

Absence of Placental Transfer of Pentasaccharide (Fondaparinux, Arixtra®) in the Dually Perfused Human Cotyledon in vitro

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Keywords

Thromboembolism, pregnancy, pentasaccharide, fondaparinux, enoxaparin, placental transfer

Summary

The synthetic pentasaccharide, fondaparinux, is the first of a new antithrombotic class: selective factor Xa inhibitors. Comparative clinical trials of fondaparinux versus heparins in prevention and treatment of venous thromboembolism are ongoing. Little is known about fondaparinux during pregnancy, as women of child-bearing potential were excluded from clinical trials. No particular safety issue, for either mother or fetus, has been reported for heparins. The objective of this study was to compare in vitro the steady state placental transfer of fondaparinux and enoxaparin at the plasma concentrations reached during acute treatment of venous thromboembolism (1.75 μg/mL and 1 anti-Xa IU/mL respectively), using antipyrine (20 mg/L) as reference. No biological activity was detectable in the fetal venous effluent during perfusion of enoxaparin-antipyrine, fondaparinux-antipyrine or control media. Furthermore, fetal venous samples did not differ significantly from fetal arterial samples. This apparent absence of placental transfer supports further evaluation of fondaparinux in pregnant women.

Introduction

Pregnant women are estimated to be at a 5-fold increased risk for developing venous thromboembolism (VTE) (1). Unfractionated heparin and especially low-molecular-weight heparins (LMWH) are the antithrombotic agents of choice for the prevention and treatment of venous thromboembolism during pregnancy (2). In contrast to heparin, coumarin derivatives (warfarin) cross the placenta and have the potential to cause both teratogenicity and bleeding in the fetus or neonate (2). Direct thrombin inhibitors, such as hirudin, hirudin derivatives, ximelagatran or argatroban have not yet been evaluated during pregnancy.

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The pentasaccharide fondaparinux is the first of a new class of synthetic antithrombotics, selective factor Xa inhibitors. Fondaparinux binds to antithrombin with high affinity (dissociation constant, Kd = 50 nM) and in a 1 : 1 stoichiometric and reversible relationship (3). According to the law of mass action, there is a dynamic equilibrium between the antithrombin-bound drug and the free fraction, as well as between the compartments containing the antithrombin molecules. This binding produces an irreversible conformational change in human antithrombin resulting in a potent inhibition of factor Xa without any effect on factor IIa (4-6). Fondaparinux does not interact with plasma proteins other than antithrombin (4). Administered by subcutaneous injection, the elimination half-life of fondaparinux ranged from 13 to 17 h (7). In four randomized, double-blind phase III trials conducted in 7344 patients undergoing major orthopedic surgery, fondaparinux (2.5 mg once daily by the subcutaneous route) was more effective than enoxaparin in preventing VTE with an overall risk reduction of more than 50% ($p < 0.001$) and a similar safety profile with regard to clinically important bleeding (8-11). In animal studies conducted with fondaparinux, quantifiable placental transfer was observed in female rabbits at the highest doses of 2 and 10 mg/kg/d, but not at 0.4 mg/kg/d. When placental transfer was observed in animals at high doses, no treatment-related abnormalities were detected, either in placentas or in fetuses (unpublished data). In humans, no data are available for placental transfer at the doses currently tested in clinical trials: 2.5 mg o.d. (0.04 mg/kg) in the prevention of VTE in orthopedic surgery or up to 10 mg o.d. (0.16 mg/kg) in the curative treatment of VTE. We therefore compared the placental transfer of fondaparinux to that of enoxaparin, a LMWH, both at the highest therapeutic plasma concentrations, using the single-pass dually perfused human cotyledon model (12, 13).

