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Choosing potential dissolution medium to study the influence of water-insoluble substances on aggregation of platelets within preclinical studies under conditions *in vitro*

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Abstract
The authors have studied the influence of dissolution media of potential antiaggregant in order to further carry out preclinical research of their impact on functional activity of platelets. There has been defined different impact of ethanol, dimethylsufoxide (DMSO), dimethylformamide (DMFA), and dioxane on the hemostasis system. It has been verified that dioxane, DMFA, and DMSO exert evident influence on adenosine diphosphate (ADP)- and collagen-induced aggregation of platelets, which is determined by turbidimetric method. The findings enable using ethanol as a solvent in maximum 50% exposure (vol/vol) in order to study the impact on aggregation of platelets of water-insoluble substances by this method.

Keywords: Blood; platelets; antiaggregation activity; DMSO; ethanol; dioxane; DMFA.

Introduction

According to scientific papers, the biological activity of a substance directly depends on the physical and chemical parameters of a molecule, including, without limitation, water solubility of a molecule. Liposoluble compounds exceed in average water-soluble equivalent in terms of biological activity—the more liposoluble the preparation is, the more capable it is of penetrating through cellular membrane (Lin and Lu, 1997). Solubility of a substance depends, on the one hand, on its chemical structure and, on the other hand, on the nature of the solvent. The hemostasis system is characterized by sensitivity and variability of the effects in response to any impact on hemostasis. Thus, the choice of a solvent for potential water-insoluble antiaggregants is to be made on an amount of minimal impact of the solvent on the hemostasis system.

Upon analyzing the impact of firstly synthesized xanthine derivatives on the hemostasis system, there appeared an issue of dissolving a whole group of water-insoluble compounds of such kind (Kamilov *et al*., 2011). There are no unilateral recommendations to choose solvents for compounds of synthetic nature. In accordance with the FDA's Guidance for Industry, Q3C (2012), we have chosen a list of potential solvents and studied their impact on functional activity of platelets under conditions *in vitro*.

Materials and Methods

Experiments have been made using the blood of healthy male donors. The research excluded the participation of subjects carrying blood with somatic, oncologic pathology, endocrine, acute, and inveterate virulent diseases as well as the smoking and using of inwardly alcoholic liquids and pharmaceuticals. The average age of the donors is 18–24 years. The blood sampling was made from cubital vein. Cubital vein tap was carried out with the system of evacuated blood collection BD Vacutainer® (Becton, Dickinson and Company, USA). For a vein blood anticoagulant, sodium citrate solution 0.109 mol/L was used. All the research was carried out within 2 hours after blood sampling. Platelet-rich plasma (PRP) samples were received by centrifuging citrated blood at 150 g for 10 minutes, and platelet-poor plasma (PPP) samples at 300 g for 15 minutes.

Platelet aggregation
The impact on functional activity of platelets was researched with the help of laser analyzer of platelet aggregation "Biola 230 LA" (Russia).

Aggregation intensity was estimated in accordance with the dynamics of light transmission of plasma when added to with aggregation-modifying drugs under Born methods (1962) and in accordance with the dynamics of size changing in generated aggregate (Gabbasov, 1992). For aggregation inducer, ADP in 20 μg/ml exposure and collagen in 5 mg/ml exposure produced by "Technologiya-Standart," Russia, was used.

To register performance aggregatogram, 240 μL of platelet-enriched plasma was put into cuvet, heated in the aggremeter incubator up to 37°C for 1–2 min, and then 10 μL of aggregation inducer was added. 10 μL of the analyzed substance was added by PRP-dosimeter under constant stirring in the course of study of solvents impact on platelets aggregation. Three minutes of incubation at 37°C was followed by 10 μL inducer solution input.

Aggregatogram analysis was carried out with the help of AGGR software, with the account of the following indices: general nature of aggregation (single-wave, two-wave, complete, non-complete, inversive, noninversive), maximal aggregation (MA) value, maximal speed of aggregation (tg α), average size of thrombocyte aggregate in relative units (MR), latent period duration in the use of collagen as an inducer (lag period) (Zhou and Schmaier, 2005).

