratory animals, reaffirming its potential as a robust antiplatelet agent. In the final phase of our study, the polypeptide demonstrated its ability to inhibit platelet aggregation in blood plasma of pregnant woman with aspirin resistance, presenting a promising avenue for innovative treatment approaches in such cases.

Conclusion. This study underscores the potential of the *Echis multisquamatis* venom-derived polypeptide as a promising antiplatelet agent, effective in diverse scenarios, including aspirin resistance. Further research and clinical trials are imperative to fully harness its therapeutic potential.

Key words: disintegrin; blood plasma; platelets; thrombosis; blood coagulation; platelet aggregation; animal model.

The pursuit of new platelet aggregation inhibitors is an urgent issue, as today's World possesses new challenges: the emergence of patient resistance to antithrombotic agents [1, 2], increased risk of blood loss [3], and genetic resistance to existing drugs [4, 5].

Platelet aggregation inhibitors from snake venoms are small proteins that bind to receptors on platelets surface with high affinity [6, 7]. Due to their specificity, these proteins don't interfering with coagulation factors, which means that platelet aggregation inhibitors from snake venoms have a lower risk of causing excessive bleeding [8, 9]. Also, because they are acting by targeting platelets receptors it helps to avoid the development of treatment resistance [10, 11]. For example, aspirin cannot be successfully applied for the treatment of patients with resistance to aspirin [12] that can be found at least in 20% of population [13].

So, study of novel antiplatelet agents, in particular polypeptides from the venom of snakes, is a promising task for the development of potential drugs with antithrombotic action. Previously platelet aggregation inhibitor was found in the venom of snake *Echis multisquamatis* [14]. The aim of our study was to approbate platelet aggregation inhibitor from *Echis multisquamatis* snake venom *in*

vitro, in vivo and *ex vivo*. In particular our goal was to study the effect of this polypeptide on platelet aggregation after intravenous injection into rat's bloodstream and also to find out whether it can suppress platelet aggregation in platelet rich blood plasma of pregnant women with resistance to aspirin.

Materials and Methods

Polypeptide that effectively inhibited platelet aggregation was purified from the venom of *Echis multisquamatis* by ion-exchange and size-exclusion chromatography and analyzed by SDS-PAGE as it was reported earlier [14].

Male Wistar rats (8 weeks old) with body weight ≅ 200 g were individually housed in separate cages and had ad libitum access to standard food and water. The drug was administered by injection into the tail vein using a 0.3 ml syringe and a needle of diameter 30 g. Rats were anesthetized by sodium thiopental. The procedures were conducted in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986) and the Law of Ukraine On the Protection of Animals from Cruelty No. 3447 of 21.02.2006. Samples of animal blood were collected by heart puncture. 3.8% sodium citrate was added to blood immediately after collection.

Somatically healthy woman with single spontaneous pregnancy with diagnosed aspirin-resistance was enrolled in the study. Blood sample was kindly provided by the Kyiv Perinatal Center and analyzed immediately. Venous blood sampling for testing was collected from a peripheral vein using vacuum systems into sterile plastic 4 ml tubes, containing 3.8% sodium citrate solution. Written informed consent to be included in the study was accepted.

Platelet rich plasma (PRP) for the aggregometry study was obtained from whole blood by centrifuging at 160 g for 30 min at 25 °C [15].

Platelet aggregation was studied by aggregometry using Solar AP2110. PRP was added to the cuvette of the aggregometer and constantly stirred by the magnet mixer. In *in vitro* and *ex vivo* studies the solution of platelet aggregation inhibitor was added to the cuvette (final concentration was 0.04 mg/ml). Platelets were activated by ADP (final concentration 12.5 μM) and $CaCl_2$ (1 mM). Change of the light transmission was monitored. The speed and the rate of platelet inhibitors were measured [16].

Statistical analysis. All measurements were performed in triplicate.

