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# Liposome-induced hypersensitivity reactions: Risk reduction by design of safe infusion protocols in pigs

Tamás Fülöp[a,1], Gergely T. Kozma[a,b,1], Ildikó Vashegyi[b,1], Tamás Mészáros[a,b], László Rosivall[c], Rudolf Urbanics[a,b], Gert Storm[d,e], Josbert M. Metselaar[d,f,**,1], János Szebeni[a,b,g,*,1]

[a] Nanomedicine Research and Education Center, Department of Pathophysiology, Semmelweis University, Budapest, Hungary
[b] SeroScience Ltd, Cambridge, MA, Budapest, Hungary
[c] Department of Pathophysiology, International Nephrology Research and Training Center, Semmelweis University, Budapest, Hungary
[d] Dept. Targeted Therapeutics, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, the Netherlands
[e] Dept. Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, the Netherlands
[f] Institute for Experimental Molecular Imaging, University Clinic and Helmholtz Institute for Biomedical Engineering, Aachen, Germany
[g] Department of Nanobiotechnology and Regenerative Medicine, Faculty of Health, Miskolc University, Miskolc, Hungary

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ABSTRACT

Intravenous administration of liposomal drugs can entail infusion reactions, also known as hypersensitivity reactions (HSRs), that can be severe and sometimes life-threatening in a small portion of patients. One empirical approach to prevent these reactions consists of lowering the infusion speed and extending the infusion time of the drug. However, different liposomal drugs have different levels of reactogenicity, which means that the optimal protocol for each liposomal drug may differ and should be identified and evaluated to make the treatment as safe and convenient as possible.

The goal of the present study was to explore the use of pigs for the above purpose, using PEGylated liposomal prednisolone (PLP) as a model drug. We compared the reactogenicities of bolus versus infusion protocols involving 2-, 3- and 4-step dose escalations for a clinically relevant total dose, also varying the duration of infusions. The strength of HSRs was measured via continuous recording of hemodynamic parameters and blood thromboxane B2 levels. We showed that bolus administration or rapid infusion of PLP caused transient changes in systemic and pulmonary blood pressure and heart rate, most notably pulmonary hypertension with paralleling rises in plasma thromboxane B2. These adverse responses could be significantly reduced or eliminated by slow infusion of PLP, with the 3-h 3-step dose escalation protocol being the least reactogenic. These data suggest that the pig model enables the development of safe infusion protocols for reactogenic nanomedicines.

## 1. Introduction

Non-IgE-mediated (pseudoallergic) hypersensitivity, or infusion reactions following i.v. administration of nanomedicines and biologicals are infrequent but salient adverse immune effects of many state-of-art pharmaceuticals, including PEGylated liposomes, such as liposomal doxorubicin (Doxil). Earlier studies provided evidence that the cardiovascular and cutaneous symptoms of human IRs to liposomes, in general, and Doxil, in particular, can be reproduced by i.v. injection of these liposomal drugs in pigs. Moreover, it has been established that the symptoms can be explained by complement activation, resulting the name “complement activation-related pseudoallergy” (CARPA) [1].

It was also shown earlier in pigs that the rate of infusion of multilamellar liposomes had a significant impact on their reactogenicity, with slowing the infusion speed leading to reduced cardiopulmonary distress [2]. As this is concordant with the human experience that slowing the infusion rate reduces the risk of HSRs [3], these observations suggest that pigs can be used to model the impact of infusion

*Abbreviations:* CARPA, complement activation-related pseudoallergy; HR, heart rate; HSRs, hypersensitivity reactions; PL, phospholipid; PAP, pulmonary arterial pressure; SAP, systemic arterial pressure

* Correspondence to: J. Szebeni, Nanomedicine Research and Education Center, Semmelweis University, Budapest 1085, Nagyvárad tér 4, Hungary.
** Correspondence to: J. M. Metselaar, Institute for Experimental Molecular Imaging, University Clinic and Helmholtz Institute for Biomedical Engineering, Aachen, Germany.
*E-mail addresses:* bart@enceladus.nl (J.M. Metselaar), Jszebeni@seroscience.com (J. Szebeni).
[1] Equal contributions.