Materials and Methods

Chemicals

Fondaparinux sodium (Arixtra®) in 0.25 mL prefilled syringes (10 mg/mL) was supplied by Sanofi-Synthelabo Research Division (Montpellier, France). Enoxaparin sodium in 0.4 mL prefilled syringes (4000 anti-factor Xa IU/0.4 mL) was supplied by Aventis Pharma (Montrouge, France). Antipyrine was purchased from Sigma Chemical corporation (St Louis, MO, USA), human serum albumin (HSA, 20% intravenous solution) 10 g/L and antithrombin (AT, Aclotine® 1000 IU, intravenous solution) 1 IU/mL from the Laboratoire français du Fractionnement et des Biotechnologies (Les Ulis, France). Calcium chloride, D(+)-glucose, disodium hydrogen phosphate, magnesium sulfate, potassium chloride, potassium dihydrogen phosphate, sodium chloride, sodium

hydrogen carbonate and sodium hydroxide were manufactured by Prolabo (Fontenay-Sous-Bois, France). All reagents and solvents were of analytical grade. The "low molecular mass heparin standard for assay biological reference preparation" (anti-Xa activities) was provided by the European Pharmacopoeia Commission (batch no. 2).

Placental Experiments

Placentas were obtained from 15 normal term pregnancies (mean ± SD: 38.6 ± 1.5 weeks of amenorrhoea) and deliveries of healthy Caucasian women aged 31.2 ± 6.1 years and weighing 72.2 ± 8.6 kg. All placental donors expressed their informed consent in writing and the study protocol was approved by a Regional Ethics Committee (Bordeaux A). After delivery, a suitable placental cotyledon was selected, and immediately canulated and perfused. The placental model used was that originally described by Panigel et al. (12) and later modified by the use of antipyrine as a reference substance (13). The time interval between delivery and the start of perfusion ranged from 15 to 30 minutes. The standard perfusion medium used was Earle's solution with HSA (10 g/L), antithrombin (1 IU/mL) (15) and glucose (1g/L) (13, 16, 17). The temperature of the preparation was carefully maintained at 37° C and the cotyledon was housed in a Plexiglas chamber at 37° C. Maternal and fetal perfusates circulated through a thermostated bath just before entering the cotyledon in order to achieve a placental perfusion temperature of 37° C.

The experimental protocol has already been described in detail (14, 17). Briefly, both fetal and maternal media were gassed (20 mL/min) with a mixture of 95% O_2 and 5% CO_2 throughout the experiments. The pH of maternal and fetal media were 7.4 ± 0.1 and 7.2 ± 0.1, respectively. The placentas were perfused at controlled flow rates (6 ± 0.6 mL/min on the fetal side and 12 ± 1.2 mL/min on the maternal side). The viability and integrity of the cotyledon was checked during phase I (0-30 min) before starting randomization and fondaparinux-antipyrine or enoxaparin-antipyrine perfusion (Fig. 1). Markers of integrity during phase I were establishment of equal inflow and outflow rates in fetal and maternal circuits, arterial pressures below 40 mmHg in both circuits and maintenance of pH in the perfusates within the physiologic ranges. These markers were monitored throughout the experiments.

Once stable perfusion had been achieved, two compounds were added to the maternal medium only (start of phase II): 20 mg/L antipyrine as a permeability and viability reference substance (14, 16, 17), and fondaparinux (1.75 mg/L) or enoxaparin (1 IU/mL) according to the allocation list. These concentrations corresponded to either the maximal plasma concentrations observed in normal volunteers receiving 10 mg fondaparinux per 24 h (the highest dose tested in the treatment of VTE) (7) or 1 mg/kg bid of enoxaparin (the recommended dose for VTE treatment) (8-11). Since antipyrine exhibits a stable and reproducible pattern of behavior from one study to another, its fetal transfer rate served as an additional means of confirming the functioning of the placenta during phase II (14). The perfusions were continued for 90 min openly, i.e., neither maternal nor fetal media were recirculated. Pharmacokinetic data were assessed during the second phase of the perfusion at 0, 4, 8, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80 and 90 min after the addition of the antithrombotic agents and antipyrine. Samples (5 mL) were collected from the fetal venous effluent and from the maternal reservoir. Only those preparations in which perfusate outflow rates were equal to perfusate inflow rates, arterial pressures remained below 40 mmHg and pH was maintained within the physiologic range on both sides were included in our study.

Determination of Antipyrine Concentrations

Antipyrine was assayed in Earle's maternal and fetal solutions by a validated high performance liquid chromatography (HPLC-UV) method with a lower and an upper limit of quantification of 1 μg/mL and 25 μg/mL, respectively (16, 17). The intra- and inter-assay relative standard deviations at 1 μg/mL were less than 2.5%. The maximum intra- and inter-assay accuracies at 1 μg/mL, expressed as percentage of bias, were below 14.5%.