Results and Discussion

Previously, a polypeptide was obtained from the venom of the *Echis multisquamatis* snake, and it was found to be an effective inhibitor of platelet aggregation. It was demonstrated that this polypeptide acts as a disintegrin or an antagonist of integrin receptors. This means that when it interacts with integrin receptors, it hinders the receptors' binding to the fibrinogen molecule, thus complicating the process of platelet aggregation and the formation of a fibrin-platelet thrombus. The researchers suggested a dosage that reduces the degree of platelet aggregation by 50% (IC50), which was 0.040 mg/ml.

In the initial phase of the study, the effect of this dosage of disintegrin was assessed *in vitro*, meaning it was added to a test tube containing platelet rich blood plasma from a control rat. Specifically, 0.01 ml of the disintegrin solution (1.2 mg/ml) was added to 0.24 ml of platelet rich blood plasma and incubated in the aggregometer cuvette, with constant automatic stirring achieved by a rotating magnet. After 3 minutes, platelet aggregation was triggered by adding 0.025 ml of $CaCl_2$ (0.025 M) and 0.025 ml of ADP (0.12 mM). In the control sample, an equivalent volume of physiological saline was added instead of the disintegrin solution. This allowed the determination of ADP-induced platelet aggregation, where platelets activated by ADP aggregated and formed microclots. The device measured the increase in the transparency of the tested suspension. In the case of inhibition of platelet aggregation, this process was delayed, resulting in reduced final transparency of the suspension.

Here we confirmed that the selected dosage effectively inhibited the platelet aggregation *in vitro* (Fig. 1).

The next stage of the research was to determine its effectiveness when administered intravenously (*in vivo*), i.e., to assess whether its efficacy would be maintained. It is confirmed that the blood volume of a rat is approximately 50 ml per kilogram of body weight [17]. Therefore, it is possible to calculate the amount of the polypeptide solution (1.2 mg/ml) needed to be injected into the bloodstream of a 200 g rat to achieve

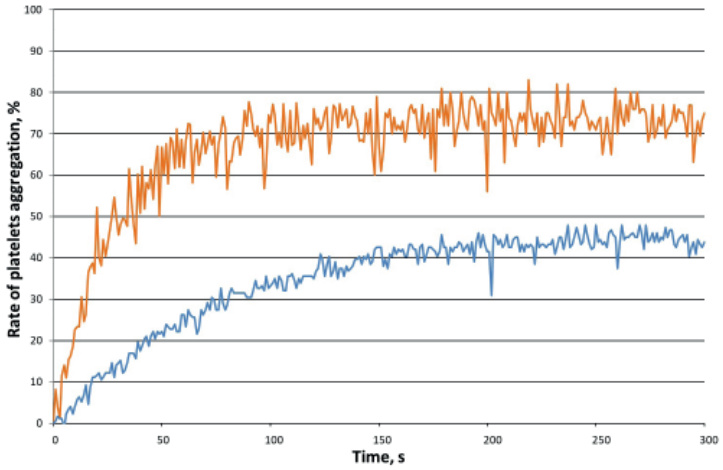

Fig. 1. **ADP-induced aggregation of platelets of a control rat in the presence of disintegrin from the venom of the *Echis multisquamatis* snake (blue line) or an equivalent volume of physiological saline (orange line)**
All measurements performed in triplicate; typical curves are presented