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**Table 1**
Parameters of PLP infusion in pigs in 3 administration protocols.

| Steps | Step 1 | | | Step 2 | | | Step 3 | | | Step 4 | | | Time |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Protocol (n pigs) | Min | Rate ml/kg/h | Total mL/kg | Min | Rate ml/kg/h | Total mL/kg | Min | Rate ml/kg/h | Total mL/kg | Min | Rate ml/kg/h | Total mL/kg | Min |
| A ($n$ = 1) | 15 | 0.24 | 0.06 | 15 | 0.60 | 0.15 | 15 | 1.20 | 0.30 | 90 | ***6.00*** | 9.00 | 135 |
| B ($n$ = 10) | 20 | 0,40 | 0.13 | 115 | ***4.00*** | 7.67 | | 0 | 0 | | | | 135 |
| C ($n$ = 5) | 40 | 0.04 | 0.03 | 20 | 0.40 | 0.13 | 120 | ***4.00*** | 8.0 | | | | 180 |

Abbreviations: mL refers to the volume of drug infused after dilution of the undiluted PLP stock (see Methods). For simplicity the 135 min infusion times are referred to as 2-h protocol thenceforth. Bold italicized entries triggered more or less pulmonary hypertension as specified below.

speed on human HSRs and to develop safe infusion protocols. The goal of the present study was to explore this possibility, using PEGylated liposomal prednisolone sodium phosphate (PLP) as model for PEGylated liposomes and testing therapeutically relevant 2- and 3-h infusion protocols versus bolus i.v. administrations. Indeed, PLP was shown earlier to cause complement activation in human serum [4], suggesting the potential for causing CARPA in man. Thus, the present experiments also served the purpose of developing a safe administration protocol for PLP.

## 2. Materials and methods

### 2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), 1,2-distearoyl-phosphatidylethanolamine-methyl-poly-ethyleneglycol conjugate-2000 (DSPE-PEG2000) and cholesterol were obtained from Lipoid GmbH, Ludwigshaven, Germany). Prednisolone sodium phosphate was from BUFA (Uitgeest, The Netherlands) and zymosan from Sigma. The thromboxane B2 (TxB2) kit was from Amersham (UK).

### 2.2. Preparation of liposomes

PEGylated liposomal prednisolone sodium phosphate (PLP) was prepared using the ethanol injection method [5] encapsulating the drug with DSPE-PEG2000, DPPC and cholesterol in a 0.15:1.85:1.00 ratio. Multiple rounds of extrusions through polycarbonate membranes (final pore sizes of 100 nm, Nucleopore, Pleasanton, USA) were performed and unencapsulated prednisolone was removed with a tangential flow filtration unit (Pall Minimate, Pall Millipore). Mean particle size was determined using dynamic light scattering and the amount of encapsulated and free (unencapsulated) prednisolone was determined with high performance liquid chromatography as described previously [5,6]. The encapsulated drug content was 2.8 mg/mL (along with 37.5 mg/mL, 50 mM phospholipid, PL) and the unencapsulated prednisolone remained under 0.02 mg/mL. The mean liposome size was 100 nm, polydispersity index ~0.1 and zetapotential: ~ −5 mV in PBS.

