Accepted Manuscript online: 2022-04-20 Article published online: 2022-08-22

Placental Passage of Protopine in an *Ex Vivo* Human Perfusion System

OPEN ACCESS

Authors
Deborah Spiess[1,2*], Vanessa Fabienne Abegg[2*], Antoine Chauveau[2], Andrea Treyer[2], Michael Reinehr[3], Mouhssin Oufir[2**], Elisa Duong[1,2], Olivier Potterat[2], Matthias Hamburger[2], Ana Paula Simões-Wüst[1]

Affiliations
1 Department of Obstetrics, University Hospital Zurich, University of Zurich, Zurich, Switzerland
2 Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland
3 Department of Pathology and Molecular Pathology, University Hospital Zurich, Zurich, Switzerland

Key words
Protopine, *Eschscholzia californica*, Papaveraceae, placental barrier, *ex vivo* cotyledon perfusion, pregnancy

received	March 2, 2022
accepted after revision	April 8, 2022
published online	August 22, 2022

Bibliography
Planta Med 2023; 89: 194–207
DOI 10.1055/a-1829-9546
ISSN 0032-0943

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

Correspondence
Prof Dr Matthias Hamburger
Department of Pharmaceutical Sciences, Division of Pharmaceutical Biology, University of Basel
Klingelbergstrasse 50, 4056 Basel, Switzerland
Phone: + 41 6 12 07 14 25, Fax: + 41 6 12 07 14 74
matthias.hamburger@unibas.ch

Correspondence
PD Dr Ana Paula Simões-Wüst
Department of Obstetrics, University Hospital of Zurich
Schmelzbergstrasse 12/PF 125, Path G51a, 8091 Zurich, Switzerland
Phone: + 41 4 42 55 51 31, Fax: + 41 4 42 55 44 30
AnaPaula.Simoes-Wuest@usz.ch

Supplementary material is available under https://doi.org/10.1055/a-1829-9546

ABSTRACT

The placental passage of protopine was investigated with a human *ex vivo* placental perfusion model. The model was first validated with diazepam and citalopram, 2 compounds known to cross the placental barrier, and antipyrine as a positive control. All compounds were quantified by partially validated U(H)PLC-MS/MS bioanalytical methods. Protopine was transferred from the maternal to the fetal circuit, with a steady-state reached after 90 min. The study compound did not affect placental viability or functionality, as glucose consumption, lactate production, and beta-human chorionic gonadotropin, and leptin release remained constant. Histopathological evaluation of all placental specimens showed unremarkable, age-appropriate parenchymal maturation with no pathologic findings.

* These authors contributed equally to the work.

** Present address: Oncodesign SA, Villebon-sur-Yvette, France

ABBREVIATIONS

96-DWP	96-deepwell plate
Cal	calibration sample
CHMP	Committee on Herbal Medicinal Products
EMA	European Medicines Agency
ESI	electrospray ionization
FITC	fluorescein isothiocyanate
FM	fetal-maternal
FM ratio	fetal-maternal concentration ratio
IS	internal standard
LLOQ	lower limit of quantification
MeCN	acetonitrile
NMD	non-psychotic mental disorder
PM	perfusion medium
QC	quality control
QCH	quality controls at high levels
QCL	quality controls at low levels
QCM	quality controls at medium levels
SS	stock solution
ULOQ	upper limit of quantification
WS	working solution
***β*-hCG**	beta-human chorionic gonadotropin

Introduction

During pregnancy, a large number of women need medical care. Pharmacotherapy in pregnant women is challenging, given that adverse effects on the embryo/fetus have to be considered [1]. The situation is exacerbated by the fact that pregnant women are, in most cases, actively excluded from clinical drug development trials. This severely reduces the number of medications labeled for use during pregnancy [2, 3]. As a consequence, clinicians often make use of the so-called off-label prescribing (i.e., they advise the use of medications in a way that diverges from the approved product information [e.g., indication, application, dosage, patient categories]) [4]. Probably for all these reasons, expectant mothers often perceive synthetic medications as potentially dangerous. They try to reduce their consumption [5, 6] and seek supposedly safe alternatives, such as phytomedicines. In a multinational study, an average of 28.9% of pregnant women reported using herbal medicines during pregnancy, with an even higher proportion of 40.6% in Switzerland [7].

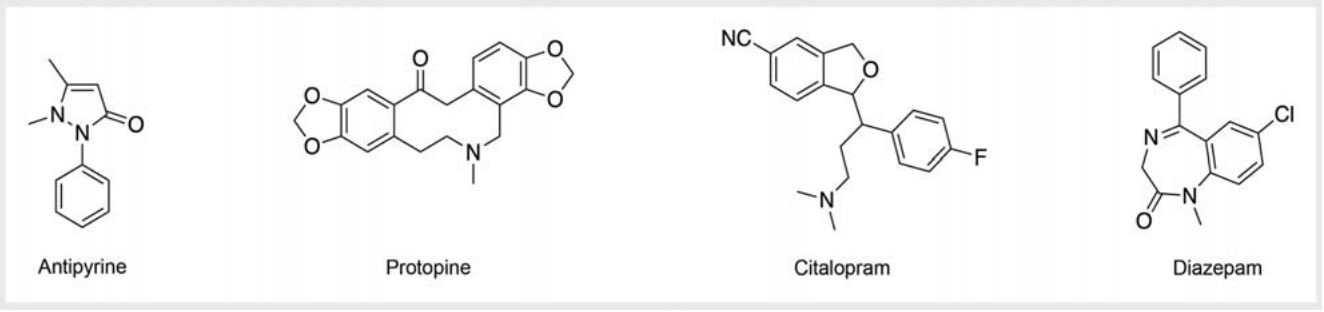
Some of the phytomedicines are used in the treatment of non-psychotic mental disorders (NMDs) in pregnancy, such as sleep disorders, restlessness, anxiety, and mild depression. A recent prevalence estimate in Switzerland reported that 16.7% of perinatal women used mental healthcare [8].

The perception of phytomedicines as safe in pregnancy [9] contradicts that studies on their safety in pregnancy are essentially lacking. For example, how much phytochemicals can pass across the placental barrier to reach the fetus is unknown. We are currently investigating the transplacental transfer of selected compounds from medicinal plants used to treat mild NMDs in pregnancy to shed some light on the matter.

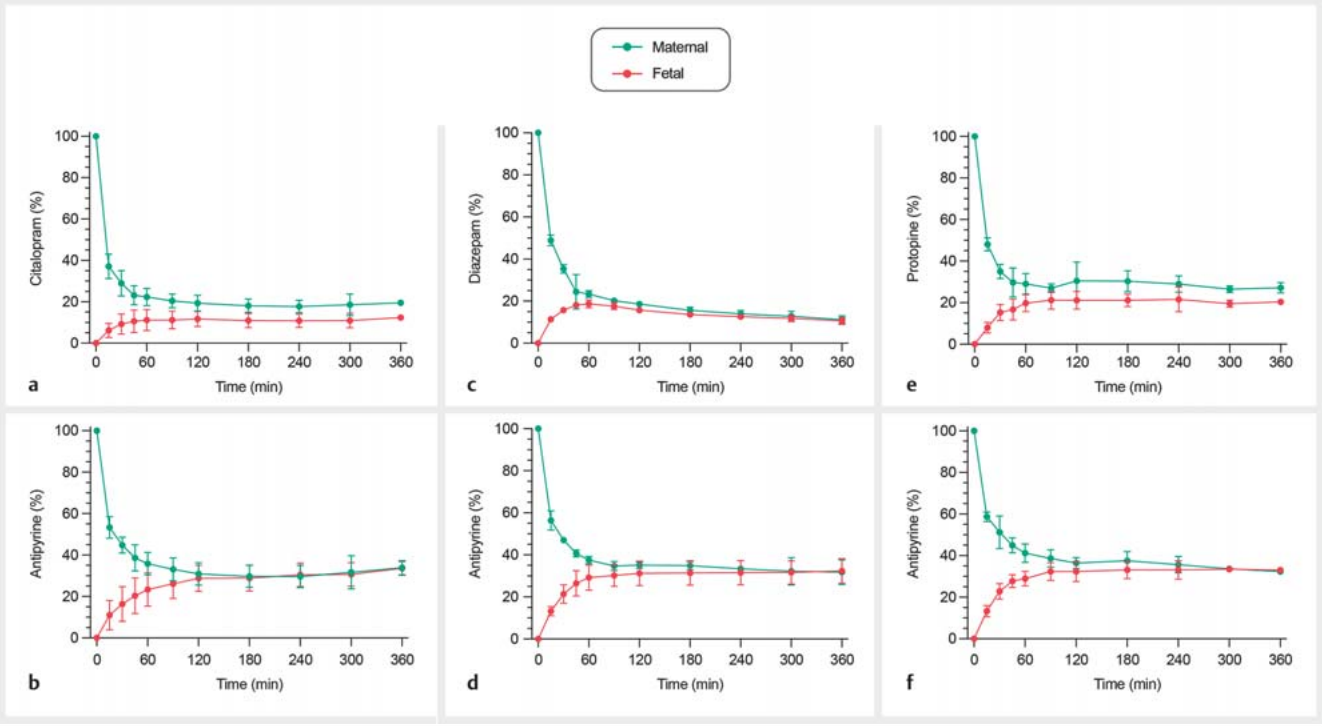
California poppy (*Eschscholzia californica* Cham., Papaveraceae) has a long tradition among indigenous people in the USA [10]. The CHMP has classified it for traditional use as a sleeping aid and for the relief of mild symptoms of mental stress [11]. Various *Eschscholzia* products are on the market, ranging from approved phytomedicines to food supplements available via the internet. They contain either powdered herbal drug or extract as the active ingredient, sometimes combined with other herbs. Very few products have been standardized for their content in total alkaloids [12–14]. California poppy contains 0.5% to 1.2% of total alkaloids, with protopine, a phytochemical that is also present in several other herbal medicines [15], being one of the major compounds [16–18]. Extracts of California poppy have shown sedative and anxiolytic effects *in vivo* [10], and these properties have been attributed to the isoquinoline alkaloids [19]. Several *in vitro* studies suggest that protopine is a CNS-active compound. Protopine was found to bind with $GABA_A$ receptors in rat synaptic membrane preparations [20, 21]. Protopine was also shown to be a ligand at $5\text{-}HT_{1A}$ receptors expressed in human CHO cell membranes [22]. The alkaloid is also an inhibitor of serotonin and noradrenaline transporters expressed in murine S6 and N1 cells, respectively [23]. The CHMP does not recommend using California poppy during pregnancy due to a lack of sufficient safety data [11].