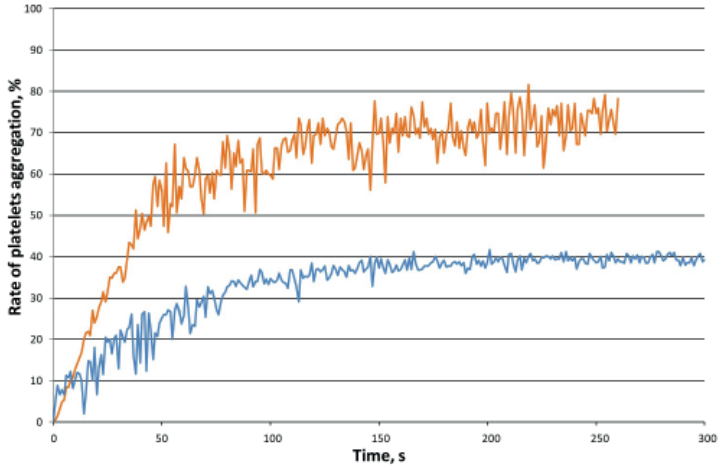

Fig. 2. **ADP-induced aggregation of platelets of a rat after the intravenous administration of disintegrin from the venom of the Echis multisquamatis snake (blue line) or an equivalent volume of physiological saline (orange line)**
All measurements performed in triplicate; typical curves are presented

an effective polypeptide concentration in the bloodstream of 0.04 mg/ml.

The volume of rat blood was 10 ml. The initial concentration of the polypeptide solution was 1.2 mg/ml, and the desired final concentration in the bloodstream was 0.04 mg/ml. Achieving this required diluting the original solution by a factor of 30. Consequently, for 10 ml of blood, 0.33 ml of the solution needed to be injected.

The polypeptide solution was administered through injection into the lateral tail vein of the rats using an insulin syringe. Control rats were injected with 0.33 ml of a physiological saline solution. Blood samples were collected by heart puncture 30 minutes after the injection.

The resulting aggregation shows that the introduction of the polypeptide significantly reduces both the speed and the degree of ADP-induced aggregation of platelets (Fig. 2).

The experiment's results demonstrate that the studied polypeptide from the venom of the *Echis multisquamatis* snake effectively inhibits platelet aggregation even under conditions of intravascular administration. The data obtained suggest promising potential for the use of the investigated disintegrin in future antithrombotic therapy.

As a final step of examinations, we approbated the inhibitory actions of the studied polypeptide *ex vivo* on the platelet rich blood plasma donated by pregnant woman with diagnosed aspirin-resistance.

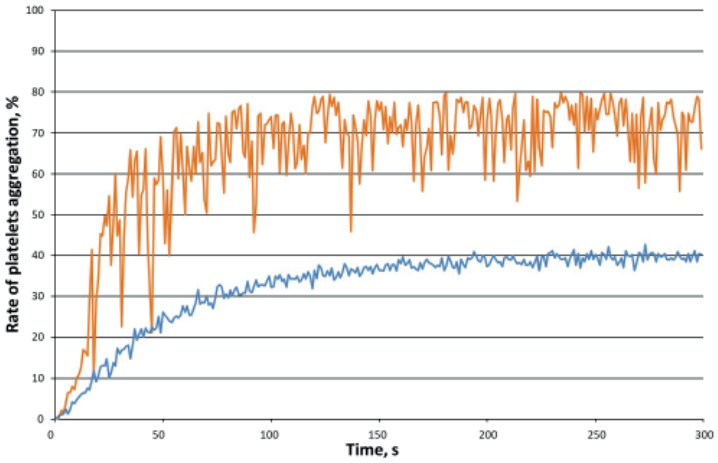

Fig. 3. **ADP-induced aggregation of platelets of a pregnant woman with diagnosed aspirin-resistance after the addition of disintegrin from the venom of the Echis multisquamatis snake (blue line) or an equivalent volume of physiological saline (orange line)**
All measurements performed in triplicate; typical curves are presented

We demonstrated that being the antagonist of integrin receptors the studied polypeptide can be effective for aspirin-resistant patients.

As a result, it can be concluded that the polypeptide derived from the venom of the *Echis multisquamatis* snake effectively reduces the aggregation ability of platelets *in vitro*. Moreover, this antiplatelet effect of the polypeptide remains unaffected under the conditions of intravenous administration to laboratory animals. Notably, it also demonstrated the ability to inhibit platelet aggregation *ex vivo* in patients with aspirin resistance.