### 2.3. CARPA studies in pigs

Details of the pig experiments were described earlier [1,7,8]. In brief, mixed breed male Yorkshire/Hungarian White Landrace pigs (2–3 months old, 20–25 kg) were obtained from the Animal Breeding and Nutrition Research Institute, Herceghalom, Hungary. Animals were sedated with Calypsol/Xilazine and then anesthetized with isoflurane (2–3% in $O_2$). Intubation was performed with endotracheal tubes to maintain free airways, and to enable controlled ventilation if necessary. The animals were breathing spontaneously during the experiments. Surgery was done after povidone iodine (10%) disinfection of the skin. In order to measure the pulmonary arterial blood pressure (PAP), a Swan–Ganz catheter (AI-07124, 5 Fr. 110 cm, Arrow Internat Inc.) was introduced into the pulmonary artery via the right external jugular vein. Additional catheters were placed into the left femoral artery to record the systemic arterial pressure (SAP), to the left external jugular vein for saline and drug administration, and to the left femoral vein for blood sampling. Before and during infusion pigs were monitored for PAP, SAP and heart rate (HR) changes, among many other parameters that are customarily measured in our model [1,7,8], but were not presented in this paper as their changes were consistent with those of PAP. The latter was expressed both in absolute and relative terms (compared to baseline), or as area under the curve during the first 15 min of the first reaction (AUC), which measure was independent of individual variation of PAP waveforms. Blood samples were collected pre-administration and at various times post-administration for the measurement of plasma TxB2.

### 2.4. PLP administration via different infusion protocols

From the 18 pigs used in this study, 3 obtained PLP as a bolus IV administration and the rest of animals were treated with different PLP infusion protocols. The total drug dose was approximately equal in all pigs, 3-4 mg/kg, which corresponded to the human therapeutic dose range. In case of infusion appropriate volumes from the stock (provided in sterile vials) were diluted in 5-6 (average 5.5) volume normal saline (NS). Upon treatment, animals were randomly selected into 3 groups differing in the speed and length of infusion (see Table 1). At the start of each experiment, animals received 5 mL NS (baseline), injected as i.v. bolus, and then they were monitored for 5–10 min before starting the infusion.

### 2.5. Statistical methods

Normality was tested by the Kolmogorov-Smirnov test. The PAP and TxB2 values at all time points were compared to their baseline (0 min) and the significance of differences were determined by non-parametric Kruskal-Wallis and Friedman test, followed by Dunn's multiple comparisons. A $p$-value of $< 0.05$ was considered to be statistically significant. Statistical analysis was performed by GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

## 3. Results

### 3.1. Effects of PLP administered as bolus

Among the common symptoms of CARPA in pigs (changes in hemodynamic, respiratory, hematological, and blood chemistry parameters and skin alterations), we previously found the hemodynamic and TxB2 alterations to be the most reproducible and quantitative. Therefore, we focused on these changes in PLP-treated pigs.

Fig. 1 shows the changes in PAP, SAP and HR caused by repetitive bolus injections of PLP; real-time tracings in 3 different pigs (panels A-C) to illustrate the individual variation. The injected doses are specified above the arrows which show the time of injection. The second injection repeated the first, and then the subsequent (3rd) dose was increased 5-fold in order to establish any change in sensitivity, and, hence, the validity of using the peak heights for quantitation of HSRs in