Research target

Sterile water, 0.9% NaCl solution, ethanol, DMSO, DMFA (*N,N*-dimethylformamide), and dioxane (diethylenedioxide). For ethanol, DMSO, DMFA, and dioxane, concentrated 95% solution (I) was used, as well as 65.0% (II), 47.5% (III), 30.0% (IV), 10.0% (V), 5.0% (VI), 1.0% (VII), and 0.1% (VIII) liquors were taken according to the volume.

Statistical analysis

The impact on functional activity of platelets was defined in six blood samples of different donors for each solvent. Data are expressed as mean $\pm$ SD. Statistical significance was evaluated by Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results

The findings showed that sterile distilled water and 0.9% NaCl solution in the amount of 4% from reactor feed do not influence platelet aggregation induced by ADP and collagen (Table 1).

Indices of other solvents impact on platelet aggregation process are shown in Figure 1 and Tables 2 and 3.

DMSO

The findings (Figure 1) show that DMSO solutions I–III totally suppress platelets aggregation induced by ADP and collagen. An experiment defined DMSO EK50, which corresponds to DMSO solution VI for ADP and collagen. DMSO solution VIII does not influence on inducer-induced aggregation of platelets. However, despite the absence of impact on MA index, this concentration enables to significantly lower the speed of aggregation and the average size of thrombocyte aggregates compared to the control (Tables 2–3).

Ethanol

Ethanol mother liquor arrests platelet aggregation induced by ADP at 26.8 $\pm$ 2.7 and collagen at 24.2 $\pm$ 2.8. Beginning from 47.5% concentration (vol/vol), there was not a registered case of impact on the MA index. But under analysis of aggregatogram received by exhibiting ethanol solution II, there is a noticeable decrease of aggregation speed and the size of average thrombocyte aggregates. Beginning from ethanol solution III, there have been no such effects (Tables 2 and 3).

DMFA

DMFA, being similar to DMSO, in the mother liquor arrests platelet aggregation induced by

Table 1: Platelet aggregation indices under the impact of sterile distilled water and 0.9% NaCl solution.

No.	Index	Control	H_2O	P1	0.9% NaCl	P1
1	ADP, mm	51.4 $\pm$ 3.8	50.2 $\pm$ 3.4	0.3	53.7 $\pm$ 2.5	0.8
2	Collagen, mm	52.9 $\pm$ 4.3	53.1 $\pm$ 3.2	0.6	54.1 $\pm$ 3.6	0.7

Note: P1-level of statistical significance of indices differences compared to the control group.