In summary, the polypeptide from the venom of the *Echis multisquamatis* snake has the potential to serve as a prototype for an effective antithrombotic agent, capable of inhibiting platelet aggregation.

Authors' contribution

MZ performed aggregometry and analyzed datasets; OP purified polypeptide, performed aggregometry; YK was in aim for animal housing, sample injection and blood collection; YS analyzed results, wrote the manuscript.

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REFERENCES

1. *Mărginean A., Bănescu C., Scridon A., Dobreanu, M.* Anti-platelet Therapy Resistance — Concept, Mechanisms and Platelet Function Tests in Intensive Care Facilities. *Journal of critical care medicine (Universitatea de Medicina si Farmacie din Targu-Mures)*. 2016, 2(1), 6–15. https://doi.org/10.1515/jccm-2015-0021
2. *Kumar A., Kao J.* Platelet resistance to antiplatelet drugs. *Recent patents on cardiovascular drug discovery*. 2009, 4(2), 98–108. https://doi.org/10.2174/157489009788452959
3. *Ferraris V. A., Ferraris S. P., Saha S. P.* Antiplatelet drugs: mechanisms and risks of bleeding following cardiac operations. *The International journal of angiology : official publication of the International College of Angiology. Inc.* 2011, 20(1), 1–18. https://doi.org/10.1055/s-0031-1272544
4. *Feher G., Feher A., Pusch G., Lupkovics G., Szapary L., Papp E.* The genetics of antiplatelet drug resistance. *Clinical genetics*. 2009, 75(1), 1–18. https://doi.org/10.1111/j.1399-0004.2008.01105.x
5. *Trenk D., Hochholzer W.* Genetics of platelet inhibitor treatment. British journal of clinical pharmacology. 2014, 77(4), 642–653. https://doi.org/10.1111/bcp.12230
6. *Echeverría S. M., Van de Velde A. C., Luque D. E., Cardozo C. M., Kraemer S., Gauna*

Pereira M. D. C., Gay C. C. Platelet aggregation inhibitors from Bothrops alternatus snake venom. *Toxicon : official journal of the International Society on Toxinology*, 2023, 223, 107014. https://doi.org/10.1016/j.toxicon.2022.107014
7. *Chérifi F., Laraba-Djebari F.* Bioactive Molecules Derived from Snake Venoms with Therapeutic Potential for the Treatment of Thrombo-Cardiovascular Disorders Associated with COVID-19. *The protein journal.* 2021, 40(6), 799–841. https://doi.org/10.1007/s10930-021-10019-4
8. *Kuo Y. J., Chung C. H., Huang T. F.* From Discovery of Snake Venom Disintegrins to A Safer Therapeutic Antithrombotic Agent. *Toxins*. 2019, 11(7), 372. https://doi.org/10.3390/toxins11070372
9. *Lazarovici P., Marcinkiewicz C., Lelkes P. I.* From Snake Venom's Disintegrins and C-Type Lectins to Anti-Platelet Drugs. *Toxins*. 2019, 11(5), 303. https://doi.org/10.3390/toxins11050303
10. *Frangieh J., Rima M., Fajloun Z., Henrion D., Sabatier J. M., Legros C., Mattei C.* Snake Venom Components: Tools and Cures to Target Cardiovascular Diseases. *Molecules (Basel, Switzerland.* 2021, 26(8), 2223. https://doi.org/10.3390/molecules26082223
11. *Van den Kerkhof D. L., van der Meijden P. E. J., Hackeng T. M., Dijkgraaf I.* Exogenous Integrin αIIbβ3 Inhibitors Revisited: Past, Present and Future Applications. *International journal of molecular sciences*. 2021, 22(7), 3366. https://doi.org/10.3390/ijms22073366
12. *Khan H., Kanny O., Syed M. H., Qadura M.* Aspirin Resistance in Vascular Disease: A Review Highlighting the Critical Need for Improved Point-of-Care Testing and Personalized Therapy. *International journal of molecular sciences.* 2022, 23(19), 11317. https://doi.org/10.3390/ijms231911317
13. *Lordkipanidzé M., Pharand C., Schampaert E., Turgeon J., Palisaitis D. A., Diodati J. G.* A comparison of six major platelet function tests to determine the prevalence of aspirin resistance in patients with stable coronary artery disease. *European heart journal*. 2007, 28(14), 1702–1708. https://doi.org/10.1093/eurheartj/ehm226
14. *Chernyshenko V., Petruk N., Korolova D., Kasatkina L., Gornytska O., Platonova T., Chernyshenko T., Rebriev A., Dzhus O., Garmanchuk L., Lugovskoy E.* Antiplatelet and anti-proliferative action of disintegrin from Echis multisquamatis snake venom. *Croatian medical journal*. 2017, 58(2), 118–127. https://doi.org/10.3325/cmj.2017.58.118
15. *Chernyshenko V., Shteinberg K., Lugovska N., Ryzhykova M., Platonova T., Korolova D., Lugovskoy E.* Preparation of highly-concentrated autologous platelet-rich plasma for biomedical use. The Ukrainian Biochemical Journal. 2019, 91(2), 19–27. https://doi.org/10.15407/ubj91.02.019
16. *Korolova D. S., Pavlenko A. O., Altorjay A., Zhuk S. I., Us I. V., Tsaryk Y., Suranyi A., Chernyshenko V. O.* Validation of the diagnostics algorithm to monitor coagulation parameters in pregnant women. The Ukrainian Biochemical Journal. 2023, 95(3), 33–41.
17. *Górska P.* Principles in laboratory animal research for experimental purposes. *Medical science monitor : international medical journal of experimental and clinical research*. 2000, 6(1), 171–180.