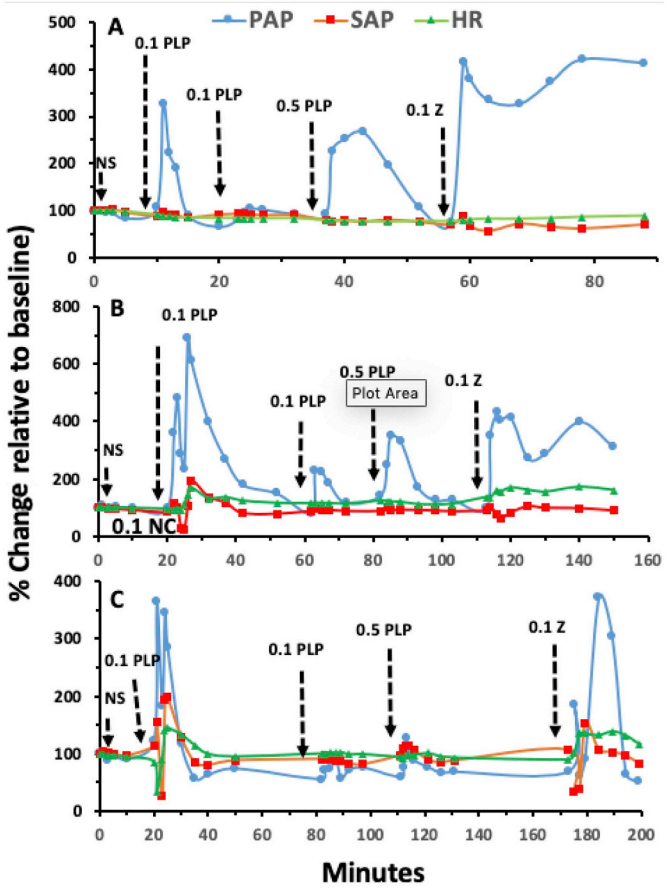


**Fig. 1.** Real-time recordings of the hemodynamic effects of PLP boluses in 3 different pigs (A-C). Pulmonary arterial pressure (PAP), systemic arterial pressure (SAP) and heart rate (HR) changes are expressed as % of baseline. The time of i.v. injections are indicated by arrows. NS, normal saline. The numbers before PLP specify the phospholipid dose (mg/kg). 0.1 Z, 0.1 mg/kg zymosan. Other details are described in the Methods.

case of repeated injections. In all 3 pigs the first bolus led to massive (300–600%) rises of PAP, which was followed by no, or smaller changes after an identical, and then larger repeat doses. Thus, the PAP response to PLP was tachyphylactic (self-limiting); therefore, only the first peak was taken as quantitative measure of the drug's reactivity. The SAP and HR showed less or no changes after each injection, while Zymosan (0.1 mg/kg), used as positive control, caused massive pulmonary hypertension in each animal. These changes were consistent with other liposome reactions described in this model [1,7,8], particularly those induced by PEGylated liposomal doxorubicin (Doxil) and its drug-free equivalent vesicle (Doxebo) [9,10]. These results therefore suggested that the reaction is due to the liposomal bilayer, and not to the drug payload. Likewise, the individual variation of first peaks is consistent with our previous results with Doxil [9,10].

### 3.2. Effects of PLP administered in infusion: impacts of infusion rate, duration and drug dose

Next we examined the reactogenicity of PLP administered in infusion using different protocols referred to as A, B and C (Tables 1 and 2). These protocols represented stepwise increases of dose rate over different time windows and different overall duration of infusion. In protocols A, B and C the dose rates were increased in 4, 2 and 3 steps, respectively, with major differences in total infusion time and dose rates at the first step.

As shown in Fig. 2A, the rises of infusion rates to 0.38 and then to

**Table 2**
Phospholipid dose rates in the different steps of different protocols.

| Protocol | Infusion steps | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| | μg phospholipid/kg/min | | | |
| A | 27.2 | ***68.2*** | 136.4 | ***681.8*** |
| B | ***44.3*** | ***454.7*** | | |
| C | 5.1 | 44.3 | ***454.5*** | |

The entries were obtained from Table 1 by dividing the total amount of phospholipid injected during the different steps by the duration of steps. The total amount of phospholipid injected was obtained from the total mL/kg entries in Table 1 multiplied by 37.5 (μg phospholipid/mL) divided by 5.5 (dilution). Bold italicized entries triggered more or less pulmonary hypertension.