A broad range of *in vitro* and *in vivo* models have been used to assess fetal exposure to exogenous compounds. Chronically cannulated sheep have been used extensively, and *in situ* placental perfusion techniques in rodents (guinea pigs, rabbits) have been established [24]. *Ex vivo* perfusion models with rats [25] and mice [26] have been used for an early screening of substance transfer across the placental barrier. However, with all animal models, the extrapolation of results to humans is limited due to functionally and anatomically large interspecies differences [24, 27, 28]. *In vitro* models utilizing well-established human placental cell lines (e.g., BeWo, Jar, JEG-3 cells) or human placental primary cells (villous trophoblasts) and explant tissue have been employed. These latter models enable the study of various factors affecting the transplacental transport, such as uptake, efflux, and metabolism. BeWo b30 cells (a clone of BeWo cells) form confluent monolayers on the semi-permeable membrane of Transwell inserts can be used as an *in vitro* model for the placental barrier. However, all cell-based placental models lack the cellular organization, compartmentalization and 3-dimensional structure of intact, physiologically active placentae [29]. The current gold standard among the placental transfer models is the *ex vivo* perfusion utilizing human placentae that are obtained immediately after delivery [30–32]. Here, the structure of the cotyledon as a functional unit of the placenta is fully preserved [33], and data obtained are highly predictive of the *in vivo* transfer [34].

We here determined the transplacental transfer of protopine, a major alkaloid in California poppy [35] and several other medicinal plants [15], side by side with compounds known to cross the placental barrier, like antipyrine, citalopram, and diazepam (▶ **Fig. 1**). The effects of protopine on the viability of placental tissue and the production of placental hormones were also investigated.



▶ **Fig. 1** Structures of connectivity control (antipyrine), the selected phytochemical (protopine), and synthetic study compounds (citalopram, diazepam).

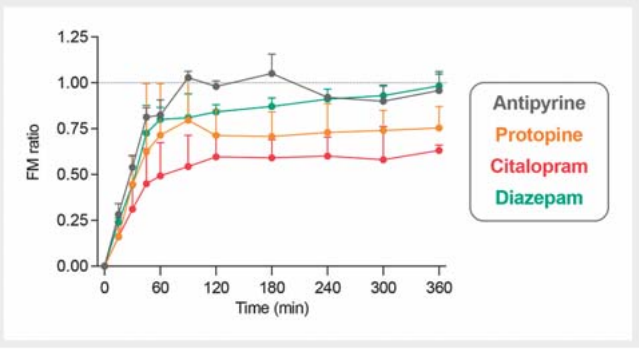


▶ **Fig. 2** *Ex vivo* human placental perfusion profiles of (**a**) citalopram (n = 4), (**c**) diazepam (n = 5), and (**e**) protopine (n = 4, except at t = 300 and 360 min, in which only 2 values are included) with corresponding connectivity control (antipyrine) transfers (**b, d**, and **f**, respectively). Concentrations are expressed as a percentage (%) of initial analyzed concentration in the maternal sample. All values are expressed as mean ± standard deviation (SD). Perfusion profiles with absolute concentrations (ng/mL) can be found in **Fig. 1S** (Supporting Information).

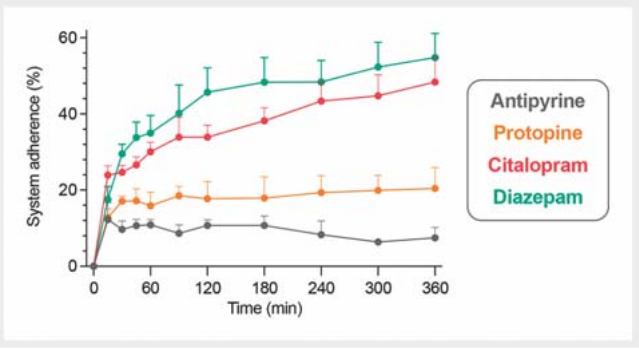
Results

As a first step, the *ex vivo* placental perfusion model – with which we wanted to study the transfer of protopine – was validated with 3 placenta-permeable compounds: antipyrine, citalopram, and diazepam (▶ **Fig. 2**). Antipyrine served as a connectivity (positive) control in all placental perfusions to verify the overlap of the maternal and the fetal side. Citalopram and diazepam crossed the human placental barrier as expected [36, 37]. After approximately 60 min, a steady-state concentration was achieved on the fetal side corresponding to 11% of the initially present citalopram at the maternal side. Thereby, a steady-state concentration of 22% was reached on the maternal side. Concentrations did not change during the following 300 min (▶ **Fig. 2a**). For diazepam, steady-state concentrations were reached after approximately 45 min, with 24% of the initially analyzed concentration and a slow decrease until 360 min (▶ **Fig. 2b**). Antipyrine also readily crossed the placental barrier and reached a steady-state concentration after approximately 120 min (▶ **Fig. 2c** and **d**). These findings aligned with previous work and confirmed that antipyrine was a suitable connectivity control [38].

Protopine was also transferred from the maternal to the fetal circuit. A gradual decrease of protopine in the maternal compartment and a concomitant increase in the fetal compartment were observed. After about 60 min, a steady-state was established in



▶ **Fig. 3** The fetal-maternal concentration ratio (FM ratio; fetal concentration divided by maternal concentration) calculated for each timepoint of protopine (n = 4), citalopram (n = 4), and diazepam (n = 5), in comparison with antipyrine (n = 3) from control perfusions. The FM ratio is 1.0 when the fetal and maternal concentrations are equal. All data are expressed as mean ± standard deviation (SD) of at least 3 independent experiments (except t = 300 and 360 min in case of protopine, and t = 360 min in case of antipyrine, in which only 2 values are included).



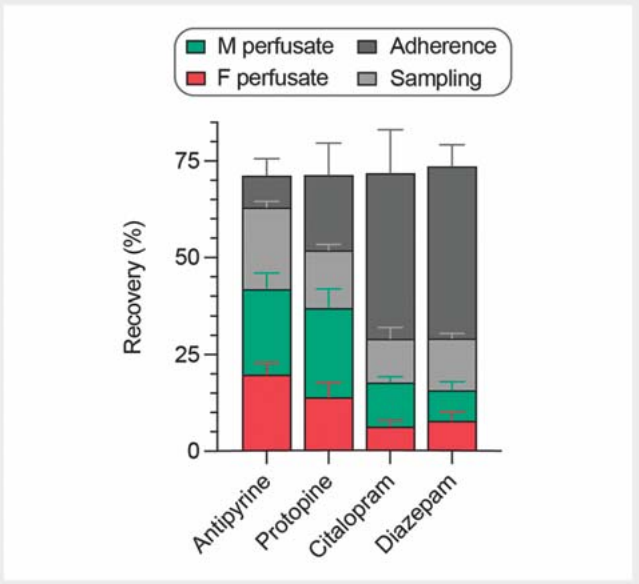
▶ **Fig. 4** Adherence of study compounds from a 360 min system adherence test (circulation of study compounds through an empty perfusion chamber comprising only the maternal circuit). All compounds were tested individually in 3 independent experiments (n = 3) and are expressed as mean ± standard deviation (SD). Displayed is the percentage (%) of compound (of initially analyzed concentration in the maternal sample) that adheres to the equipment: antipyrine (7.4 ± 4.7%), protopine (20.4 ± 9.5%), citalopram (48.4 ± 12.9%), and diazepam (54.8 ± 12.7%) at 360 min.

the 2 circuits, with virtually no change over the remaining 300 min (approximately 27% [maternal] vs. 20% [fetal] of initially analyzed concentration; ▶ **Fig. 2e**). The overlap of maternal and fetal circuits was again confirmed with antipyrine reaching an equilibrium after 120–240 min (▶ **Fig. 2f**). Perfusion profiles with absolute concentrations (ng/mL) can be found in **Fig. 1S** (Supporting Information).

If the fetal-maternal concentration ratio (FM ratio; ▶ **Fig. 3**) of protopine is considered, no concentration equilibrium was apparent in the fetal and maternal compartments at any point of the placental perfusion (FM ratio of 0.75 after 360 min). The profiles of the synthetic compounds are shown for comparison. In citalopram, an equilibrium between fetal and maternal concentrations was never reached (FM ratio of 0.63 after 360 min). As for diazepam, the FM ratio was comparable to that of antipyrine (0.98 vs. 0.95 at 360 min).