Monitoring of Antithrombotic Concentrations

The concentrations of each antithrombotic agent were determined on the basis of the corresponding anti-Xa activities by a validated method using a chromogenic assay (18). Concentrations in fetal and maternal media were determined on a STA® automatic analyzer (Stago, Asnières, France) using Stachrom kit reagents (Stago, Asnières, France). The lower limits of quantification were 0.032 mg/L for fondaparinux and 0.066 anti-Xa IU/mL for enoxaparin. The limits of detection were 0.008 mg/L for fondaparinux and 0.008 anti-Xa IU/mL for enoxaparin. It can be stated that, in the event of fetal transfer, this cannot exceed 0.5% and 0.8% for fondaparinux and enoxaparin, respectively. Antithrombin concentrations were assayed with an antithrombin chromogenic assay (ATIII Chrom®, Biomerieux, Marcy l'Etoile, France).

Data Analysis and Statistical Analysis

The placental transfer of the antithrombotic drugs and antipyrine was assessed by the following parameters:

– the fetal transfer rate (Tf) expressing the transplacental transfer of a substance in relation to its circulating maternal concentration (14):

$$Tf\ (\%) = C_{Fv} \times 100 / C_{Ma}$$

where C_{Fv} = concentration in the fetal vein, and C_{Ma} = concentration in the maternal artery.

– the clearance index (CI) defined as the ratio of the fetal transfer rate of the antithrombotic drug ($Tf_{antithrombotic\ drug}$) to that of antipyrine ($Tf_{antipyrine}$):

$$CI = Tf_{antithrombotic\ drug} / Tf_{antipyrine}$$

The CI was used to overcome possible differences in the extent of drug transfer among individual placental units (14).

The stability was assessed by comparing the concentrations of fondaparinux, enoxaparin and antipyrine in the maternal reservoir at the end of the ex-