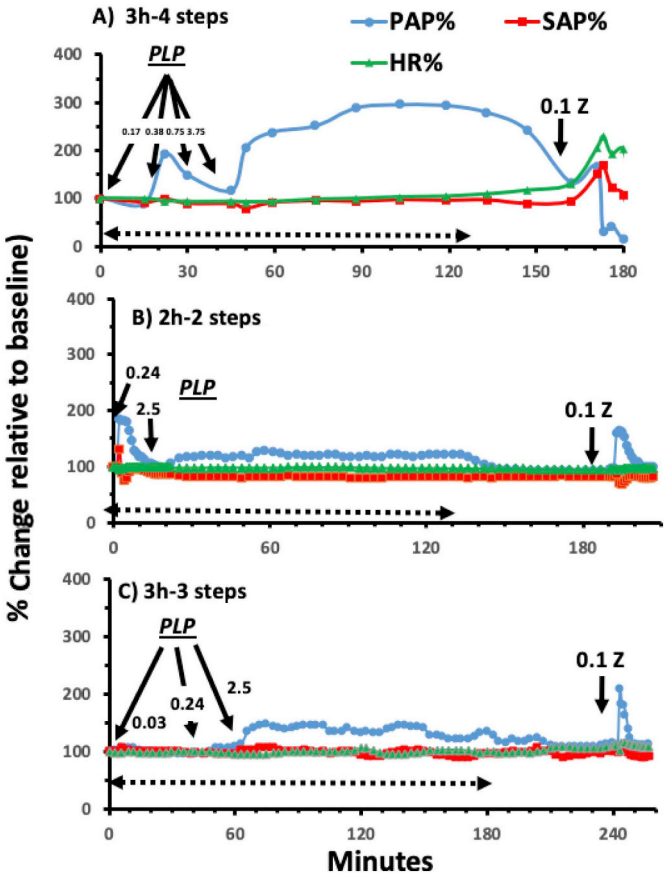


**Fig. 2.** Real-time recordings of the hemodynamic effects of PLP infusion according to 3 administration protocols, specified in Tables 1 and 2. All other details are the same as in Fig. 1, except that dotted lines are used to show the length of infusion and the rates of infusion are specified, instead of infusion dose (Fig. 1 was bolus treatment). Protocol A was applied only in 1 pig. Panels B and C show typical data from 9 and 4 animals infused with the 2- and 3-step protocols, respectively, with 1–1 outlier (identified by the Kolmogorov-Smirnov test) excluded from both groups.

3,75 mg PL/kg/min) at the 2nd and 4th step in protocol A triggered major, permanent (up to 300%) rise of PAP, indicating significant cardiopulmonary distress. Since our aim was to prevent these changes, this protocol was not tested in further animals. In protocols B and C, applied in 10 and 5 pigs, respectively (Figs. 2B and C), the increases of infusion rates to 2.5 mL/kg/h at the 2nd or 3rd steps, respectively, also caused permanent pulmonary hypertension, but these were less prominent (20% and 50% rises, respectively, Figs. 2B and C). The 0.24 mg phospholipid/kg/min initial infusion in protocol B still caused a

**Table 3**
Initial (within 10 min) rise of PAP induced by the 2-step (B) and 3-step (C) dose escalation infusion protocols for PLP administration.

| Protocol | Pig n | Mean % of baseline | SD | SEM |
|---|---|---|---|---|
| B (2-step) | 9 | 140.0[a] | 39.8 | 12.6 |
| C (3-step) | 4 | 105.7 | 2.8 | 1.4 |

[a] Significant difference relative to baseline and protocol C, Mann Whitney P: 0.007.

moderate but significant rise of PAP (Fig. 2B, Table 3), while an 8-fold reduction of the initial dose rate in protocol C was reaction free at the first and second steps of dose escalation, with minor, biologically negligible rise of PAP after the 3rd infusion step (Fig. 2C, Table 3).

These data taken together suggest that stepwise infusion protocols can be free of major cardiopulmonary distress, provided the infusion does not exceed certain threshold rates, which are different for the first and subsequent infusion steps. Under the conditions of our study these thresholds values were 0.24 and 2.5 mg phospholipid/kg/min for the initial and subsequent infusion periods, respectively.

### 3.3. Features of hemodynamic and thromboxane A2 responses in the 2- and 3-step dose escalation protocols

Figs. 3A and B shows the time courses of PAP changes in pigs infused with PLP according to protocols B and C, respectively, along with the plasma TxB2 readings at different times. In keeping with Fig. 2, the mean values of both parameters were higher in "B pigs" compared to "C pigs", however, the SD values were also higher, suggesting greater variation of response in protocol B. The figure also shows clear concordance between the rises and falls of PAP and TxB2, which is consistent with the causal role of TxA2 in liposome-induced pulmonary hypertension in pigs [7].

