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Is Bisphenol S a safer alternative to Bisphenol A in terms of potential fetal exposure ? Placental transfer across the perfused human placenta.

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27 Declaration of interest : None.

28

Abbreviations

BPS : Bisphenol S ; BPSG Bisphenol S glucuronide ; BPA : Bisphenol A ; BPAG : Bisphenol A glucuronide ; LLOQ : lower limit of quantification ; P-gp : P glycoprotein.

29 **Highlights**

30 - The placenta is more efficient to restrict fetal exposure to BPS than to BPA.
31 - Materno-fetal transfer of Bisphenol S glucuronide was almost non-existent.
32 - It is likely that BPS and its metabolite are effluxed out of the fetal
33 compartment.

Abstract

The aim of our study was to evaluate the bidirectional transfer of Bisphenol S (BPS) and its main metabolite, BPS Glucuronide (BPSG), using the model of perfused human placenta and to compare the obtained values with those of Bisphenol A (BPA) and BPA Glucuronide.

Fourteen placentas at term were perfused in an open dual circuit with deuterated BPS (1 and 5 µM) and non-labelled BPSG (2.5 µM) and a freely diffusing marker antipyrine (800 ng/ml) in the presence of albumin (25 mg/ml). In a second experiment, the potential role of P-glycoprotein in the active efflux of BPS across the placental barrier was studied using the well-established P-glycoprotein inhibitor, PSC833 (2 and 4 µM).

Placental transfer of BPS was much lower than that of BPA in both directions. The placental clearance index of BPS in the materno-fetal direction was three times lower than in the opposite direction, strongly suggesting some active efflux transport. However, our results show that P-glycoprotein is not involved in limiting the materno-fetal transfer of BPS. Placental transfer of BPSG in the fetal compartment was almost non-existent indicating that, in the fetal compartment, BPSG originates mainly from feto-placental metabolism. The feto-maternal clearance index for BPSG was 20-fold higher than the materno-fetal index.

We conclude that the blood-placental barrier is much more efficient in limiting fetal exposure to BPS than to BPA, indicating that the placenta has a crucial role in protecting the human fetus from BPS exposure.

Keywords : Bisphenol S, Bisphenol S Glucuronide, human placental transport, endocrine disruptor, Bisphenol A.

1. Introduction

Widespread human exposure to bisphenol A (BPA) and serious concern about its harmful effects on human health have led to the ban or restriction of BPA production and use (ECHA, 2017; French government, 2012; USEPA, 2014), and its replacement by structural analogues. Bisphenol S (BPS), one of the principal BPA-alternative chemicals, is increasingly used for the production of epoxy resins and paper products and as an anticorrosive agent in epoxy glues and a reagent in polymer reactions (Wu et al., 2018). Recent human biomonitoring studies revealed the presence of BPS in 89.4 % of urine samples from the general U.S. population (Lehmler et al., 2018), and in 67.8 % of urine samples from a large population (n=1396) of pregnant women in the Netherlands (Philips et al., 2018). In addition, BPS was found in four maternal (0.03-0.07 ng/ml) and seven cord serum samples (0.03-0.12 ng/ml) from a population of 61 mother-newborn pairs in China, suggesting that BPS can cross the placental barrier (Liu et al., 2017). Due to the structural similarity of BPS to BPA and its extensive use in our daily life, concern about the safety of BPS has become an important issue. Recent *in vitro* and *in vivo* data showed that BPS has equal or even greater toxicological effects, with an endocrine-disrupting effect similar, at least in part, to that of BPA (Eladak et al., 2015; Goldinger et al., 2015; Rochester and Bolden, 2015; Roelofs et al., 2015; Rosenmai et al., 2014; Siracusa et al., 2018). Furthermore, *in vivo* exposure to BPS during development has been reported to disrupt reproductive, metabolic and developmental endpoints through endocrine pathways in zebrafish, rodents and sheep (Hill et al., 2017; Ji et al., 2013; Naderi et al., 2014; Pu et al., 2017; Qiu et al., 2016). This highlights the vulnerability of the developmental period to BPS exposure (Catanese and Vandenberg, 2017; Crump et al., 2016).

84 Data about human fetal exposure to BPS are to date extremely scarce.
85 Toxicokinetic investigations in the pregnant ewe showed that only 0.4 % of the
86 maternal dose was transferred to the fetus. During the late stage of pregnancy, the
87 fetus is very efficient in metabolizing BPS into its main metabolite, BPS glucuronide
88 (BPSG), that remains trapped in the fetal compartment because of its inability to
89 cross the placenta. The elimination of fetal BPSG thus requires its back conversion
90 into bioactive BPS in the feto-placental unit. Furthermore, the restricted feto-maternal
91 placental passage of BPS leads to an accumulation of both BPS and BPSG in the
92 fetal compartment (Grandin et al., 2018). Thus, the placental transfers of BPS and
93 BPSG are crucial in determining exposure of the fetus to BPS and its conjugate.
94 However, the direct extrapolation of animal studies to humans is hindered by
95 structural differences in the placenta between species. This highlights the need to
96 develop models to predict fetal exposure relevant to humans. An *ex vivo* system of
97 perfused isolated human cotyledon (vascular functional unit of the placenta) in an
98 open-circulating system had been used to quantify the transplacental transfer of BPA
99 (Corbel et al., 2014). This relevant model was therefore used in the current study to
100 investigate the placental transfer of BPS and its main metabolite, BPSG.