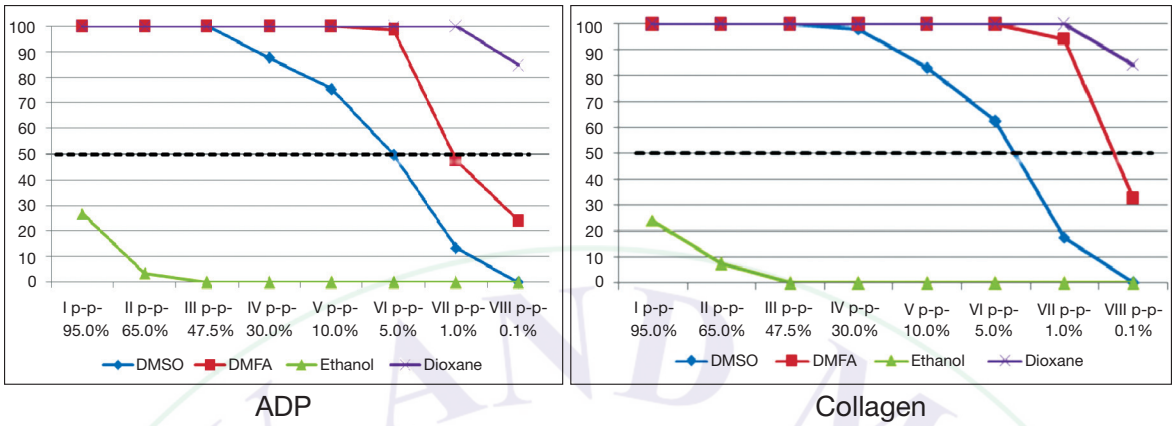

Figure 1: Dependency indices of antiaggregation activity of DMSO, DMFA, ethanol, and dioxane from concentration under platelet aggregation induced by ADP and collagen.

Table 2: ADP indices of platelet-induced aggregation under the influence of 1% liquors of DMSO, DMFA, ethanol, and dioxane (vol/vol).

No.	Index	Control	DMSO	P1	DMFA	P1	Ethanol	P1	Dioxane	P1
1	ADP, mm	51.4 ± 3.8	40.6 ± 3.2	0.003	26.9 ± 2.9	0.001	53.7 ± 3.1	0.4	0.0 ± 0.0	0.001
2	MR, o.e.	6.7 ± 1.4	4.2 ± 1.2	0.001	2.4 ± 0.7	0.0008	5.7 ± 1.8	0.3	0.0 ± 0.0	0.001
3	tg α, sec.	44.7 ± 0.3	34.6 ± 1.1	0.002	24.4 ± 1.2	0.00006	41.8 ± 1.6	0.4	0.0 ± 0.0	0.001

Note: P1-level of statistical significance of indices differences compared to the control group.

both inducers up to 100%. It is worth mentioning that DMFA totally arrests platelet aggregation induced by ADP and collagen up to 5% concentration (vol/vol). The effective concentration of 50% determined experimentally corresponds to solution VIII–95-fold dilution of the mother liquor. Moreover, in dilution 1:950 (solution VIII), there is a steady antiaggregational activity that is equal to 24.2% for ADP and 32.7% for collagen (Tables 2 and 3).

Dioxane

According to the findings, dioxane exerts the most apparent influence on functional activity of platelets. Aggregation is totally interrupted even over dioxane in 95-fold dilution. The analysis of

Table 3: Indices of collagen-induced platelet aggregation under 1% liquors of DMSO, DMFA, ethanol, and dioxane (vol/vol).

No.	Index	Control	DMSO	P1	DMFA	P1	Ethanol	P1	Dioxane	P1
1	Collagen, mm	52.9 ± 4.3	43.5 ± 1.9	0.006	4.2 ± 1.1	0.0001	53.7 ± 3.2	0.7	0.0 ± 0.0	0.001
2	Lag period, sec.	62.7 ± 3.6	72.8 ± 3.1	0.004	85.5 ± 4.3	0.01	60.8 ± 2.8	0.5	0.0 ± 0.0	0.001
3	MR, o.e.	6.5 ± 0.9	4.8 ± 0.3	0.01	0.5 ± 0.3	0.004	5.9 ± 0.7	0.8	0.0 ± 0.0	0.001
4	tg α, sec.	38.5 ± 0.9	29.5 ± 1.1	0.007	21.8 ± 1.2	0.003	37.5 ± 0.5	0.6	0.0 ± 0.0	0.001

Note: P1-level of statistical significance of indices differences compared to the control group.

solvent VIII showed that dioxane keeps influencing on platelet aggregation.