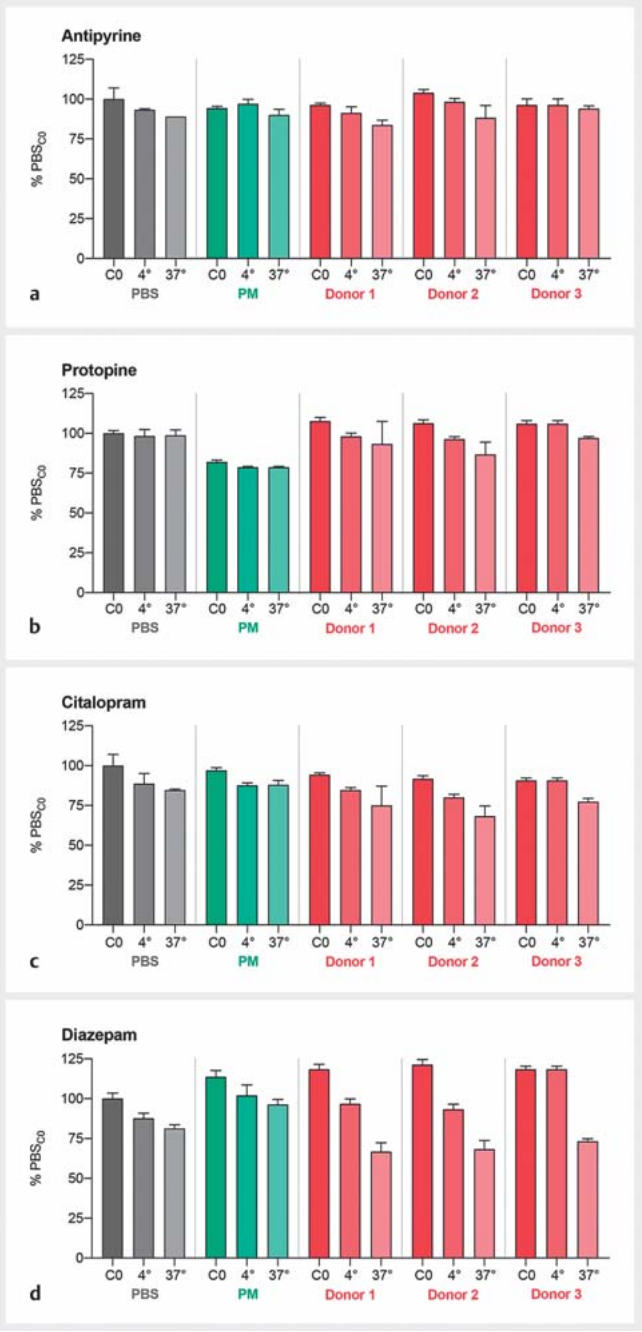
The 360 min system nonspecific adherence tests (empty perfusions; ▶ **Fig. 4**), which were performed without placenta and only in the maternal circuit, revealed that only minor proportions of protopine and the connectivity control antipyrine were lost over 360 min (20.4% and 7.5% of initially analyzed concentration, respectively). The relative amount of citalopram and diazepam which adhered to the perfusion equipment after 360 min, was significantly higher with 48.4% and 54.8%, respectively.

Several aspects must be considered for the recovery calculations (▶ **Fig. 5** and **Table 1S**, Supporting Information) of study compounds during placental perfusions. Looking at the final distribution of the compounds in the 2 compartments (fetal and maternal circuit) after 360 min of the perfusion, we found they all passed the placental barrier and were distributed in the following proportions (fetal vs. maternal): antipyrine (19.8% vs. 22.0%), protopine (14.0% vs. 23.1%), citalopram (6.4% vs. 11.4%), and diazepam (7.9% vs. 7.9%). As shown in **Table 1S** (Supporting Information), 11.4–21.2% of the substance was removed by sampling during perfusion, corresponding to one-sixth to one-third



▶ **Fig. 5** Recovery of study compounds in the human *ex vivo* placental perfusion system, expressed as percentage (%) of initial amount analyzed in the maternal sample at the beginning of the perfusion. The final recovery was calculated as the sum of compound present in fetal and maternal perfusates at the end of a perfusion and the amounts sampled during the perfusion from fetal and maternal perfusates. The loss of compound by binding to the perfusion model was accounted for by the system adherence test (empty perfusion). All data are represented as mean ± standard deviation (SD) of 3 to 5 independent experiments.

of the final recovery. In addition, it is crucial to include the results from the 360 min system adherence test (empty perfusion). While only small losses were seen for antipyrine and protopine, considerable amounts of citalopram and diazepam adhered to



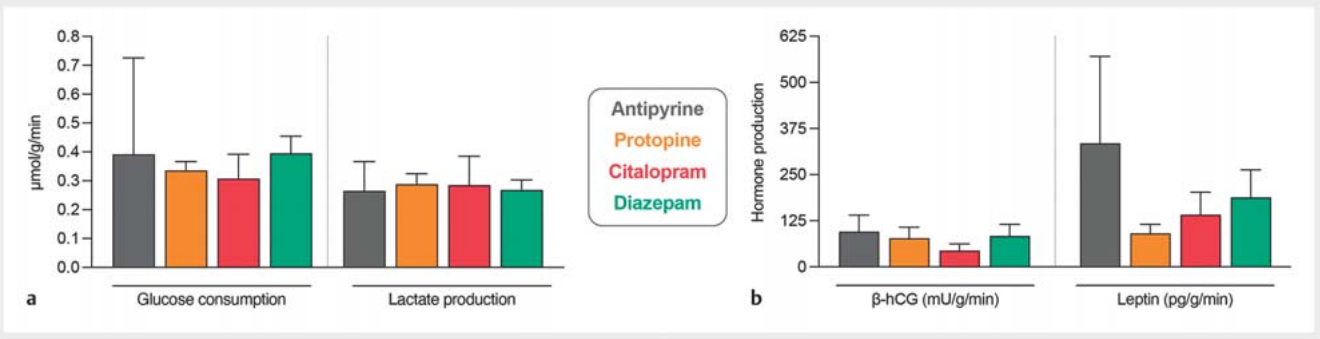
▶ **Fig. 6** Stability data of study compounds (**a**–**d**) expressed as a percentage (%) of the initial concentration (C0) in PBS. The stability test was performed for 360 min at 2 different temperatures (4 °C and 37 °C) and 3 different matrices (PBS, perfusion medium (PM), and placental homogenates from 3 different donors). Differences due to matrix effects were excluded in a separate experiment (see **Fig. 2S**, Supporting Information). Samples were processed via solid phase extraction or protein precipitation before analysis. All data are represented as mean ± standard deviation (SD).

the equipment and tubing after 360 min. Using the system adherence test to assess the final recovery, we obtained the following values for antipyrine (71.2 ± 7.2 %), protopine (71.4 ± 8.6 %), citalopram (71.9 ± 5.2 %), and diazepam (73.6 ± 4.8). Finally, the fraction unbound to homogenates ($f_{u,hom}$) of placental tissue was assessed to account for potential loss of compound in the placenta itself (**Table 2S**, Supporting Information). The $f_{u,hom}$ was equal to 1.0 for antipyrine, followed by protopine (0.48 ± 0.04), citalopram (0.21 ± 0.01), and diazepam (0.09 ± 0.007). Thus, $f_{u,hom}$ followed the pattern of the system adherence test, reflecting the lipophilic nature of the compounds.

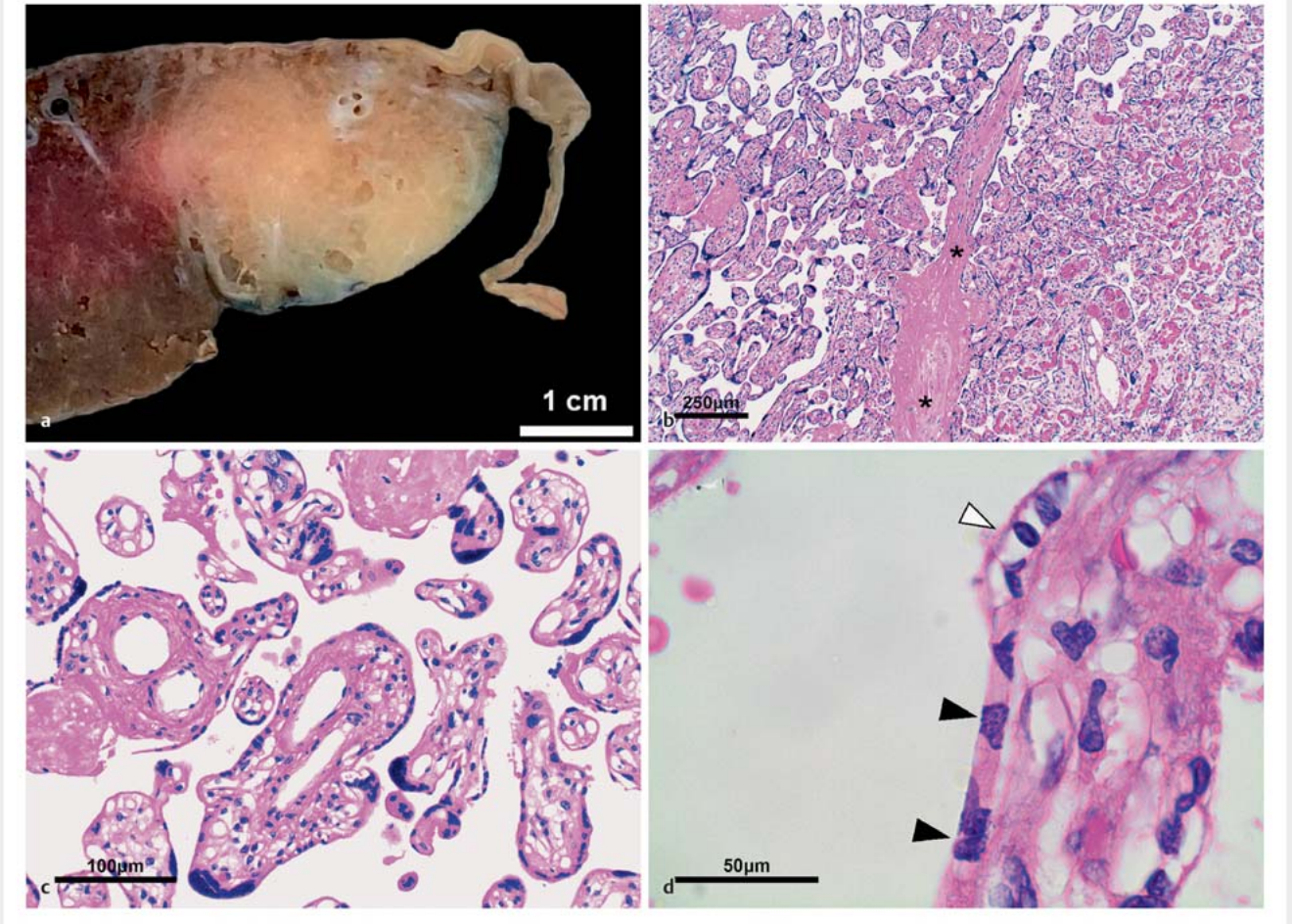
The stability of study compounds was tested over 360 min in 3 different matrices (PBS, perfusion medium (PM), and 3 placental homogenates [Donors 1–3] at 2 temperatures (4 °C, 37 °C). The stability data of antipyrine, and citalopram were very comparable in the 2 matrices PBS and PM, while protopine (in PM) and diazepam (in PBS) were slightly less stable over 360 min at 4 °C and 37 °C (▶ **Fig. 6**). A degradation in homogenate at 37 °C was observed with diazepam (66.7 %, 68.3 %, and 73.3 % respectively). The use of 3 different placental homogenates (donors) resulted in comparable values for all test substances. Differences due to matrix effects were excluded in a separate experiment (see **Fig. 2S**, Supporting Information).