101 Our first objective in this study was to evaluate the transplacental transfer of
102 BPS and BPSG in both the materno-fetal and feto-maternal directions, using the
103 human perfused cotyledon and to compare these values with those of BPA and BPA
104 glucuronide (BPAG) determined previously with the same model (Corbel et al., 2014).
105 The limited materno-fetal transfer of BPS prompted us to examine, in a second
106 experiment, the possible involvement of an efflux transporter in limiting the materno-
107 fetal placental transfer of BPS. The P-glycoprotein (P-gp or ABCB1), the most
108 abundant member of the ATP-binding cassette protein transporter family, is

109 expressed in the apical membrane of the syncytiotrophoblast, a placental epithelial
110 structure at the interface with maternal blood (Ceckova-Novotna et al., 2006), and is
111 involved in the efflux transport of a wide variety of xenobiotics and thereby in
112 protection of the fetus (Joshi et al., 2016; Syme et al., 2004). Our second objective in
113 this study was therefore to test the hypothesis that P-gp might be involved in limiting
114 BPS materno-fetal transfer (Mölsä et al., 2005; Rahi et al., 2009; Sudhakaran et al.,
115 2005).

116 ## 2. Materials and Methods

117 Placenta cotyledons were perfused in an open double circuit using a method
118 modified from that of Schneider *et al.*, (Schneider et al., 1972) as previously
119 described (Corbel et al., 2014). Placentas (547 ± 132 g) were collected from HIV-
120 seronegative women (age range : 28-42 years) between July and December 2017
121 after uneventful and single pregnancy and after vaginal (n=3) or caesarean (n=7)
122 delivery in the CHU Paule de Viguier, Toulouse, France. The study received
123 institutional approval (DC-2013-1950) and each patient gave written informed
124 consent to participate in the study.

125 ### *2.1. Chemicals*

126 BPS-d4 (purity ≥ 97 %) and BPSG (purity ≥ 97 %) were purchased from
127 Toronto Research Chemicals (Toronto, Canada). BPS was dissolved in ethanol at a
128 concentration of 1 mg/ml and BPSG was dissolved in isotonic saline at 10 mg/ml.
129 Antipyrine and PSC833 (purity ≥ 98 %) were purchased from Sigma-Aldrich (Saint-
130 Quentin Fallavier, France) and were dissolved at 10 mg/ml and 4.9 mg/ml in
131 apyrogenic water and DMSO, respectively. All solutions were stored at -20°C until

132 use. Bovine serum albumine (BSA, Fraction V, purity ≥ 96 %) and Earle's Balanced
133 Salt solution were purchased from Euromedex (Souffelweyersheim, France).

134 All materials for placenta perfusion, including the materials used for the
135 preparation of solutions, sampling, processing and analysis, were in glass or BPS-
136 free plastic.

137 ***2.2. Placental perfusion***

138 The study was designed to evaluate the simultaneous placental transfer of
139 BPS and BPSG, both in the materno-fetal and feto-maternal directions (Figure 1).
140 The maternal and fetal perfusion solutions were prepared with Earle medium
141 supplemented with 25 g/L of BSA to reflect the physiological plasma protein
142 concentration at late pregnancy (Larsson et al., 2008). BPS was used in deuterated
143 form (BPS-d4) in order to distinguish the d4-labelled BPS added to the media from
144 non-labelled BPS potentially resulting from BPSG hydrolysis.

145 Antipyrine (800 ng/ml), a free passive diffusion reference substance which is
146 not bound to plasma proteins, and the test substances, BPS-d4 and BPSG, were
147 added to the maternal or to the fetal reservoir to study the materno-fetal or feto-
148 maternal transfers, respectively. BPS-d4 was infused at 1270 ng/ml (5 µM) and 254
149 ng/ml (1 µM) in the maternal and fetal medium, respectively, while BPSG was infused
150 at the same concentration (1065 ng/ml, 2.5 µM) in both compartments (Figure 1).
151 Because a low BPS placental transfer was previously observed in the pregnant
152 sheep model (Grandin et al., 2018), high nominal concentrations of both BPS-d4 and
153 BPSG were used to ensure their effective detection in the opposite compartment
154 after placental transfer.

155 Each perfusion lasted 90 min. The pH was adjusted continuously throughout
156 the perfusion to 7.41 ± 0.013 and 7.31 ± 0.011 for the maternal and fetal perfusion
157 media, respectively. Maternal and fetal exudates were collected every 5 min after
158 adding the test substances and the volumes were measured. BPS-d4 and BPSG
159 materno-fetal (feto-maternal) transfers were determined after simultaneously adding
160 antipyrine, BPS-d4 and BPSG to the maternal (fetal) reservoir at time 0. Control
161 samples (1 ml) were collected from the fetal and maternal inflow reservoirs before
162 addition of these substances. Samples were then collected from the maternal (fetal)
163 inflow reservoir immediately after adding the test molecules and at 30, 60 and 90
164 min, from the maternal (fetal) outflow perfusate at 0, 30, 60 and 90 min, and from the
165 fetal (maternal) outflow perfusate at time 0 and every 5 min up to 90 min, to evaluate
166 the materno-fetal (feto-maternal) transfer~~)~~.