Discussion

The findings show that almost all potential solvents have impact on platelet aggregation. A regular exception is distilled sterile water and 0.9% NaCl liquor. The selected research-based volume of these substances does not influence functional activity of platelets that enable to consider them as interchangeable solvents for water-soluble compounds. However, the purpose of this work is to find a solvent for water-insoluble compounds. DMSO is used traditionally as a solvent for water-insoluble compounds. In fact, the available scientific literature contains sufficient number of works where DMSO is selected as a solvent for first synthesized substances (Bojić *et al*., 2011; Lazzarato *et al*., 2011). There are many fundamental and clinical research works on the influence of DMSO on hemostasis under conditions *in vitro* and *in vivo* (Fratantoni and Poindexter, 1983; Asmis *et al.*, 2010). According to Fratantoni and Poindexter's findings, DMSO within 1–10% concentration arrests aggregation and the reaction of platelet migration induced by collagen, thrombin, and arachidonic acid. Further (Asmis *et al.*, 2010), the ability of DMSO to arrest platelet adhesion also in 0.5% concentration and aggregation done by arachidonic acid without affecting platelet aggregation induced by high dose of ADP, collagen, ristocetin, and adrenalin was defined. This DMSO effect is determined by the ability to inhibit cyclooxygenase-1 (COX-1). Therefore, the findings and literature analysis show that DMSO arrests functional activity of platelets even in 950-fold (0.1%) dilution, and that makes consider this solvent adverse for preclinical research of impact of water-insoluble compounds on hemostasis.

DMFA and dioxane are equally aggressive to vessel-thrombocytic hemostasis component by arresting physiological response of platelets to aggregation agonist. Even in high dilution DMFA/dioxane: H_2O = 1:18 (vol/vol), there is virtually no platelet aggregation under the impact of ADP and collagen. Only the use of 0.1% (vol/vol) liquor of these solvents enables to register platelet aggregation, which does not exceed 30% of control values. Size indices of average thrombocyte aggregates and aggregation speed show apparent inhibition impact of these solvents. Therefore, despite good solvency of DMFA and dioxane, their usage as solvents is unfeasible even in proportion with water at more than 0.1% (vol/vol) because of apparent inhibition impact on aggregation function of platelets.

Ethanol impact is sufficiently studied under conditions *in vitro* and *in vivo*. According to the paper (Klatsky *et al*., 1990), moderate abuse of ethanol (1–2 standard drinks per day or 10–20 g/d) is connected with lower risk of cardiovascular disease. Haut and Cowan were the first to define the ethanol affect on functional activity of platelets, showing that ethanol being added to human platelets under conditions *in vitro* taken inward by volunteers or injected intravenously remarkably lowered platelet aggregation in response to thrombin, collagen, adrenalin, and ADP (Haut and Cowan, 1974). Research papers that are supposed to define ethanol impact mechanism showed its influence on the signal transfer system cyclic adenosine monophosphate (cAMP) connected with membrane, membrane phospholipin decomposition, arachidonic acid metabolism, and metabolism and calcium exchange (Salem and Laposata, 2005). However, a number of researchers defined apparent dependence of ethanol effects from concentration and impact lifetime. Further, Renaud and others showed the following dependence in 1600 male volunteers—the higher the level of abuse, the lower the aggregation response of platelets (Rand *et al*., 1987). Dose-dependency of ethanol effects on hemostasis and its physiological display enable to suppose the presence of concentration of minimal impact on platelet aggregation. Indeed, findings show that ethanol exerts less apparent influence on functional activity of platelets in comparison with other solvents. Concentrations where ethanol does not influence functional activity of platelets have been found. Such additional parameters as lag period, aggregation speed, and average size of thrombocyte aggregates in these concentrations also remain on the level of control values. Thus, the most physiological, exerting minimal impact on hemostasis, among the analyzed solvents for water-insoluble compounds is ethanol in concentration not exceeding 50% (vol/vol).

Conclusion

The findings enable using ethanol as a solvent in maximum 50% exposure (vol/vol) in order to

study the impact on aggregation of platelets of water-insoluble substances by this method.

Ethical Approval

The research has been approved by the ethics committee of our university. Every volunteer has been informed about the purposes, tasks of the research and agreed to take part in it.

Conflict of Interests

The authors of this article assure that open publishing of the research findings take into account interests of all its participants.

Authors' Contributions

All authors contributed equally to this work.

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