The placental perfusion model can not only be used to characterize the transplacental compound transfer and investigate the possible effects of the compounds on placental viability and hormonal production. All placentae viability and metabolic activity were constant in our case, as neither glucose consumption nor lactate production was affected by the study compounds (▶ **Fig. 7a**). With antipyrine (from control perfusions, **Fig. 3S**, Supporting Information), the total glucose consumption and lactate production during 360 min were 0.39 and 0.27 µmol/g/min, respectively. Perfusions with protopine, citalopram, and diazepam showed comparable values, indicating that they did not impair placental viability and metabolic activity. As an additional measure for placental function, the production of beta-human chorionic gonadotropin (*β*-hCG) and leptin was determined and expressed as the release rate per min and weight of cotyledon (g) (▶ **Fig. 7b**). The tissue of all placentae retained its functionality throughout the *ex vivo* perfusion period. A *β*-hCG production of 95.7 mU/g/min and leptin production of 334.8 pg/g/min were observed in control perfusions with antipyrine (from control perfusions, **Fig. 3S**, Supporting Information). Protopine did not inhibit *β*-hCG and leptin production in a statistically significant way, even though leptin production was somewhat lower in the presence of all study compounds.

To establish the human *ex vivo* placental perfusion model, we introduced a detailed histopathological examination of the perfused tissue. Representative macroscopic and microscopic images of the transition between perfused and nonperfused tissue, and details of the perfused area and an individual villus with an outer layer of trophoblast cells are shown (▶ **Fig. 8**). The most important histopathological criteria for quality control (QC) were: (i) macroscopically, an evident effect of the perfusion on the cotyledon, in contrast to the nonperfused tissue (▶ **Fig. 8a**); microscopically, (ii) a difference between perfused and nonperfused tissue, the latter being characterized by a narrow intervillous space and blood-filled capillaries (▶ **Fig. 8b**); (iii) the perfused area had dilated intervillous space, bloodless capillaries, and regular (mature) villi (▶ **Fig. 8c**); and (iv) the proportion of vacuolated (degenerated) trophoblast cells was between 0–20 % (▶ **Fig. 8d**).



▶ **Fig. 7** Assessment of tissue viability and functionality during the *ex vivo* human placental perfusion. **a** Comparison of glucose consumption and lactate production of the selected phytochemical (protopine) and synthetic compounds (citalopram, diazepam) with antipyrine from control perfusions. Displayed are the changes between beginning and end of the perfusion in fetal and maternal circuits. Normalized by the total perfusion time (min) and perfused cotyledon weight (g). **b** Comparison of beta-human choriogonadotropin (*β*-hCG) and leptin tissue production of perfusions with the selected phytochemical (protopine), synthetic compounds (citalopram, diazepam), and antipyrine (from control perfusions). The net release rate of placental hormones is displayed during the placental perfusion, normalized by the total perfusion time (min) and perfused cotyledon weight (g). All data are represented as mean ± standard deviation (SD) of 3 to 5 independent experiments.



▶ **Fig. 8** Histopathological evaluation of placental tissue as additional quality control. **a** Macroscopic image of a representative placental specimen from the transitional area of nonperfused and perfused tissue (left and right, respectively; scale bar 1 cm). **b** Overview of the perfused vs. nonperfused area (left and right, respectively; scale bar 250 µm). The septum (asterisk) represents the (incomplete) partition separating the cotyledons. The blood-filled capillaries and the narrow intervillous space, which is also partly filled with blood, are clearly visible on the right. **c** Perfused parenchyma showing mostly empty capillaries and empty, dilated intervillous space. Parenchyma shows inconspicuous, regular mature villi (scale bar 100 µm). **d** Close-up of a villus from the perfused area showing vacuolated degenerate and regular trophoblast cells (white and black arrowheads, respectively; scale bar 50 µm).

▶ **Table 1** Detailed histopathological evaluation assessing the microscopic effects of human *ex vivo* placental perfusions with protopine (n = 4), and the damage of placental tissue in perfused areas compared to nonperfused areas.

	Experiment number	Protopine			
		1	2	3	4
Microscopic effects of perfusion (in perfused tissue) in %	Sharp transition perfused/nonperfused	Y	Y	Y	Y
	Emptiness of villous vessels	90	80	95	95
	Blood-filled villous vessels in nonperfused	90	95	100	95
	Dilatated (recognizable) villous vessels	95	50	60	ND
	Intervillous spaces without blood	100	95	95	95
	Blood-filled intervillous space in nonperfused	50	30	5	20
	Dilatated intervillous space	80	40	80	50
	Dilatated intervillous space in nonperfused	30	10	30	40
	Hydropic changes	15	10	5	5
	Hydropic changes in nonperfused	1	0	0	0
Damage of placental tissue (in perfused tissue) in %	Thrombi in villous vessels	1	5	0	5
	If so: in nonperfused too?	1	1	–	1
	Thrombi in vessels of stem villi	0	0	0	0
	If so: in nonperfused too?	–	–	–	–
	Vacuolated trophoblast in villi	0	20	1	1
	If so: in nonperfused too?	–	0	0	0

ND = not determined; Y = yes.

Initial histopathological examinations showed that, in our hands, perfusion was effectively taking place and did not seriously damage the perfused cotyledon. These examinations were then performed after every perfusion experiment with study compounds to demonstrate successful perfusion (including connection of the fetal and maternal perfused area) and assess possible deleterious effects of the compound on the tissue compared to nonperfused tissue.

The histopathological evaluation of the placental specimens showed that the only macroscopically discernible effect of the perfusion was the pale tissue, which was apparent in all placentae. In all cases, the microscopic examination (▶ **Table 1**) of the tissue sections revealed a clear transition between perfused and nonperfused tissue. The villous vessels of the perfused side were ≥ 80 % empty (the nonperfused area was ≥ 90 % blood-filled) and mostly dilated. The intervillous spaces of the perfused tissue were also ≥ 95 % bloodless (the nonperfused area was ≤ 50 % blood-filled) and mostly dilated (40–80 %), in contrast to the nonperfused side (10–40 %). Hydropic villous changes were found more often in perfused (5–15 %) than in nonperfused areas (0–1 %). Histopathological examinations showed that the endothelium in perfused and nonperfused tissue was still viable after 360 min of perfusion. There were also no ruptures of villous vessels or extravasation into villous stroma in perfused and nonperfused areas. In addition, no thrombi could be detected in vessels of stem villi. The percentage of thrombi in villous vessels (0 % or 1 %) was the same in perfused and control tissue (nonperfused), whereas 2 cotyledons perfused with protopine showed a slightly higher proportion of villous thrombi compared to control (5 % vs. 1 %). Trophoblast vacuolization in the perfused areas occurred in a small proportion of 0–20 %. Overall, protopine did not cause apparent damage of placental tissue according to the assessment of endothelium, vascular rupture, thrombi, and trophoblast vacuolization. In addition, no signs of inflammation were found in any of the perfused areas of the placentae examined, as neither bacteria nor neutrophils were present in the villous vessels and intervillous spaces. The assessment of global placental pathology was also inconspicuous, with no signs of fetal or maternal malperfusion, an absence of villous immaturity, chronic/acute villitis, chronic deciduitis and chorioamnionitis, and no bacteria in the nonperfused area of the placenta.

Discussion

The present data show that protopine was rapidly transferred from the maternal to the fetal circuit, and no evidence for metabolization was found. However, the FM ratio of protopine was lower than that of antipyrine (0.75 vs. 0.96), and no equilibrium between maternal and fetal concentrations was reached. This finding was similar to the results obtained with citalopram. Whether the absence of an equilibrium is due to active transplacental transport of protopine deserves further investigation. In our experiments, the transplacental transfer of antipyrine, citalo-

pram, and diazepam was comparable with previously reported data [36–38].

The use of placental perfusion and U(H)PLC-MS/MS methods are strengths of the present work. Placental perfusion is the gold standard when studying the transfer of compounds from the mother to the fetus. A limitation of this model is that substance transfer at term may be overestimated compared to that in the premature placenta of early pregnancy [28]; in addition, the model cannot mimic the mother's drug absorption, distribution, metabolism, and excretion. Only U(H)PLC-MS/MS methods to detect very low concentrations enabled the work on this project, since clinically relevant concentrations of phytochemicals from (multicomponent) extracts are known/expected to be very low.

Ex vivo placental perfusions allow determining the transfer of study compounds and assessing placental viability and function. Control perfusions with antipyrine showed similar values for the placental viability (glucose consumption, lactate production) and functionality (β-hCG and leptin production) as previously reported for this model [32]. None of the study compounds altered the glucose consumption, lactate production, and β-hCG accumulation, and only a statistically nonsignificant decrease in leptin production was observed. These results indicated that none of the compounds impaired placental performance. The histopathological evaluation of perfused tissues (cotyledons) was in line with these results. No pathological findings were observed, and all placental specimens showed only unremarkable, age-appropriate parenchymal maturation. Trophoblastic vacuolization of villi was the only perfusion-induced change observed. Despite the ischaemic periods up to 60 min, no increase in damage of placental tissue could be observed with time (▶ **Table 1** and **Table 3S**, Supporting Information). Neither the number of thrombi in villous vessels nor the vacuolization of trophoblasts in villi was increased.