Fig. 4 shows the correlation between PAP and TxB2 values in protocol B and C pigs. Consistent with the significant rise and substantial individual variation of PAP in B animals (Table 3), the paired PAP-TxB2 values segregated into two groups; 1/3 ($n$ = 3) of pigs displayed relatively high PAP and TxB2 values that showed significant correlation (Fig. 4, upper regression line), while 2/3 ($n$ = 6) of the animals showed minor or no changes relative to baseline. This suggests that protocol B represents a borderline in terms of risk for initial pulmonary hypertension, leading to the conclusion that the protocol cannot be considered as reaction-free. In contrast, all "C pigs" showed minor or no change of initial PAP and TxB2 with no statistical difference relative to baseline. These observations, although in a small number of animals, suggest that the serendipitously tested 8–10-fold

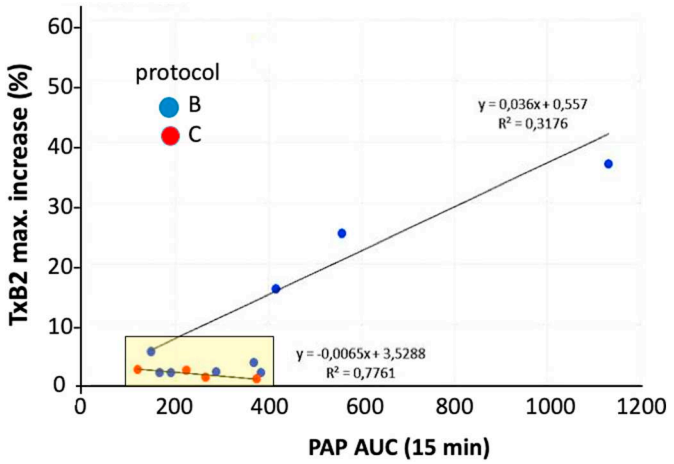


**Fig. 4.** Correlation between the first 15 min PAP AUC values and maximal increases in the TxB2 blood concentration (percentage of 0′ value) in 4/9 animals treated with protocol B. The values in the shaded rectangle represent small, biologically irrelevant changes.