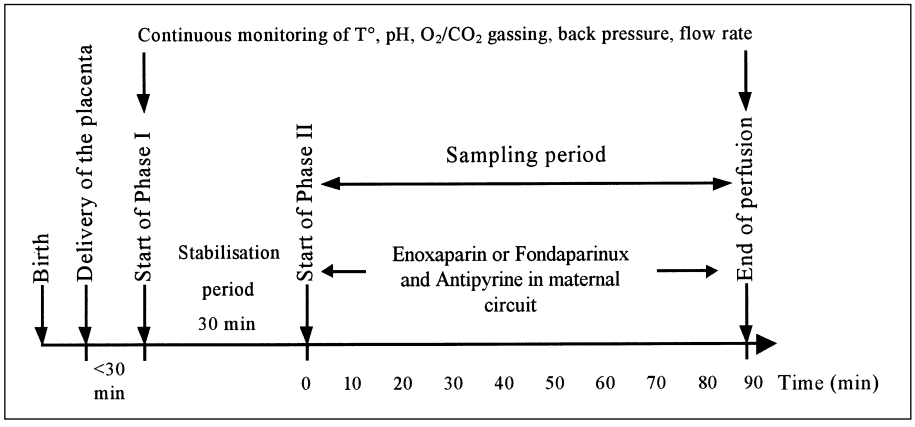

Fig. 1 Perfusion scheme for human isolated cotyledon

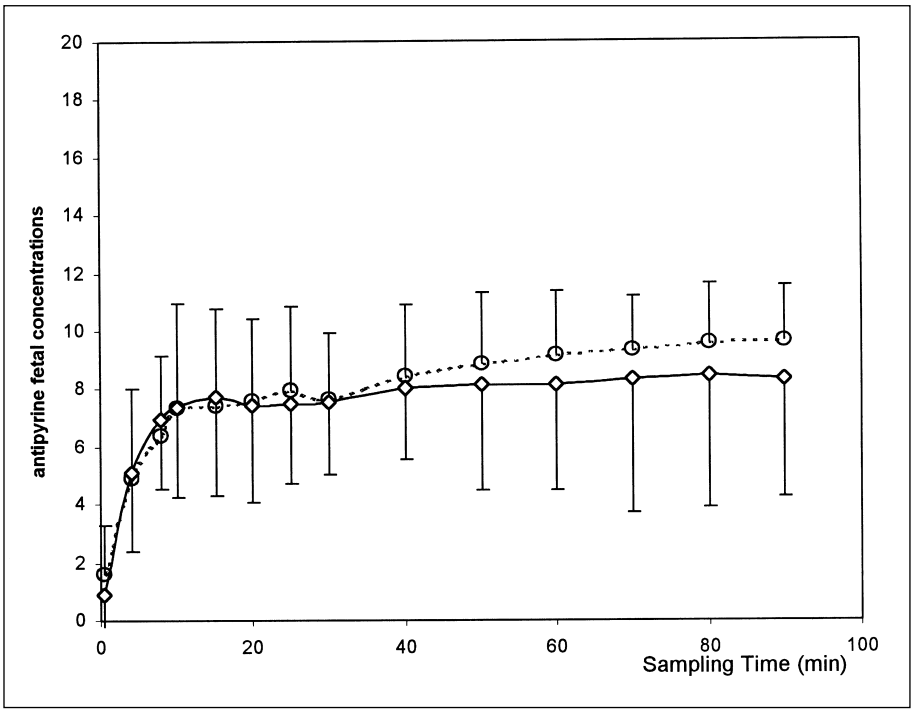

Fig. 2 Mean fetal venous concentrations of antipyrine as a function of time for the enoxaparin (○) and fondaparinux experiments (✧) (bars indicate standard deviations)

periments with those of the freshly prepared solutions. The statistical analysis was performed by Fisher's analysis of variance for the means (Intercooled Stata® version 7.0). Control over the quality of each perfusion was maintained on the basis of the following criteria: mean $Tf_{antipyrine}$ greater than 18%, with a relative standard deviation of the maternal concentrations of antipyrine of less than 7.5% and a relative standard deviation of the fetal concentrations of antipyrine of less than 10% (16, 17).

The steady state concentrations of antipyrine, fondaparinux and enoxaparin in the fetal and the maternal compartments then allowed calculations of the $Tf_{antipyrine}$ $Tf_{fondaparinux}$ and $Tf_{enoxaparin}$ at each sampling time. The means of Tf determined during the steady state phase of each experiment were used as synthetic variables. A one-way analysis of variance was used to test for differences between treatments (fondaparinux versus enoxaparin). The alpha risk was fixed at 0.05.

Results

To ensure an optimal comparison of the fetal transfer rates of the two antithrombotic agents, we looked for placentas with antipyrine fetal concentration-time profiles that could be superimposed (Fig. 2). Out of the 15 placentas obtained, two placentas were perfused with Earle's medium without any antithrombotic drug and served as references to investigate the baseline anti-Xa activity (control samples) of the model. Accordingly to the protocol, 12 placentas were considered suitable for comparing the placental transfer of fondaparinux and enoxaparin.

In vitro Placental Transfer

Earle's solution permitted appropriate solubilisation of both fondaparinux and enoxaparin. In the two groups of the study, the concentrations of antipyrine, fondaparinux, enoxaparin and antithrombin in the maternal reservoir were stable throughout the experiments (one-way ANOVA; NS). The results observed at steady state (means ± SD) are presented in Table 1. The set-up and steady state concentrations of antipyrine in the fetal venous samples are depicted in Figure 2.

No biological activity was detectable in the fetal venous effluent irrespective of whether enoxaparin-antipyrine, fondaparinux-antipyrine

Table 1 Fetal transfer rate (Tf) and antithrombotic agent concentrations observed in the dually perfused human cotyledon model

Drugs (number of experiments)	Drugs concentrations in maternal reservoir	Antipyrine fetal transfer rate (range)	Drug concentrations in fetal venous effluent
Fondaparinux (n=6)	1.673 ± 0.069 mg/L	37.55 ± 7.95 % (23.44 %– 51.83 %)	Below limit of quantification (<0.032 mg/L)
Enoxaparin (n=6)	0.909 ± 0.069 IU/mL	40.94 ± 7.70 % (29.72 % – 53.17 %)	Below limit of quantification (<0.066 IU/mL)

Results are expressed as means ± standard deviations.

or antithrombotic-free medium was perfused in the maternal artery. Furthermore, there was no significant difference when the fetal venous samples of perfusate were compared to the fetal arterial samples (one-way ANOVA: NS). Consequently, only the $Tf_{antipyrine}$ could be calculated (Table 1). At steady state, there were no significant between-group differences for the concentrations of antipyrine (one-way ANOVA; NS), $Tf_{antipyrine}$ (one-way ANOVA; NS) and anti-Xa activity in fetal venous samples (one-way ANOVA; NS). Under these conditions, there was no apparent fetal transfer of either fondaparinux or enoxaparin.