It was crucial to calculate the recovery of study compounds in the best possible way to validate the findings. The amounts in the fetal and maternal compartments, the amounts removed by sampling, and the loss due to adsorption in the maternal circuit could be considered. Study compounds may also be taken up by cells/membranes and may be metabolized by placental enzymes. To minimize adsorption to the perfusion system, we used only tubes recommended for pharmaceutical and medical applications, and we shortened the tubing in the model. Moreover, in placental homogenates, fraction unbound, and stability of compounds were determined. Nevertheless, the calculated recoveries are likely to be underestimated, given that adsorption in the fetal circuit could not be measured.

To further assess safety of California poppy during pregnancy, transplacental passage of additional phytochemicals have to be investigated, together with testing in additional models. We are currently using a range of *in vitro* assays to characterize cytotoxic and genotoxic effects, and the influence on metabolic and differentiation processes of whole extracts [39] and protopine (Spiess et al., manuscript under review). From a safety perspective, these results will be particularly relevant for compounds that can cross the placental barrier and, therefore, have access to the fetal circulation, as is the case for protopine. In addition, we are investigating the intestinal and hepatic metabolization of several phytochemicals.

The human placental *ex vivo* perfusion model was successfully implemented and used for the first time with a phytochemical. The *ex vivo* placental perfusion model will now be used also for transport studies with relevant phytochemicals in other medicinal plants used for the treatment of mild NMDs in pregnancy.

Materials and Methods

Chemicals, reagents, and study compounds

All solvents were of U(H)PLC grade. Merck KGaA supplied MeCN, and MeOH was purchased from Reuss-Chemie AG. Purified water was obtained from a Milli-Q integral water purification system.

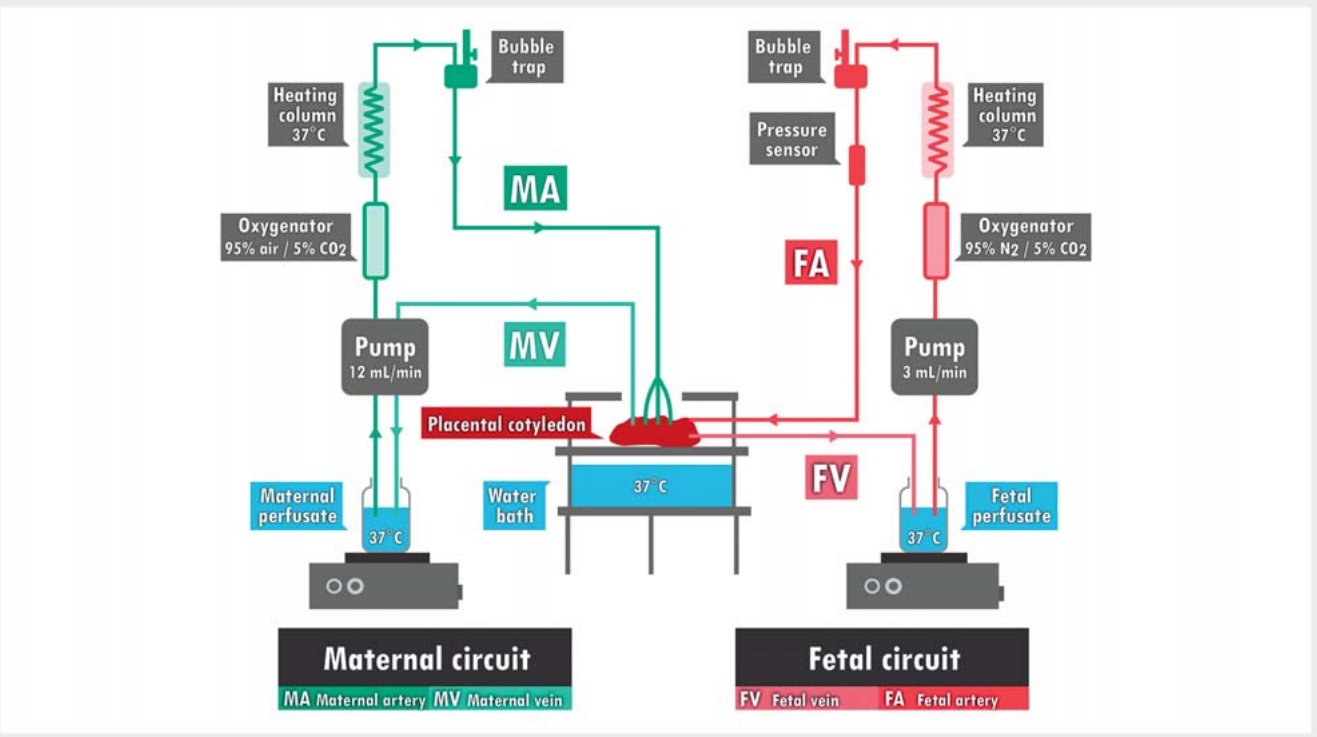
Scharlau supplied DMSO, and formic acid was from BioSolve. Antipyrine and BSA were purchased from Sigma-Aldrich and antipyrine-D3 from HPC Standards GmbH. Protopine HCl was purchased from Extrasynthese SAS, and verapamil HCl from Sigma-Aldrich. Citalopram HBr, diazepam, and diazepam-D5 were purchased from Lipomed AG, and citalopram-D4 HBr was obtained from CDN Isotopes.

Ex vivo human placental perfusion

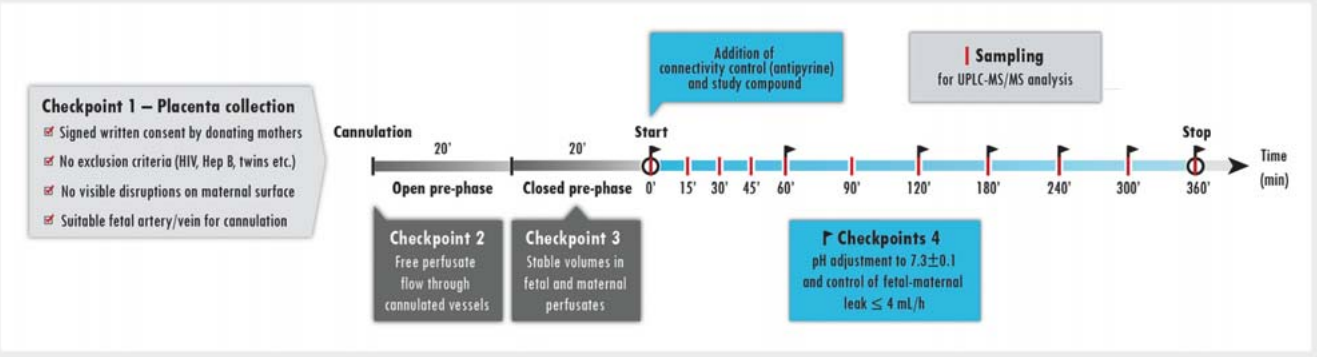
Placentae collection. Placentae were collected in collaboration with the Department of Obstetrics of the University Hospital of Zurich, Switzerland. Only placentae from women undergoing elective caesarean section from uncomplicated term pregnancies (37–41 wk) were considered. Each pregnant woman signed informed written consent before delivery for the use of placentae for research. This procedure (including consent form) was approved by the Ethics Committee of the Canton of Zurich (KEK-StV73 Nr. 07/07; March 21, 2007). Exclusion criteria included: twin and/or complicated pregnancy, smoking, substance abuse, and patients positive for HIV, HBV and SARS-CoV-2.

Equipment and experimental procedure of perfusion. A slightly modified *ex vivo* human placental perfusion model [32, 38] was used to study the transfer of the study compounds across the placental barrier (▶ **Fig. 9**). A cotyledon of the placenta (lobule) was perfused with 2 reconstructed circuits representing the maternal and fetal sides. Both sides consisted of an artery transporting the perfusate to the cotyledon and a vein that returns the perfusate to the original reservoir. Two heating magnetic stirrers were added to ensure physiological conditions and to prevent uneven distribution of study compounds.

The time course of a perfusion experiment, including the preparatory phases, checkpoints and samplings is shown in ▶ **Fig. 10**. After obtaining a suitable placenta (checkpoint 1), the fetal artery and associated vein of a selected cotyledon were cannulated and mounted in a perfusion chamber within 60 min after delivery. The selection of a suitable cotyledon for perfusion was based on a thorough visual examination of the villous structures and associated fetal vessels. Cotyledons with a ragged maternal surface (visible disruptions; macroscopic tissue trauma), evidence of basal plate fibrin deposition (on the maternal surface), suspected placental infarction, or too little fetal membrane (on the disk of the placenta) were not considered for perfusion. The chorionic artery was cannulated (⌀ 1.2 mm cannula) first, following the chorionic vein cannulation (⌀ 1.5–1.8 mm cannula). A surgical



▶ **Fig. 9** Placental perfusion setup consisting of maternal (left) and fetal (right) circuit. Both perfusates are placed on magnetic stirring and heating devices and transported to the placental cotyledon (lobule) via arteries with the aid of peristaltic pumps operated at different flow rates (maternal: 12 mL/min; fetal: 3 mL/min). Flow heaters (heating columns) and a water bath below the perfusion chamber keep the temperature at 37 °C. On the way to the placental cotyledon, the perfusates are gassed by oxygenators with non-identical compositions (maternal: 95% air/5% CO_2; fetal: 95% N_2/5% CO_2) and freed from any air bubbles by bubble traps. A sensor in the fetal artery records the pressure (and temperature). Two veins return the perfusates to the corresponding reservoirs.