167 At the end of the perfusion, the isolated cotyledon was rinsed with phosphate-
168 buffered saline (pH 7.4) at 4°C for 20 min. This washing solution was collected. All
169 samples were immediately chilled in ice and centrifuged for 10 min at 3000*g* and 4°C
170 to discard placental cells and the supernatant was collected and stored at -20°C until
171 assayed.

172 The involvement of P-gp, in limiting the materno-fetal clearance of BPS, was
173 determined by measuring the materno-fetal transfer of BPS-d4 before and after the
174 addition of a P-gp inhibitor. PSC833 was selected because it is a specific inhibitor of
175 P-gp efflux protein and its inhibitory effect during *ex vivo* placental open perfusion
176 had already been observed within the range of concentrations used in this study
177 (Rahi et al., 2007; Sudhakaran et al., 2008). It was added to the maternal reservoir
178 after 40 min of perfusion. First, the placental passage of BPS-d4 and BPSG was
179 evaluated before and after the addition of PSC833, at a concentration of 2µM (one

180 placenta). As the materno-fetal transfers of BPS-d4 and BPSG were not affected by
181 this inhibitor under these conditions, the experiment was repeated with a higher
182 concentration of PSC833, 4µM, and only with BPS-d4.

183 Placental samples (about 1 g) were taken from a non-perfused cotyledon and
184 from the perfused cotyledon (white area) after the washing perfusion and stored at -
185 80°C, to determine the tissue concentrations of BPS and BPSG.

186 ***2.3. Placental tissue homogenization***

187 Placental tissue was reduced to 1 to 9 mm^2 fragments and aliquoted into 100
188 mg-fractions. These fractions were then ground for 4 min at 30 Hz using a vibrating
189 tissue homogenizer (MM 400, Retsch, Eragny sur Oise, France) in liquid nitrogen-
190 refreshed containers to avoid sample defrosting. Samples were stored at -80°C until
191 assayed.

192 The extent of potential BPSG hydrolysis during the homogenization step was
193 determined, in a preliminary experiment, by processing 5 blank liver samples spiked
194 with BPSG-d8. The mean hydrolysis rate of BPSG-d8 after the tissue
195 homogenization procedure was 1.27 ± 0.24 % and was therefore considered
196 negligible. This tissue homogenization procedure was then applied to placenta
197 samples.

198 ***2.4. Analytical procedure***

199 BPS-d4, BPSG and antipyrine were extracted from the perfusion medium and
200 placental tissue by adding 200 µL of an acetonitrile/zinc sulfate mixture (50:50,
201 vol:vol) containing internal standards (BPS at 50 ng/ml, BPSG-d8 and antipyrine-d3
202 at 500 ng/ml in perfusion medium and BPS-d4 at 100 ng/mL and BPSG-d8 at 1000
203 ng/mL in placental tissue). Samples were mixed for 5 min at 10°C and 1400 rpm

204 (MB-102, Bioer, Hangzhou, China) and then centrifuged at 20000*g* and 4°C for 20
205 min.

206 Antipyrine, BPS-d4 and BPSG concentrations in the perfusion medium and in
207 placental tissue were determined by liquid chromatography coupled with tandem
208 mass spectrometry detection using a previously described adapted method (Grandin
209 et al., 2017). In the perfusion medium, the calibration curves ranged from 10 to 1000
210 ng/ml, 1 to 100 ng/ml and 5 to 1000 ng/ml, for antipyrine, BPS and BPSG,
211 respectively. In placental tissue, the calibration curves ranged from 5 to 1000 ng/ml
212 for both BPS and BPSG. The mean intra- and inter-day coefficients of variation for
213 the three concentration levels were below 15 %. In perfusion medium, the lower limits
214 of quantification (LLOQs) were validated at 10 ng/ml for antipyrine, 1 ng/ml for BPS-
215 d4 and 5 ng/ml for BPSG. In placental tissue, the LLOQs were validated at 5 ng/g for
216 both BPS and BPSG.

217 BPS (but not BPS-d4) and BPSG were measured in cotyledon samples
218 (around 100 mg) before and after perfusion.

219 Because of the very low passage of BPSG in the materno-fetal direction, an
220 analytical method using dansyl chloride derivation, which enabled a higher sensitivity
221 (LLOQ at 0.25 ng/ml) to be obtained (Wang et al., 2013), was developed to measure
222 BPSG in the fetal outflow perfusate.

223 ***2.5. Calculation method and statistical analysis***

224 Only the concentrations at steady-state for antipyrine, BPS-d4 and BPSG,
225 were used to calculate the transfer rate, the extraction rate, the placental clearance
226 and the clearance index, according to the Challier formulas (Challier, 1985; Challier
227 et al., 1977), as previously described (Corbel et al., 2014). The transfer rate was

228 calculated for each steady state time point as the ratio between the concentrations of
229 BPS/BPSG in the receiving compartment to the concentration in the entrance
230 compartment expressed as a percentage. The clearance index was the ratio of the
231 transfer rate of BPS or BPSG to that of antipyrine. The transplacental clearance of
232 BPS/BPSG was calculated as the ratio of the concentration in the receiving
233 compartment multiplied by its flow rate to the concentration in the entrance
234 compartment. The extraction rate was the fraction of the dose (corresponding to the
235 concentration multiplied by its flow rate) transferred from the entrance compartment
236 to the receiving one (Figure 1). In the materno-fetal direction, the perfusions were
237 validated if the rate of antipyrine transfer was above the generally accepted threshold
238 of 20 % (Gavard et al., 2009), whereas a threshold antipyrine transfer rate higher
239 than the 15 %, previously reported by Challier et al. (1977), was chosen to validate
240 the feto-maternal perfusions.