АПРОБАЦІЯ ІНГІБІТОРА АГРЕГАЦІЇ ТРОМБОЦИТІВ З ОТРУТИ *Echis multisquamatis in vitro, in vivo* ТА *ex vivo*

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Інгібітори агрегації тромбоцитів, отримані з отрути змій, є перспективними антитромботичними засобами, які водночас зменшують ризик кровотечі та дозволяють уникнути резистентності, як, наприклад, у пацієнтів, стійких до аспірину. Наше дослідження мало на меті оцінити ефективність інгібітора агрегації тромбоцитів, отриманого з отрути змії *Echis multisquamatis*, у різних умовах, включаючи *in vitro, in vivo* та *ex vivo*.

Методи. Досліджено поліпептид з отрути *Echis multisquamatis*, очищений за допомогою розробленого протоколу хроматографії, на кількох моделях. Цей поліпептид додавали до збагаченої тромбоцитами плазми крові або вводили внутрішньовенно щурам. Вплив на агрегацію

тромбоцитів оцінювали за допомогою агрегатометрії, зосереджуючись на індукованій ADP агрегації.

Результати та обговорення. Результати показали, що концентрація порліпептиду 0,040 мг/мл значно знижувала агрегацію тромбоцитів *in vitro*. Примітно, що ця доза також виявилася ефективною при внутрішньовенному введенні лабораторним тваринам, підтверджуючи потенціал поліпептиду як надійного антиагрегантного агента. На завершальному етапі дослідження поліпептид продемонстрував свою здатність пригнічувати агрегацію тромбоцитів у плазмі крові вагітної жінки з резистентністю до аспірину, що є багатообіцяючим для створення інноваційних підходів до лікування в таких випадках.

Висновок. Це дослідження підкреслює потенціал поліпептиду, отриманого з отрути *Echis multisquamatis*, як перспективного антиагрегантного агента, ефективного, зокрема, і за резистентності до аспірину. Для повного використання його терапевтичного потенціалу потребуються подальші дослідження та клінічні випробування.

Ключові слова: дезінтегрин; плазма крові; тромбоцити; тромбоз; зсідання крові; агрегація тромбоцитів; модель на тваринах.