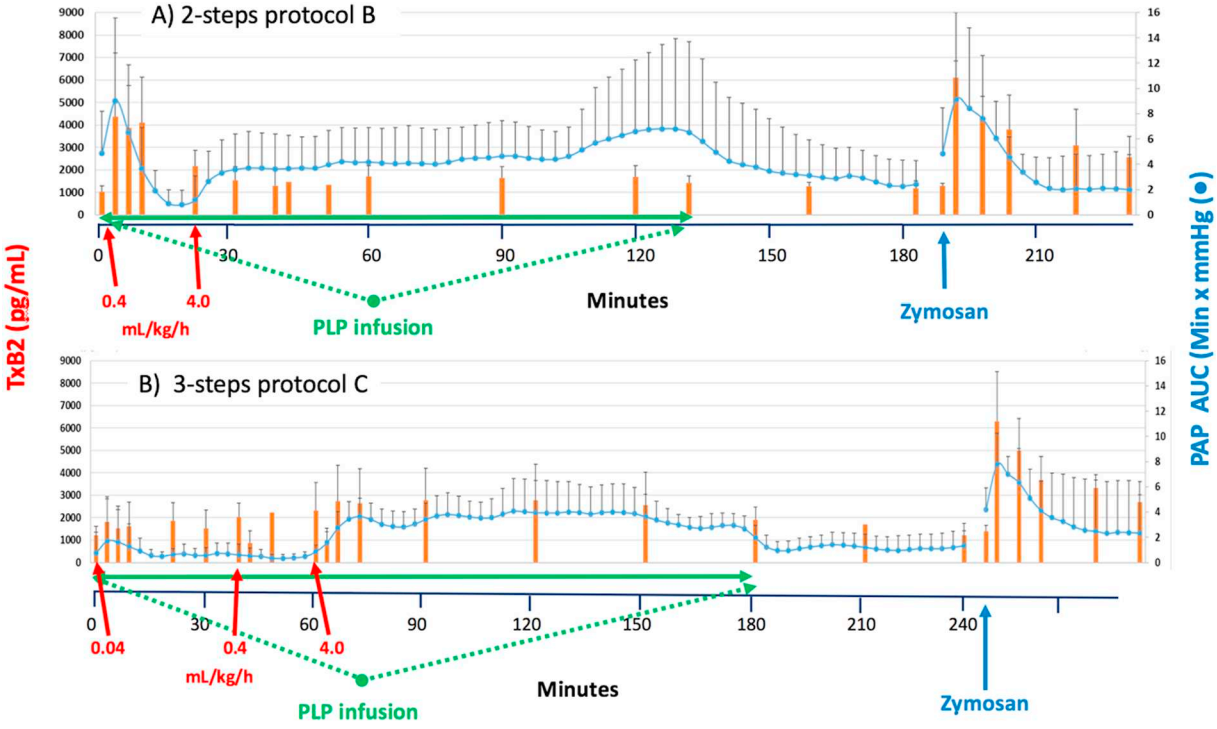


**Fig. 3.** Time course of TxB2 (red bars) and PAP (blue line) changes (Mean ± SD SD) in pigs treated with PLP with the 2-step (B) ($n$ = 9) and 3-step (C, $n$ = 4) infusion protocols. Red arrows point to the time when the infusion was started at the rate specified by the number (in mL/kg/h). Green shows the duration of infusion. Zymosan was administered at 0.1 mg/kg. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reduction of initial (and first step-up) infusion rates that we applied in infusion protocol C versus B minimized the risk of TxB2 release and consequent initial pulmonary hypertension. Thus, under the experimental conditions of this study the infusion parameters in protocol C provided the best administration protocol in terms of risk for hyperacute (within minutes) HSRs. It should be emphasized, however, that this relative "safety" applies only to the first, initial reaction, as the cause and biological relevance of gradually developing 2–3-fold constant rise of TxB2 and pulmonary hypertension after 1 h infusion remains to be established.

## 4. Discussion

Infusion reactions have been observed ever since infusion therapy has been implemented in modern medicine, yet even today it represents a safety issue for many drugs and drug candidates [11–13]. Their mechanism is poorly understood, but it is clear that multiple immunological pathways are involved, the relative contributions of which may vary from case to case. One pathway gaining recent attention involves complement activation, a possible trigger mechanism of HSRs to nanomedicines and biologicals [14]. Complement activation can trigger HSRs by at least two pathways, via release of anaphylatoxins [15], and also via opsonization of the trigger agent enhancing its binding to (with or without uptake by) macrophages or other complement-receptor containing allergy mediating cells, which respond with secretion of bioactive mediators. Both phenomena have been observed in pigs [16,17]. However, a portion of acute physiological changes corresponding to HSR may be triggered independent of complement activation, such as the liposome-induced acute hypertension in mice [18]. Thus, while a variety of symptoms can be generated by one single "hit" on allergy mediating cells, the broadest vision HSRs raises the possibility of two or more "hits", suggesting that the variety of symptoms is due to the individual variation of these "hits" [19].

The current, standard approach of preventing HSRs is premedication of the patient with steroids, antihistamines and other anti-inflammatory drugs, and administration of the drug in slow infusion. There is no doubt that these measures are effective, without them a large number of drugs could not be used in infusion therapy. However, these methods are not full-proof, either, as occasionally, despite all attention and effort, severe HSRs occur and cause death. Just focusing on PEGylated pharmaceuticals, over the past few years three were withdrawn from clinical use partly because of severe HSRs: PEGylated EPO-mimetic peptide (*Peginesatide, Omontys*®) [20], PEGylated urate oxidase (*Pegloticase, Krystexxa*®) [21,22] and a PEGylated IXa blocker RNA aptamer *(Pegnivacogin, Revolixys*®) [23]. These facts lend importance to studies that try to understand these reactions and develop new ways of their prevention.