241 The mass balance was calculated as the ratio of the sum of the quantities of
242 substrate in the perfusion media, the tissue (for BPSG only) and the PBS washings to
243 the measured amount of the substrate in the maternal or fetal reservoir, as previously
244 described (Corbel et al., 2014).

245 Data are expressed as means (± SD). The statistical analyses were done
246 using the R®software (R development core team, 2005).

247 For both the feto-maternal and materno-fetal transfers, the mean placental
248 transfers of BPS, BPSG and antipyrine at each time point along the experiment were
249 compared by a three-way ANOVA with the substrate and the perfusion time as fixed
250 effect factors. The placenta and the double interactions between substrate, perfusion
251 time and placenta were random-effect factors.

252 For each substance, the materno-fetal and feto-maternal clearance indices
253 were compared using a three-way ANOVA with the direction (materno-fetal and feto-
254 maternal), the substrate and their interaction as fixed-effect factors and the placenta
255 nested in the direction as a random-effect factor.

256 The BPS clearance indices in absence or presence of the P-gp inhibitor were
257 compared using a three-way ANOVA with the inhibitor, the perfusion time and their
258 interaction as fixed-effect factors, and with the placenta and the perfusion time
259 nested in the inhibitor as random-effect factors.

260 ## 3. Results

261 The overall mean transfer rates of antipyrine, for all the validated perfusions
262 (n=10), were 34.2 ± 10.1 % and 20.7 ± 4.84 % in the materno-fetal and feto-maternal
263 directions, respectively. The wet weight of perfused cotyledons was 29.5 ± 17.2 g.
264 The average flow rates were 12.7 ± 1.2 ml/min in the maternal circulation and 5.3 ±
265 0.46 ml/min in the fetal circulation and remained stable throughout the perfusion.

266 Before perfusion, BPS was quantified in two placentas out of nine, at
267 concentrations close to the BPS LLOQ (6.66 and 5.90 ng/g), which probably reflect
268 the environmental exposure to BPS, whereas no BPSG was detected in any
269 placenta. BPS-d4, BPSG and antipyrine were never detected in control perfusion
270 medium samples collected in the maternal and fetal reservoirs before the beginning
271 of the perfusion.

272 For all 10 placentas, the overall mean recovery (± SD) of infused substances
273 at the end of the perfusion, in the fetal and maternal perfusates and in the cotyledon
274 (BPSG only) were 85.8 ± 6.03 % (range 75.2-92.9 %) and 84.7 ± 5.12 % (range 79.9-
275 90.1 %) for BPS-d4 perfusion and 90.2 ± 9.54 % (range 78.3-99.2 %) and 81.6 ± 1.22

276 % (range 80.6-83.0 %) for BPSG perfusions in the materno-fetal and in the feto-
277 maternal directions, respectively.

278 BPS was never detected in any perfusate following BPSG perfusion in either
279 the feto-maternal or materno-fetal directions.

280 ***3.1. Placental transfer of BPS and BPSG***

281 Figure 2A depicts the mean (± SD) time courses of the materno-fetal placental
282 transfer of BPS-d4, BPSG and antipyrine at the concentrations of 1270 ng/ml,
283 1065 ng/ml and 800 ng/ml (n=7), respectively. The materno-fetal transfer rates (mean
284 ± SD, range) of BPS (3.18 ± 2.64 %, 0.527-8.12 %, n=7) and BPSG (0.339 ±
285 0.264 %, 0.113-0.717 %, n=4) were about 10-fold lower for BPS and 100-fold lower
286 for BPSG than the transfer of freely diffusible antipyrine (34.2 ± 10.1 %, 23.8-52.4 %,
287 n=7, $p < 0.001$, Table 1). The mean ± SD (range) materno-fetal clearances were
288 0.174 ± 0.151 ml/min (0.0239-0.450 ml/min) and 0.0181 ± 0.0149 ml/min (0.006-
289 0.0398 ml/min) for BPS and BPSG, respectively. In the materno-fetal experiment,
290 2.49 ± 2.36 % and 0.228 ± 0.221 %, respectively, of the BPS-d4 and the BPSG
291 added to the maternal perfusate, were transferred across the placental barrier. A low
292 fraction of the BPSG dose (0.112 ± 0.0754 %, ranging from 0.0489 to 0.216 %)
293 remained in the cotyledon after BPSG perfusion.