Discussion

When administered during any trimester of pregnancy, LMWH and UFH have been shown to be devoid of any particular risk for either the fetus or the mother (19). The incidence of adverse outcomes in fetuses or infants of women without comorbid conditions who received an antithrombotic treatment during pregnancy was similar to that observed in the general population of pregnant women not receiving antithrombotic treatment (19). Thus, LMWHs are emerging as an alternative to UFH during pregnancy due to their increased half-life, improved bioavailability and more predictable dose response (2, 19, 20).

In healthy pregnant women, it is generally assumed that the placental transfer of substances increases during the fetal period as the placental membrane becomes progressively thinner and provides a larger area of exchange by the presence of microvilli. Our trial provides therefore appropriate experimental conditions for comparing maximal placental transfer and potential fetal exposure in healthy pregnant women. However, a limitation of this model, investigating term placentas, is that it yields no data concerning placental transfer at earlier stages of gestation (14). In addition, as placental transfer may be altered in women with complicated pregnancies associated with thrombosis of placental capillaries, our results should be extrapolated cautiously to pathological pregnancies (14, 21).

Techniques for dual perfusion of isolated human placental cotyledons have been well validated by several studies using different substances (14). The physiological relevance of the experimental design of the perfused human placenta has been previously discussed (17). The main attribute of this model is that it demonstrates transport independent of fetal uptake accumulation and metabolism, and each experiment can be validated by virtue of an internal control substance such as antipyrine. Antipyrine is a well-accepted marker of flow-limited placental transfer and is used because it does not bind to either human serum albumin or tissues (14). The $Tf_{antipyrine}$ values obtained in our study were close to those reported in earlier studies, suggesting the validity of our experimental conditions (14, 16, 17).

Antithrombin, human serum albumin, antipyrine, fondaparinux and enoxaparin concentrations were stable throughout the 1.5-h duration of the experiments. Since selective binding to antithrombin occurs with enoxaparin and fondaparinux, only their unbound concentrations in maternal perfusates are immediately available for placental transfer. Accordingly, antithrombin was included in the perfusion media at physiological maternal and fetal concentrations of 1 IU/mL (15).

Fondaparinux is a small single chemical entity with a molecular weight of 1728 daltons. It is a pure factor Xa inhibitor and is devoid of any other significant effect on coagulation factors at therapeutic concentrations. Thus, the concentration of fondaparinux can be measured in mg/l using a specific validated chromogenic assay based on anti-Xa activity (18). Consequently, the absence of anti-Xa activity in the fetal venous effluent evidenced the absence of placental transfer of fondaparinux in vitro. In contrast, enoxaparin is an animal-sourced preparation comprising various molecules with heterogeneous molecular weights (mean molecular weight, 4170 Da). Since it was assumed that the lowest molecular weight fractions of enoxaparin could be transferred without inducing any biological activity (22), or fetal effect (2), and that the placenta produces heparinase (22), enoxaparin was not radiolabelled. In this context, the use of radiolabelled agents may lead to false conclusions, and biological activities seem preferable. Accordingly, the maternal arterial concentrations of enoxaparin and fondaparinux were based on patient samples withdrawn 4 to 6 h following the morning subcutaneous injection.

Considering first, that fetal exposure to drugs is required to elicit a direct fetal or neonatal effect and second, that antithrombotic agents containing the active pentasaccharide sequence are reported to have been safely administered in pregnancy (19-21), these results provide clinical perspectives relevant to pregnant patients. Due to its selective binding to antithrombin, fondaparinux may make monitoring easier in late pregnancy when an apparent "heparin resistance" occurs because of increased fibrinogen and factor VIII. Absence of apparent placental transfer gives support to further evaluation of fondaparinux sodium (Arixtra®) in pregnant patients who require antithrombotic therapy.

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