▶ **Fig. 10** Overview of the *ex vivo* human placental perfusion with checkpoints for a successful experiment. Checkpoint 1 involved obtaining a suitable placenta, written informed consent from the donor, and ensuring that no exclusion criteria were present. The quality of placental tissue and presence of fetal vessels suitable for cannulation were checked. After cannulation, it is important to verify free flow of perfusion medium (PM) through the fetal vessels (checkpoint 2), leading to a 20 min open preliminary (pre-) phase (nonrecirculating), followed by a 20 min closed pre-phase (recirculating). During the latter, the volumes of maternal and fetal perfusates have to remain stable in order to begin the main experiment (checkpoint 3). The main experiment started with circulating PM containing the connectivity control (antipyrine) and study compound at the desired concentration (O denoting the experiment's beginning and end). At defined timepoints (0, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 min), samples were taken from the maternal and fetal reservoir for analysis. At the beginning of the experiment and after each hour, the pH and the fetal-maternal (FM) leak were measured.

suture (orange or green PremiCron HR17, USP3/0, B. Braun Medical AG) was used to fix the cannulas. The experiment was performed using PM (modified composition [32] using cell culture medium 199 from Sigma-Aldrich) circulating through the fetal and maternal circuit using digitally controlled peristaltic pumps (Ismatec) at a rate of 3 and 12 mL/min, respectively. The tubing consisted of a fetal artery (∅ 1.52 mm) and maternal artery tube (∅ 2.06 mm), a maternal vein tube (∅ 2.29 mm), and connecting tubes (∅ 1.60 mm, all PharMed Ismaprene from Ismatec). The fetal perfusate was gassed with 95% N_2/5% CO_2 throughout the perfusion and the maternal perfusate with 95% air/5% CO_2 instead. The perfusion included a 20 min open preliminary (pre-) phase with nonrecirculating PM (with discarded venous outflow) to allow the placental tissue to recover from the ischemic period after the delivery and to flush out the remaining blood from the villous vasculature and intervillous space (checkpoint 2). In the following recirculating closed pre-phase (20 min; with the venous outflow leading back to the corresponding reservoir), the perfusate volumes on the maternal and fetal sides were monitored. Stability of volumes (50 mL PM in each reservoir) ensured the integrity of the circuits and the absence of leaking in the fetal-to-maternal circuit (checkpoint 3). The main experiment was then initiated by replacing the fetal and maternal perfusate simultaneously with equal starting volumes of 100 mL fresh PM, with the addition of the study compound and antipyrine (as a connectivity control) to the perfusate of the maternal circuit, both at a final concentration of 5 µM (≙ 941 ng/mL antipyrine, 1767 ng/mL protopine, 1622 ng/mL citalopram, and 1424 ng/mL diazepam). In both reservoirs, the pH was adjusted to a physiological range (7.3 ± 0.1) at the beginning of the experiment (and adjusted after every hour of perfusion), and the fetal-maternal (FM) leak was not allowed to exceed a value of 4 mL/h (checkpoint 4). Samples (3–4 mL) for analysis were taken at defined timepoints over a 360 min period (0, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 min). Samples were stored at − 80 °C for bioanalytical analysis in 0.8 mL glass micro-inserts (VWR) immediately after centrifugation at 800 rpm/1837 rcf (Centrifuge Sigma 6–16; 4 °C, 10 min) to remove residual erythrocytes. Additional QC measures included a fetal perfusion pressure ≤ 70 mmHg throughout the perfusion and an antipyrine equilibrium between fetal and maternal circuit after 240–360 min. For an overview of the experimental conditions and characteristics of the placentae used see **Table 3S** (Supporting Information).

Fetal capillary integrity marker analysis. For establishing and validating the *ex vivo* placental perfusion model, initial experiments were performed using fluorescein isothiocyanate (FITC)-dextran (40 kDa, Sigma-Aldrich) as a test substance for assessing the integrity of fetal capillaries (**Fig. 4S**, Supporting Information). FITC-dextran was added to the fetal circuit at a final concentration of 200 µg/mL. The concentration in the fetal and maternal samples was detected by a Cytation 3 fluorescence microplate reader (BioTek Instruments; excitation wavelength 490 nm; emission wavelength 520 nm). The samples (undiluted) were added to black Nunc MaxiSorp microtiter plates.

Perfusion system adherence test (empty perfusion). Before starting the perfusions with human placentae, a system adherence test (circuit of study compounds through an empty perfusion chamber comprising only the maternal circuit) was performed for a total of 360 min. This test was used to assess the dissolution and adherence of new, yet unknown, compounds to the perfusion equipment and mainly to the tubing system to evaluate the final recovery better. Study compounds were, therefore, directly dissolved in PM at a final concentration of 5 µM. All study compounds were tested individually in 3 independent experiments (n = 3).

Viability and functionality of placental tissue. We measured glucose and lactate concentration in fetal and maternal samples at the beginning and end of every perfusion. This was done to determine the glucose consumption and lactate production throughout a perfusion as indicators of tissue viability and metabolic activity using an automated blood gas system (ABL800 FLEX). The production of 2 placental hormones – β-hCG and leptin – was monitored to assess tissue functionality *ex vivo* by standard ELISA as described previously by Malek et al. [40, 41]. The only deviation following the protocol was the use of a dilution solution consisting of 1% BSA instead of 2% BSA.

Histopathological evaluation. Each placental specimen was pathologically examined as an additional QC. For this purpose, representative tissue sections – each from the perfused and nonperfused placental portion, and from the transitional area – were removed immediately after each perfusion, fixed in 4% paraformaldehyde for at least 24 h, and then processed according to the standards of routine histopathological diagnosis of the Department of Pathology and Molecular Pathology (University Hospital of Zurich). Briefly, the fixed tissue sections were embedded in paraffin, cut into 2–3 µm thick sections, then stained with standard hematoxylin and eosin stain and with a modified Gram stain (according to Braun-Brenn). The latter was used to test for bacterial contamination in the perfused area [42]. Tissue from the nonperfused specimens was examined for general placental pathologies described in routine diagnostics [43, 44]. The quality of perfusion was correlated based on the blood void and width of the intervillous (maternal) space and fetal blood vessels in the chorionic villi, with particular attention to the presence of intravascular thrombi. To test whether tissue damage might have occurred due to perfusion, we sought and compared general signs of degeneration such as vacuolization of the cytotrophoblast, the viability of the villous vascular endothelium, and the formation of hydropic villous changes with the tissue condition of the nonperfused area. All microscopic effects studied and damage to placental tissue in the perfused area are reported in relative amounts (% of total).

LC-MS/MS analysis

Instrument and chromatographic conditions. U(H)PLC-MS/MS analyses were performed on an Agilent 6460 Triple Quadrupole MS system connected to a 1290 Infinity LC system consisting of a binary capillary pump G4220A, column oven G1316C, and multisampler G7167B. Quantitative analysis by MS/MS was performed with electrospray ionization (ESI) in MRM mode. Desolvation and nebulization gas was nitrogen. MS/MS data were analyzed with Agilent MassHunter Workstation software version B.07.00. The temperature of the autosampler was 10 °C. An Acquity UPLC HSS T3 column (100 mm × 2.1 mm; 1.8 µm) (Waters Corp.) was used

for separation of the analyte and the internal standard (IS), except for diazepam and its IS diazepam-D5, where a Kinetex column (100 mm × 2.1 mm; 1.7 µm) (Phenomenex) was used. Analysis of citalopram was performed on an Acquity UPLC system consisting of a binary pump, autosampler, column heater, which was connected to an Acquity TQD (all Waters Corp.). Desolvation and nebulization gas was nitrogen. The autosampler temperature was set at 10 °C, and the column temperature at 45 °C. MS/MS data were analyzed with MassLynx software version 4.1. U(H)PLC gradients, internal standards, and MRM transitions can be found in **Table 4S** and **5S** (Supporting Information).

Standards and stock solutions. Stock solutions (SS) of the analytes and the ISs were prepared at 0.2–1 mg/mL. Working solutions (WS1) of the analytes (50 µg/mL for antipyrine, and 100 µg/mL for all other compounds in DMSO) and of the ISs (50 µg/mL in MeOH) were obtained by dilution of the corresponding SSs. Calibration samples (Cals) of the analytes within the range of 5–500 ng/mL (antipyrine), 5–250 ng/mL (protopine), 10–1000 ng/mL (citalopram HBr and diazepam), and QCs at low (QCL), medium (QCM), and high (QCH) levels were obtained from serial dilutions of the WS in the corresponding matrix (PM). The concentrations of the QCs were defined as (i) 3-fold the lowest concentration for QCL; (ii) the highest concentration divided by 2 for QCM; and (iii) 80 % of the highest concentration for QCH. All SSs were stored at − 80 °C. Cals and QCs were freshly prepared before analysis. Before each experiment, a second working solution (WS2) of the IS was prepared by further diluting the WS1 in MeOH. Details for the calibration curves can be found in **Fig. 5S–8S** (Supporting Information) and in **Tables 6S–9S** (Supporting Information).

Sample extraction in placental perfusion medium for antipyrine, diazepam, citalopram, and protopine. To 200 µL of the analyte in the PM were added 100 µL of the IS, 200 µL BSA (60 g/L), and 800 µL ice cold MeCN (1000 µL for antipyrine). The mixture was briefly vortexed at room temperature on an Eppendorf Thermomixer (1400 rpm) and finally centrifuged for 20 min at 13 200 rpm/16 100 rcf at 10 °C (Centrifuge 5415R, Eppendorf). A total of 1100 µL (1300 µL for antipyrine) supernatant was collected and transferred into a 96-deepwell plate (96-DPW) and dried under nitrogen gas flow (Evaporex EVX-96, Apricot Designs). Reconstitution was done with 200 µL of 65 % mobile phase A (purified water with 5 % MeCN and 0.1 % formic acid; in the case of diazepam water and 0.1 % formic acid only) and 35 % mobile phase B (MeCN with 0.1 % formic acid) followed by 45 min shaking on the Eppendorf MixMate. The injection was done in full loop mode (2 µL) from the 96-DWP.

Method qualification. The bioanalytical fit-for-purpose methods had been developed and qualified only based on some validation tests (within- and between-series imprecision and inaccuracy, as well as carry-over had been assessed to validate the methods), following the current guidelines for industry [45, 46].