294 Figure 2B shows the time-courses (mean ± SD) for the feto-maternal placental
295 transfer of BPS-d4, BPSG and antipyrine. The feto-maternal transfer rates (mean ±
296 SD, range) of BPS (5.85 ± 1.22 %, 4.73-7.15 %, n=3) and BPSG (3.97 ± 0.919 %,
297 3.02-4.85 %, n=3) were respectively about 3.5-fold and 5-fold lower than that of
298 antipyrine (20.7 ± 4.84 %, 15.2-24.3 %, n=3, $p < 0.001$) (Table 1). The mean ± SD
299 (range) feto-maternal clearances were 0.717 ± 0.144 ml/min (0.584-0.870 ml/min) for
300 BPS and 0.487 ± 0.108 ml/min (0.374-0.590 ml/min) for BPSG. In the feto-maternal

experiment, 4.46 ± 0.702 % of BPS and 13.2 ± 2.60 % of BPSG added to the fetal perfusate were transferred across the placental barrier. Only 0.360 ± 0.125 % (ranging from 0.244 to 0.492 %) of the BPSG dose remained in the perfused cotyledon.

The overall clearance indices of BPS and BPSG were much lower in the materno-fetal direction than in the opposite direction (3.4 and 23-fold, respectively, $p < 0.001$) (Table 1).

3.2. Effect of PSC833, a P-gp inhibitor, on the materno-fetal placental transfer of BPS

The materno-fetal rate of BPS transfer (2.56 ± 2.36 versus 2.30 ± 2.08 %, after and before the addition of PSC833, respectively), and consequently, its transplacental clearance (0.146 ± 0.137 versus 0.131 ± 0.126 ml/min) and clearance index (0.073 ± 0.051 versus 0.0717 ± 0.0543) were not significantly increased in the presence of PSC833, an inhibitor of P-gp (Figure 3). In addition, PSC833 had no significant effect on the transplacental transfer of antipyrine (31.7 ± 6.9 versus 29.5 ± 4.94 %).

4. Discussion

Fetal life represents a critical window during which its developmental processes are extremely sensitive to endocrine disruption. The lack of data concerning exposure of the human fetus to BPS, a potential endocrine disruptor, highlights the urgent need to investigate the materno-feto-placental exchanges of BPS. In the present study, the open-circuit perfused cotyledon model was used to examine the passage of BPS and its main metabolite BPSG across the human placenta.

325 Antipyrine is a useful reference substance for assessing the transfer rate of
326 lipid-soluble xenobiotics (Challier et al., 1983; Schneider et al., 1979). The rates of
327 antipyrine transfer observed in this study, were similar to the values reported in
328 previous studies (Berveiller et al., 2012; Ceccaldi et al., 2008; Corbel et al., 2014;
329 Gavard et al., 2009). By expressing BPS clearance as a fraction of antipyrine
330 clearance (clearance index), several hemodynamic factors in the perfusion system
331 can be corrected, thus allowing comparisons between different substrates.

332 The results reported here show that the placental passage of BPS is very
333 limited, with a feto-maternal BPS clearance index 3.4-fold higher than the materno-
334 fetal clearance index (0.290 and 0.0852, respectively). Despite the structural
335 similarities of BPS and BPA, these BPS clearance indices were about 3 and 10-fold
336 lower than those of BPA (BPA feto-maternal clearance index = 0.81, BPA materno-
337 fetal clearance index = 0.84) (Corbel et al., 2014). These observations suggest that
338 the efficiency of BPS transport differed from that of BPA which crosses the placenta
339 by passive diffusion (Corbel et al., 2014). It can be hypothesized that BPS placental
340 transport does not rely solely on weak diffusional permeability but might involve a
341 differentially active transport.

342 The main physico-chemical properties that determine the placental
343 permeability of a substance are lipophilicity, polarity, size and hydrogen-binding
344 capacity (Giaginis et al., 2009). The molecular weights of BPA and BPS are similar
345 (228 and 250, respectively). Moreover, BPA is a highly lipid soluble molecule (LogP
346 3.43) that is unionized at physiological pH. Its placental transfer by passive diffusion
347 is therefore rapid and solely limited by the placenta perfusion rate, independently of
348 placental permeability. In contrast, the lipid solubility of BPS is low (LogP 1.65) and
349 the ionized and unionized forms are in equilibrium at physiological pH (pKa1 = 7.42,

350 pKa2 = 8.03, Choi and Lee, 2017). Thus, the low placental permeability of BPS
351 compared to BPA could be due to its relative polarity and to its low lipophilicity
352 (Garland, 1998; Syme et al., 2004).

353 Alternatively, the different materno-fetal and feto-maternal transfer of BPS
354 across the placenta might be explained by the activity of an unidirectional efflux
355 transporter. We hypothesized that the placental efflux transporter P-gp could be
356 involved in the limited transfer of BPS to the fetal circulation. Indeed, P-gp facilitates
357 the active efflux of a wide range of xenobiotics on the placental barrier (Ceckova-
358 Novotna et al., 2006). Inter-individual variability in the expression of P-gp (Hutson et
359 al., 2010; Pollex and Hutson, 2011) was taken into account by conducting the kinetic
360 studies such that each placenta was used as its own control, by adding the P-gp non-
361 competitive inhibitor, PSC833, at the middle time of the perfusion. PSC833
362 (valspodar), a cyclosporine D analog, has been shown to be one of the most potent
363 and least toxic P-gp inhibitors (Friedenberg et al., 2006; May et al., 2008; Mölsä et
364 al., 2005; Rahi et al., 2007; Sudhakaran et al., 2008). Despite these controlled
365 experimental conditions, our data did not support the role of P-gp in the placental
366 efflux of maternal BPS. However, we cannot rule out the impact of P-gp on the feto-
367 maternal clearance of BPS or the involvement of other efflux transporters on the
368 active efflux of BPS, such as the multidrug-resistance-associated proteins and breast
369 cancer-resistance proteins, located in the maternal-facing brush border membrane.
370 Further investigations are needed to identify the specific transporters responsible for
371 protecting the fetus from exposure to BPS.