The above goals can most efficiently be achieved by using appropriate animal models. The pros and cons of the pig model was recently reviewed [1], and one of the conclusions was that the high sensitivity of the model makes it an efficient preclinical screening test for anaphylactoid reactogenicity of nanoparticle-based drugs and other agents [1]. It was also emphasized that it is a disease model, that of hypersensitive man, and that it can be used both for hazard identification and mitigation [1]. Nevertheless, the pig model was recently questioned on the basis that the prevalence of HSRs does not reproduce the average human reaction rate, the mechanisms are different and that the cardiovascular changes represent a "global response", i.e., the model has no capability to differentiate among nanoparticles in terms of reactogenicity [24–26]. Hence, it was judged as "misleading" that "should not be advertently promoted" for safety evaluation [24–26]. However, these arguments were contradicted by a study spearheaded by the lead author of the above critical reviews [27], showing that the PAP response in the same pig model used here can quantify and differentiate the reactogenicity of polystyrene nanoparticles on the basis of their shape. Thus -it was concluded-, changing the shape of nanoparticles represents a new strategy for combatting HSRs [27]. In fact, a large number of research studies provide evidence for the utility of the model to predict acute immune reactivity [28–33], and there is also example for the use of the model in the pharmaceutical industry, in developing safe administration protocols for nucleotide-containing lipid nanoparticles, such as the first FDA-approved gene therapeutic agent, Patisiran (Onpattro®) [34].

The present study represents an addition to the list of studies utilizing the pig model for risk identification and mitigation, measuring the CARPAgenicity of PLP and developing the safest infusion protocol for its administration.

Our finding that bolus injection of PLP in pigs caused very similar, tachyphylactic (self-limiting) HSR as bolus injection of Doxil suggests that the differences between the two liposome formulations in terms of encapsulated drug and bilayer composition are not critical for triggering reactogenicity. The common denominator that may control the reaction is the pegylated liposome surface, i.e. the phospholipid bilayer coated with ~5% PEG2000.

Furthermore, the finding that the slowest, 3-step dose escalation protocol was the safest in terms of cardiovascular reactivity is in line with the well-known reaction-lessening effect of slow infusion of reactogenic nano-biopharmaceuticals. For the case of PLP, an initial infusion rate of 0.04 ml/kg/h over 40 min turned out to be reaction-free as opposed to 0.4 ml/kg/h over 20 min, suggesting that the no observed adverse effect level (NOAEL) is in the 0.04–0.4 ml/kg/h range, at least under the conditions of this study wherein the drug was infused at a 5-6-fold dilution in the vehicle buffer. This translates to 5-44 microgram PL/kg/min (Table 2). Whether or not these numbers hold up for other nanomedicines in pigs, or in man, remains to be established in further studies.

The observation that intra-liposomal prednisolone did not inhibit the rise of PAP or TxB2 at times when macrophages or other allergy mediating cells might have taken up PLP during the course of infusion (e.g. the reactions to zymosan) suggests that the immune suppressive effect of prednisolone is not effective against PLP-induced HSR, at least within 2–3 h under the conditions of this study.

As for the mechanism by which slow infusion might mitigate the reactogenicity of liposomal and other nanoparticulate drugs, the "anaphylatoxin balance concept" [35] represents one possible explanation. According to this theory, the blood level of anaphylatoxins, C3a and C5a, is determined by their generation via complement activation and clearance by cellular uptake and metabolism by carboxypeptidases [36]. If massive anaphylatoxin formation exceeds its clearance, which is much slower, its blood level may rapidly spike to reach a threshold where the allergy mediating cells release their mediators. In contrast, slow formation of anaphylatoxins during slow infusion may be coped with by clearance, keeping the concentration of anaphylatoxins below the HSR threshold.

In summary, the present data, together with numerous other studies provide support for using the porcine CARPA model for assessing the reactogenicity of PEGylated nanomedicines, such as PLP, and for developing safe infusion protocols for their administration. These protocols may vary for different PEGylated and non-PEGylated nanoparticles with different reactogenicities, and the pig model might help in fine-tuning the optimal parameters. Nevertheless, further studies are needed to establish the concordance of pig and human symptoms of HSRs to different nanoparticles, and thus validate extended use of the model in preclinical safety testing.

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