Within- and between-series imprecision and inaccuracy. Six replicates of 5 QCs (LLOQ [lower limit of quantification: 5 ng/mL], QCL, QCM, QCH, ULOQ [upper limit of quantification: 500 ng/mL]) were processed and injected into the U(H)PLC-MS/MS. Three validation runs on 3 different days were performed to ensure reproducibility. In each run, the imprecision (CV%) of each QC series had to be below 15 % (20 % for LLOQ) within the series. The inaccuracy (RE%) had to be within ± 15 % of the nominal values (± 20 % at the LLOQ). After these 3 runs, CV% and RE% were calculated between the series by determining the overall mean ± standard deviation (SD) for each QC level. The acceptance criteria were the same as for within-series acceptance criteria. Additional information on quality control can be found in **Tables 10S–13S** (Supporting Information).

Carry-over. The carry-over of analyte and IS in each analytical run was determined by injecting a blank sample immediately after ULOQ in both sets of calibrators. The mean carry-over in the blank sample from the 2 calibrators sets should not exceed 20 % of the signal of the LLOQ for the analyte and 5 % for the IS [46]. Details of the carry-over assessment of all study compounds can be found in **Tables 14S–17S** (Supporting Information).

Recovery/mass balance of study compounds in the placental perfusion system

The final recovery of each study compound after the perfusion was calculated with the following equations (Eqs. 1 and 2):

$$\text{Final recovery } (\%) = \frac{\left[C_{M,t_{end}} \times V_{M,t_{end}} + C_{F,t_{end}} \times V_{F,t_{end}} + \sum_{t=t_0}^{t_{end}} (C_{S,t} \times V_{S,t})\right]}{C_{M,t_0} \times V_{M,t_0}} \times \frac{100}{100 + (100 - EP)} \quad (1)$$

$$\text{where EP} = \frac{\left[C_{EP,t_{end}} \times V_{EP,t_{end}} + \sum_{t=t_0}^{t_{end}} (C_{S,t} \times V_{S,t})\right]}{C_{EP,t_0} \times V_{EP,t_0}} \times 100 \quad (2)$$

in which $C_{M/F/S}$ is maternal/fetal/sample concentration (ng/mL), $V_{M/F/S}$ is maternal/fetal/sample volume (mL), t_0 and t_{end} are the beginning and end of the perfusion. The final recovery (%) is the sum of amount of study compound in maternal and fetal perfusates at the end of a perfusion, and samples (S) collected in relation to the initial amount of study compound measured in the maternal perfusate, including the mean amount adhered from 3 system adherence tests (empty perfusion, EP, in %).

Fraction unbound and stability assay in placental homogenate

Preparation of placental homogenate. Placental homogenates were prepared on a Precellys 24 Tissue Homogenizer (cycle: 5000 rpm, 2 × 20 sec) in 2 mL tubes containing 1.4 mm zirconium oxide beads (Precellys). To 1 g placental tissue, 4 mL PBS (without Ca^{2+} or Mg^{2+}; Dominique Dutscher) were added, resulting in a 5-fold dilution (v/w). After homogenization, the tubes were centrifuged for 5 min at 4 °C (1000 rpm), and the supernatant was collected. Samples were kept on ice throughout the whole procedure.

Determination of fraction unbound in placental homogenate. Fraction unbound was determined by membrane dialysis on a RED device (ThermoFisher) with membranes of a 6–8 kDA molecular weight cut-off. A 100-fold concentrated DMSO SS of test compounds was added to the placental homogenates, yield-

ing a final concentration of 2 µM of compound and 1 % DMSO. According to manufacturer's instructions, 200 µL of the spiked homogenates were added to the donor chamber, and 350 µL of blank buffer were added to the receiver chamber. Samples were collected after equilibration (240 min on an orbital shaker at 600 rpm, 37 °C). Samples were analyzed by U(H)PLC-MS/MS. Fraction unbound was calculated as follows [47]:

$$\text{Diluted } f_{u,d} = \frac{\text{Receiver Area Ratio}}{\text{Donor Area Ratio}} \quad (3)$$

and

$$\text{Undiluted } f_u = \frac{1/D}{(1/f_{u,d} - 1) + 1/D} \quad (4)$$

where D is the dilution factor of 5 as stated above.

Stability of compounds in placental homogenate. The stability of the compounds was assessed over 360 min (to match the time of placental perfusion experiments) in PBS, PM, and placental homogenates. Homogenates spiked with study compounds were prepared as described above. After compound spiking in the different matrixes, samples were either immediately processed for U(H)PLC-MS/MS analysis (C0) or kept at 4 °C and 37 °C for 360 min on an orbital shaker (600 rpm) before processing for U(H)PLC-MS/MS analysis. Stability was expressed as follows [47]:

$$\text{Stability as \% remaining} = \frac{\text{Area Ratio at 4°C or 37°C at 6h}}{\text{Area Ratio at C0}} \times 100\% \quad (5)$$

Data processing and calculations

Concentrations in placental perfusion profiles (▶ **Fig. 2**) and system adherence (▶ **Fig. 4**) are expressed as a percentage (%) of initial analyzed concentration in the maternal sample at the beginning of the perfusion. Note that the recovery values were calculated differently (see Eqs. 1 and 2).

The FM ratio (Eq. 6; ▶ **Fig. 3**) was calculated for each point and plotted against the perfusion time (min). Glucose consumption (Eq. 7) and lactate production (Eq. 8) are displayed as the sum of changes (from the perfusion beginning [t_0] to the end [t_{end}]) of total content (µmol) in both fetal and maternal circuits, normalized by the total perfusion time (min) and perfused cotyledon weight (W_{cot}; g). The net release rate of placental hormones β-hCG (mU) and leptin (pg) (Eq. 9) during the placental perfusion was normalized by the total perfusion time (min) and perfused cotyledon weight (g) as well.

$$\text{FM ratio, t} = \frac{C_{F,t}}{C_{M,t}} \quad (6)$$

$$\text{Glucose consumption} = \frac{(C_{M,t_0} \times V_{M,t_0} - C_{M,t_{end}} \times V_{M,t_{end}}) + (C_{F,t_0} \times V_{F,t_0} - C_{F,t_{end}} \times V_{F,t_{end}})}{t_{end} \times W_{cot}} \quad (7)$$

$$\text{Lactate production} = \frac{(C_{M,t_{end}} \times V_{M,t_{end}} - C_{M,t_0} \times V_{M,t_0}) + (C_{F,t_{end}} \times V_{F,t_{end}} - C_{F,t_0} \times V_{F,t_0})}{t_{end} \times W_{cot}} \quad (8)$$

$$\text{Hormone production} = \frac{C_{M,t_{end}} \times V_{M,t_{end}} + C_{F,t_{end}} \times V_{F,t_{end}}}{t_{end} \times W_{cot}} \quad (9)$$

Statistical data analysis

For glucose consumption, lactate production, β-hCG, and leptin production, multiple group comparisons were performed using the Brown-Forsythe and Welch ANOVA tests, followed by the Dunnett's T3 multiple comparisons posthoc test (with individual variances computed for each comparison) with GraphPad Prism (version 9.1.0 for macOS; GraphPad Software). Data are expressed as mean ± SD of at least 3 independent experiments (if not otherwise indicated). Probability values $^*p \le 0.05$ were considered statistically significant from the control group.

Supporting Information

Perfusion profiles of all study compounds with absolute concentrations (ng/mL), compound recoveries, fraction unbound of compounds to the placental homogenate, homogenate matrix effects, perfusion profile of antipyrine from control perfusions, characteristics of placentae used, data from individual perfusions in detail, assessment of the suitability of a fetal capillary integrity marker, and details on the U(H)PLC-MS/MS bioanalytical methods are available as Supporting Information.

Contributors' Statement

APSW, MH, and OP designed the study. DS established, validated, and conducted the placental perfusion experiments, performed data analysis/interpretation, and wrote the first complete version of the manuscript under the supervision of APSW. VFA and AC developed and validated the bioanalytical methods, and VFA performed all analyses. MO and AT supervised method development, and AT performed stability testing and determination of fraction unbound. MR performed the histopathological examinations. ED was assisting in placental perfusions with protopine. All authors were involved in data interpretation and reviewing of the manuscript. All authors agreed with the final version.

Acknowledgements

The authors thank the staff of the Maternity Ward of the University Hospital of Zurich and all donating mothers for their cooperation. Alexandra Dolder is gratefully acknowledged for her assistance with the placental perfusion experiments and measuring samples' glucose, lactate, and hormone content. Thanks to Dr. Tina Bürki-Thurnherr and Pius Manser (EMPA, Switzerland) for the support with the placental perfusion model. Financial support was provided by the Swiss National Science Foundation (Sinergia project CRSII5_177260; Herbal Safety in Pregnancy). Other members of the "Herbal Safety in Pregnancy" project are gratefully acknowledged for discussions.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] Gedeon C, Koren G. Designing pregnancy centered medications: Drugs which do not cross the human placenta. Placenta 2006; 27: 861–868

[2] Abduljalil K, Badhan RKS. Drug dosing during pregnancy – opportunities for physiologically based pharmacokinetic models. J Pharmacokinet Pharmacodyn 2020; 47: 319–340

[3] Gupta M, Petsalis M, Powers K, Chen HY, Chauhan SP, Wagner S. Randomized clinical trials in obstetrics-gynecology registered at ClinicalTrials. gov: Characteristics and factors associated with publication. Eur J Obstet Gynecol Reprod Biol 2020; 251: 223–228

[4] Swissmedic. Pregnancy, obstetrics and lactation. Medicines for pregnant and breastfeeding women. 2017. Accessed April 8, 2021 at: https://www.swissmedic.ch/swissmedic/en/home/humanarzneimittel/market-surveillance/fokusthemen/pregnancy-breastfeeding.html