372 Taking into account the placental blood flows reported in the literature
373 (Ferrazzi et al., 2001; Syme et al., 2004), the estimated maternal and fetal BPS
374 placental clearances were 8.72 and 37.3 mL/min, respectively. The estimated human

375 maternal placental clearance of BPS is close to that previously evaluated *in vivo* in
376 the pregnant ewe (5.22 mL/min, Grandin et al., 2018). By contrast, the placental
377 clearance of BPS by the human fetus, which represents only about 12 % of the
378 umbilical blood flow at term (Ferrazzi et al., 2001), is 8-fold higher than that estimated
379 on the pregnant ewe (4.58 mL/min, corresponding to only 0.5 % of the umbilical
380 blood flow) (Grandin et al., 2018). The lower fetal placental clearance in ovines
381 compared to humans might be explained by a more limited placental permeability in
382 sheep due to inter-species differences in placental structure and transport protein
383 expression (Ceckova-Novotna et al., 2006; Joshi et al., 2016).

384 In the pregnant ewe *in vivo*, once BPSG attained the fetal compartment, it
385 remains trapped and can be reactivated into BPS (Grandin et al., 2018).
386 Characterizing the mechanisms underlying fetal exposure to BPSG is therefore
387 critical. Our results using the human placental perfusion model indicated that the
388 materno-fetal passage of BPSG is almost non-existent (mean BPSG materno-fetal
389 clearance index of 0.0085). This result is consistent with the absence of BPSG
390 transfer from mother to fetus demonstrated in the *in vivo* pregnant sheep model
391 (Grandin et al., 2018). Therefore, the BPSG found in the fetal compartment *in vivo*
392 might likely be of feto-placental origin. Moreover, the placental transfer of BPSG in
393 the feto-maternal direction was about two-fold lower than that of BPAG (Corbel et al.,
394 2014), consistently with the higher hydrophilicity of BPSG (LogP = -0.33) than that of
395 BPAG (LogP = 1.12). Furthermore, the differential transfer of BPSG, twenty fold
396 higher in the feto-maternal direction than in the opposite direction, suggests the
397 involvement of a transport system that restricts the fetal exposure to BPSG. Several
398 ATP-dependent transporters, including multi-drug resistance proteins 1 and 2 (MRP-
399 1, MRP-2) and Organic Anion Transporting Polypeptide transporters (OATP),

400 predominantly expressed in the syncytiotrophoblast, are known to transport
401 glucuronide conjugates (Vähäkangas and Myllynen, 2009).

402 All together, our data suggest that fetal exposure to BPS and to BPSG could
403 be more limited than to BPA and BPAG for the same maternal exposure, suggesting
404 that the human fetus might be better protected from exposure to BPS than to BPA. In
405 agreement with this result, a biomonitoring study (Ihde et al., 2018) including 30
406 maternal/fetal pairs in the USA, showed that BPS was never detected in the fetal
407 cord blood, even though 60 % of the mothers were exposed to BPS. However, our
408 data need to be treated with caution, because this human isolated cotyledon model,
409 unlike the *in vivo* sheep model, does not incorporate the nonplacental toxicokinetic
410 factors that determine fetal exposure, such as maternal and fetal BPS metabolism
411 and relative maternal and fetal BPS protein binding. Another limitation is that the
412 present results refer to placenta at term and cannot necessarily be extrapolated to
413 earlier stages of gestation. Indeed, the placenta undergoes considerable changes
414 throughout gestation (Syme et al., 2004). At term, the placental transfer layer is at its
415 thinnest. In addition, the expression of certain efflux transporters, such as P-gp, is
416 modified throughout gestation (Ceckova-Novotna et al., 2006; Joshi et al., 2016). All
417 these physiological processes that determine fetal exposure will therefore require
418 further investigations to be able to predict fetal exposure to BPS and to BPSG.

419 ## 5. Conclusions

420 In conclusion, this is the first study to document the placental transports of both BPS
421 and its main metabolite, BPSG, and to compare them with those of BPA and BPAG,
422 using the human placental perfusion model. BPS was shown to cross placenta
423 bidirectionally, but to a much lower extent than BPA, particularly in the materno-fetal
424 direction. This suggests the involvement of a membrane efflux transporter which

425 impedes BPS transfer to the fetus. However, the implication of placental P-gp as a
426 possible contributor to the low materno-fetal placental transfer of BPS could not be
427 demonstrated, suggesting that BPS is probably effluxed by another transporter.

428 Although BPSG could be detected *in vivo* in the fetal compartment, our results
429 indicate that materno-fetal transfer of BPSG is almost non-existent. This suggests
430 that fetal exposure to the BPS conjugate could result from fetal metabolism, at least
431 during late gestation. Once in the fetal compartment, it is likely that BPS and BPSG
432 are extruded, albeit slowly, since the BPS and BPSG feto-maternal clearance indices
433 were considerably higher than the corresponding materno-fetal clearance indices. All
434 together, our data clearly show that fetal exposure to BPS is more efficiently limited
435 by the blood-placental barrier than fetal exposure to BPA. Nevertheless, all the
436 toxicokinetic factors, *i.e.* placental and non placental, determining fetal exposure will
437 need to be considered to ensure a meaningful evaluation of the risk of fetal exposure
438 to BPS in humans and to determine whether the human fetus is better protected from
439 exposure to BPS than to BPA.

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447 ***Competing financial interests***

448 The authors declare no conflicts of interest.

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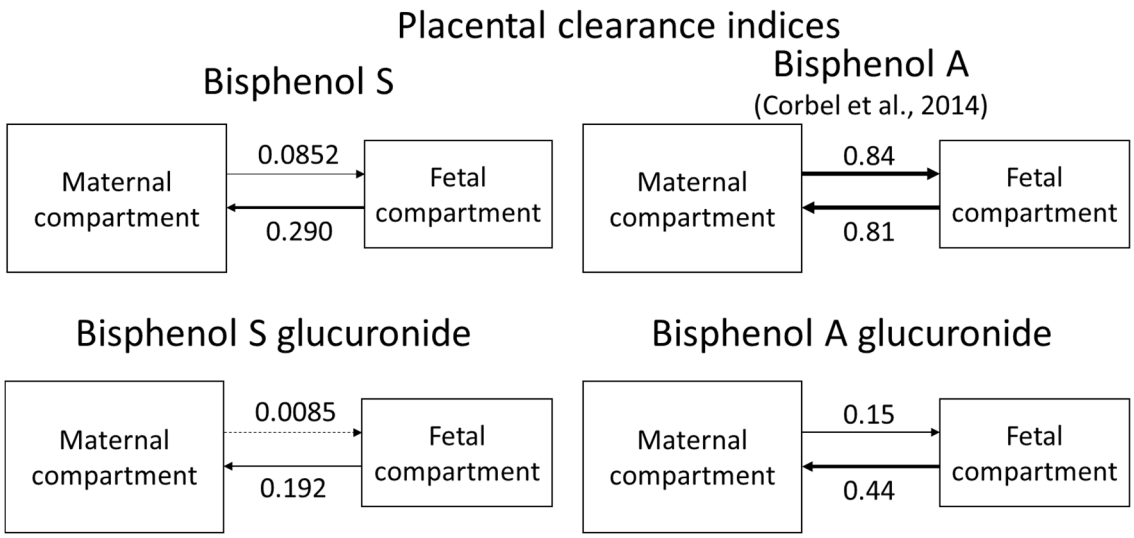
643 **Graphical abstract**



644

645 **Figure legends**

646 Figure 1 : Two-compartment model of the open-configuration double circuit placental
647 perfusion used to evaluate (A) the materno-fetal transfer (TR_{MF}) and (B) the feto-
648 maternal transfer (TR_{FM}) of antipyrine (800 ng/mL), BPS-d4 (1270 ng/mL, 5µM or
649 254 ng/mL, 1µM) and BPSG (1065 ng/mL, 2.5 µM). C_{output} and $C_{output'}$ were the
650 maternal and fetal outflow concentrations, respectively. Q_M and Q_F were the maternal
651 and fetal system flow rates, respectively.