[5] Bornhauser CB, Quack Lötscher KC, Seifert B, Simões-Wüst AP. Diet, medication use and drug intake during pregnancy: Data from the consecutive Swiss Health Surveys of 2007 and 2012. Swiss Med Wkly 2017; 147: w14572

[6] Randecker E, Gantner G, Spiess D, Quack Lötscher KC, Simões-Wüst AP. What pregnant women are taking: Learning from a survey in the Canton of Zurich. 2020. Accessed April 13, 2021 at: https://smw.ch/op-eds/post/what-pregnant-women-are-taking-learning-from-a-survey-in-the-canton-of-zurich

[7] Kennedy DA, Lupattelli A, Koren G, Nordeng H. Herbal medicine use in pregnancy: Results of a multinational study. BMC Complem Altern M 2013; 13: 355

[8] Berger A, Bachmann N, Signorell A, Erdin R, Oelhafen S, Reich O, Cignacco E. Perinatal mental disorders in Switzerland: Prevalence estimates and use of mental-health services. Swiss Med Wkly 2017; 147: w14417

[9] Holst L, Wright D, Haavik S, Nordeng H. Safety and efficacy of herbal remedies in obstetrics – review and clinical implications. Midwifery 2011; 27: 80–86

[10] Rolland A, Fleurentin J, Lanhers MC, Younos C, Misslin R, Mortier F, Pelt JM. Behavioural effects of the American traditional plant *Eschscholzia californica*: Sedative and anxiolytic properties. Planta Med 1991; 57: 212–216

[11] European Medicines Agency. EMA/HMPC/680372/2013 – European Union herbal monograph on *Eschscholzia californica* Cham., herba. 2015. Accessed November 23, 2021 at: https://www.ema.europa.eu/en/documents/herbal-monograph/final-european-union-herbal-monograph-eschscholzia-californica-cham-herba_en.pdf

[12] European Medicines Agency. EMA/HMPC/680375/2013 – Assessment report on *Eschscholzia californica* Cham., herba. 2015. Accessed November 23, 2021 at: https://www.ema.europa.eu/en/documents/herbal-report/final-assessment-report-eschscholzia-californica-cham-herba_en.pdf

[13] Medicinal product information search platform (AIPS). 2021. Accessed December 27, 2021 at: https://www.swissmedicinfo.ch

[14] B'Onaturis. Eschscholtzia BIO. Accessed April 9, 2021 at: https://www.bionaturis.ch/BIO-Kapseln/Eschscholtzia-BIO-225-mg-120-Kapseln.html

[15] Guinaudeau H, Shamma M. The protopine alkaloids. J Nat Prod 1982; 45: 237–246

[16] Guédon D, Cappelaere N, Simanek V. HPLC analysis of the main alkaloids from Eschscholtzia californica Cham. Phytochem Anal 1990; 1: 77–82

[17] Tomè F, Colombo ML, Caldiroli L. A comparative investigation on alkaloid composition in different populations of *Eschscholtzia californica* Cham. Phytochem Anal 1999; 10: 264–267

[18] Fedurco M, Gregorová J, Šebrlová K, Kantorová J, Peš O, Baur R, Sigel E, Táborská E. Modulatory effects of *Eschscholzia californica* alkaloids on recombinant $GABA_A$ receptors. Biochem Res Int 2015; 2015: 617620

[19] Hanus M, Lafon J, Mathieu M. Double-blind, randomised, placebo-controlled study to evaluate the efficacy and safety of a fixed combination containing two plant extracts (*Crataegus oxyacantha* and *Eschscholtzia californica*) and magnesium in mild-to-moderate anxiety disorders. Curr Med Res Opin 2004; 20: 63–71

[20] Kardos J, Blasko G, Simonyi M. Enhancement of gamma-aminobutyric acid receptor binding by protopine-type alkaloids. Arzneimittelforschung 1986; 36: 939–940

[21] Häberlein H, Tschiersch KP, Boonen G, Hiller KO. Chelidonium majus L.: Components with *in vitro* affinity for the $GABA_A$ receptor. Positive cooperation of alkaloids. Planta Med 1996; 62: 227–231

[22] Gafner S, Dietz BM, McPhail KL, Scott IM, Glinski JA, Russell FE, McCollom MM, Budzinski JW, Foster BC, Bergeron C. Alkaloids from *Eschscholzia californica* and Their Capacity to Inhibit Binding of [^{3}H]8-Hydroxy-2-(di-*N*-propylamino)tetralin to $5\text{-}HT_{1A}$ Receptors *in Vitro*. J Nat 2006; 69: 432–435

[23] Xu LF, Chu WJ, Qing XY, Li S, Wang XS, Qing GW, Fei J, Guo LH. Protopine inhibits serotonin transporter and noradrenaline transporter and has the antidepressant-like effect in mice models. Neuropharmacology 2006; 50: 934–940

[24] Ala-Kokko T, Myllynen P, Vähäkangas K. *Ex vivo* perfusion of the human placental cotyledon: Implications for anesthetic pharmacology. Int J Obstet Anesth 2000; 9: 26–38

[25] D'Errico JN, Fournier SB, Stapleton PA. *Ex vivo* perfusion of the rodent placenta. Jove-J Vis Exp 2019; 147: e59412

[26] Goeden N, Bonnin A. *Ex vivo* perfusion of mid-to-late-gestation mouse placenta for maternal-fetal interaction studies during pregnancy. Nat Protoc 2013; 8: 66–74

[27] Leiser R, Kaufmann P. Placental structure: in a comparative aspect. Exp Clin Endocrinol 1994; 102: 122–134

[28] van der Aa EM, Peereboom-Stegeman JHJC, Noordhoek J, Gribnau FWJ, Russel FGM. Mechanisms of drug transfer across the human placenta. Pharm World Sci 1998; 20: 139–148

[29] Myllynen P, Vähäkangas K. Placental transfer and metabolism: An overview of the experimental models utilizing human placental tissue. Toxicol In Vitro 2013; 27: 507–512

[30] Panigel M, Pascaud M, Brun JL. [Radioangiographic study of circulation in the villi and intervillous space of isolated human placental cotyledon kept viable by perfusion]. J Physiol (Paris) 1967; 59: 277

[31] Malek A, Obrist C, Wenzinger S, von Mandach U. The impact of cocaine and heroin on the placental transfer of methadone. Reprod Biol Endocrin 2009; 7: 61

[32] Grafmüller S, Manser P, Krug HF, Wick P, von Mandach U. Determination of the transport rate of xenobiotics and nanomaterials across the placenta using the *ex vivo* human placental perfusion model. Jove-J Vis Exp 2013; 76: e50401

[33] Vähäkangas K, Myllynen P. Experimental methods to study human transplacental exposure to genotoxic agents. Mutat Res 2006; 608: 129–135

[34] Hutson J, Garcia-Bournissen F, Davis A, Koren G. The human placental perfusion model: A systematic review and development of a model to predict *in vivo* transfer of therapeutic drugs. Clin Pharmacol Ther 2011; 90: 67–76

[35] Manda VK, Ibrahim MA, Dale OR, Kumarihamy M, Cutler SJ, Khan IA, Walker LA, Muhammad I, Khan SI. Modulation of CYPs, P-gp, and PXR by Eschscholzia californica (California Poppy) and Its Alkaloids. Planta Med 2016; 82: 551–558

[36] Heikkinen T, Ekblad U, Laine K. Transplacental transfer of citalopram, fluoxetine and their primary demethylated metabolites in isolated perfused human placenta. BJOG 2002; 109: 1003–1008

[37] Myllynen P, Vahakangas K. An examination of whether human placental perfusion allows accurate prediction of placental drug transport: studies with diazepam. J Pharmacol Toxicol 2002; 48: 131–138

[38] Schneider H, Dancis J, Panigel M. Transfer across perfused human placenta of antipyrine, sodium, and leucine. Am J Obstet Gynecol 1972; 114: 822–828

[39] Spiess D, Winker M, Chauveau A, Abegg VF, Potterat O, Hamburger M, Gründemann C, Simões-Wüst AP. Medicinal plants for the treatment of mental diseases in pregnancy: An *in vitro* safety assessment. Planta Med 2021. doi:10.1055/a-1628-8132

[40] Malek A, Sager R, Lang AB, Schneider H. Protein transport across the *in vitro* perfused human placenta. Am J Reprod Immunol 1997; 38: 263–271

[41] Malek A, Willi A, Müller J, Sager R, Hänggi W, Bersinger N. Capacity for hormone production of cultured trophoblast cells obtained from placentae at term and in early pregnancy. J Assist Reprod Gen 2001; 18: 299–304

[42] Mathiesen L, Mose T, Morck TJ, Nielsen JK, Nielsen LK, Maroun LL, Dziegiel MH, Larsen LG, Knudsen LE. Quality assessment of a placental perfusion protocol. Reprod Toxicol 2010; 30: 138–146

[43] Turowski G, Berge L, Helgadottir L, Jacobsen EM, Roald B. A new, clinically oriented, unifying and simple placental classification system. Placenta 2012; 33: 1026–1035

[44] Khong TY, Mooney EE, Ariel I, Balmus NC, Boyd TK, Brundler MA, Derricott H, Evans MJ, Faye-Petersen OM, Gillan JE. Sampling and definitions of placental lesions: Amsterdam placental workshop group consensus statement. Arch Pathol Lab Med 2016; 140: 698–713

[45] U.S. Food and Drug Administration. Bioanalytical method validation: Guidance for industry. 2018. Accessed March 18, 2021 at: https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf

[46] European Medicines Agency. EMA/CHMP/ICH/172948/2019 – ICH guideline M10 on bioanalytical method validation. 2019. Accessed April 28, 2021 at: https://www.ema.europa.eu/en/documents/scientific-guideline/draft-ich-guideline-m10-bioanalytical-method-validation-step-2b_en.pdf

[47] Riccardi K, Ryu S, Tess D, Li R, Luo L, Johnson N, Jordan S, Patel R, Di L. Comparison of fraction unbound between liver homogenate and hepatocytes at 4 °C. AAPS J 2020; 22: 91