652

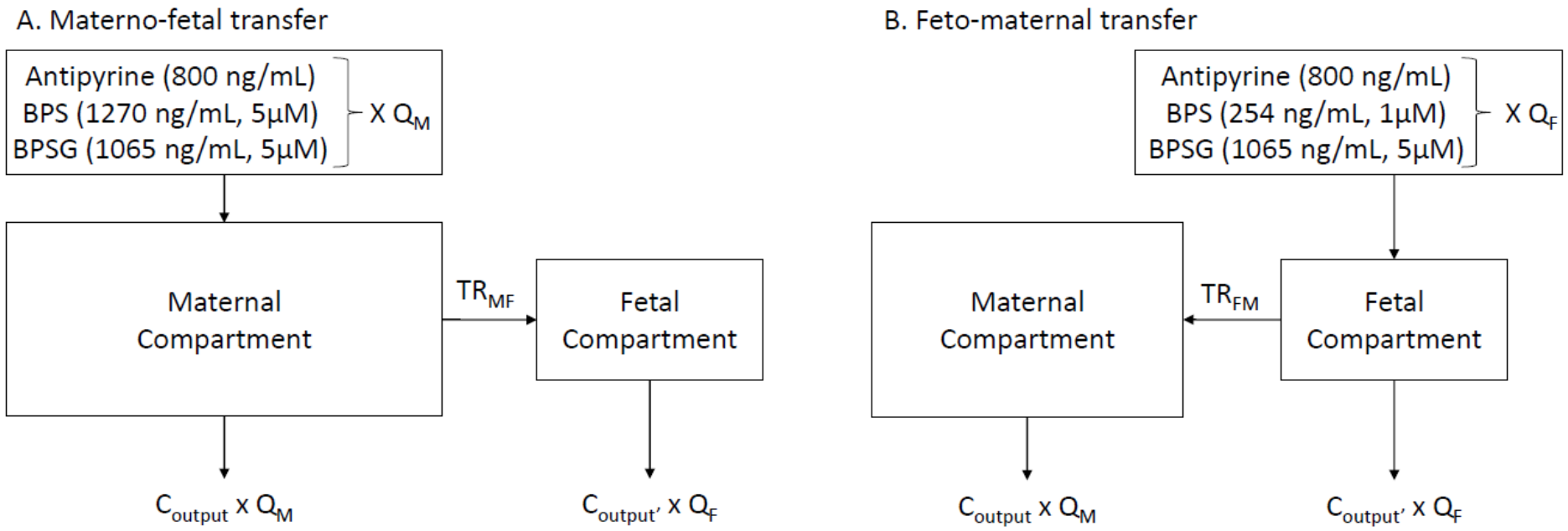

A. Materno-fetal transfer
Antipyrine (800 ng/mL)
BPS (1270 ng/mL, 5µM)
BPSG (1065 ng/mL, 5µM)
X Q_M
Maternal Compartment
TR_{MF}
Fetal Compartment
C_{output} x Q_M
$C_{output'}$ x Q_F
B. Feto-maternal transfer
Antipyrine (800 ng/mL)
BPS (254 ng/mL, 1µM)
BPSG (1065 ng/mL, 5µM)
X Q_F
Maternal Compartment
TR_{FM}
Fetal Compartment
C_{output} x Q_M
$C_{output'}$ x Q_F

Figure 2 : Time courses of the mean (± SD) materno-fetal (A, n=7) and feto-maternal (B, n=3) transfer rates of antipyrine (○, left y axis), BPS-d4 (●) and BPSG (Δ) (right y axis) during the perfusion.

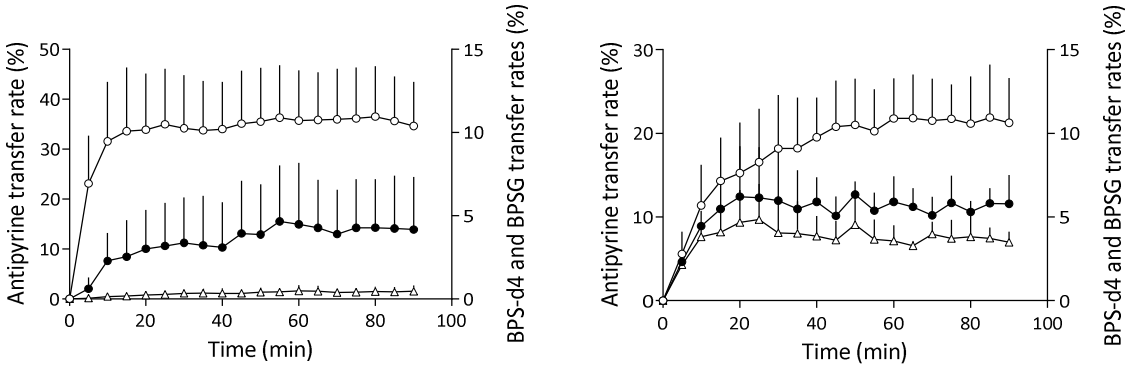
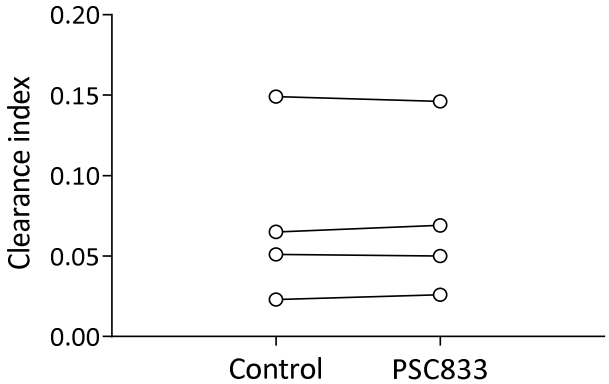


Figure 3 : Mean materno-fetal placental clearance indices of BPS before (control condition) and after addition of the P-glycoprotein inhibitor, PSC833. The lines join the data from the same placenta (n=1 at 2 µM and n=3 at 4 µM of PSC833).



Table

Table 1 : Materno-fetal and feto-maternal BPS and BPSG perfusion parameters.

Direction	Placenta	Transfer rate (%) [1]			Clearance index[2]		Placental clearance (ml/min) [3]		
		Antipyrine	BPS	BPSG	BPS	BPSG	Antipyrine	BPS	BPSG
Materno-	Mean	34.2	3.18	0.339	0.0852	0.00852	1.83	0.174	0.0181
fetal	SD	10.1	2.64	0.264	0.0515	0.00479	0.619	0.151	0.0149
Feto-	Mean	20.7	5.85	3.97	0.290	0.196	2.53	0.717	0.487
maternal	SD	4.84	1.22	0.919	0.0469	0.0247	0.579	0.144	0.108

[1]The ratio of the concentration in the receiving compartment to the concentration in the entrance compartment expressed as a percentage. [2]The molecule-studied transfer rate divided by the antipyrine transfer rate. [3]The concentration in the receiving compartment multiplied by its flow rate to the concentration in the entrance compartment.