1 **Just Accepted**

2 This article has been peer reviewed and accepted for publication. It is in production
3 and has not been edited, so may differ from the final published form.

4 Endogenous lysophosphatidic acid participates in 5 vascularization and decidualization at the maternal- 6 fetal interface in the rat

7 Micaela Sordelli , Jimena Beltrame , Elsa Zotta , Natalia Gomez , Ganna Dmytrenko ,
8 Maria Sales , Sandra Blois , Carlos Davio , SilvinaPerez
9 Martinez , Ana Franchi , Maria Ribeiro

10 Abstract

11 Lysophosphatidic acid (LPA) influences several female reproductive functions through
12 G protein-coupled receptors. LPA contributes to embryo implantation via
13 lysophosphatidic type 3 receptor (LPA3). In this study, we investigated the participation
14 of endogenous LPA signaling through LPA3 in vascularization and decidualization, two
15 crucial events at the maternal-fetal interface. Pregnant rats were treated with
16 diacylglycerol pyrophosphate (DGPP), a highly selective antagonist of LPA3, in day 5
17 of gestation. DGPP treatment produced aberrant embryo spacing and increased
18 embryo resorption. Also, LPA3 antagonist decreased the cross sectional length of the
19 uterine and arcuate arteries and induced histological anomalies in the decidua and
20 placentas. Marked hemorrhagic processes, infiltration of immune cells and tissue
21 disorganization were observed in the decidual and placental tissues from resorpted
22 sites. The mRNA expression of interleukin 10 (Il-10), vascular endothelial growth factor
23 (Vegf-a) and vascular endothelial growth factor receptor 1 (Vegf-r1), three
24 vascularization markers, was reduced in resorpted sites from day 8. Our results show
25 that the disruption of endogenous LPA signaling by blocking LPA3 modified the
26 development of uterine vessels with consequences in the formation of the decidua and
27 placenta and in the growth of the embryos.

28 RD16235 Accepted 12 January 2017

29 © CSIRO 2017

30

31

32

33 **Endogenous lysophosphatidic acid participates in vascularization and decidualization**

34 **at the maternal-fetal interfase in the rat.**

35 Micaela S Sordelli[1*], Jimena S Beltrame[1*], Elsa Zotta[2], Natalia Gomez[3], Ganna Dmytrenko[4],

36 María Elena Sales[4], Sandra M Blois[5], Carlos Davio[3], Silvina Perez Martinez[6], Ana M Franchi[7],

37 María L Ribeiro[1].

38 [1]Laboratorio de Fisiología y Farmacología de la Reproducción, CEFYBO (CONICET –

39 Facultad de Medicina, UBA), Buenos Aires, Argentina, [2]Laboratorio de Fisiopatología

40 Molecular, Cátedra de Fisiopatología, Departamento de Cs. Biológicas, Facultad de

41 Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina, [3]Laboratorio

42 de Farmacología de Receptores, Departamento de Farmacología, Facultad de Farmacia y

43 Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina, [4]Laboratorio de

44 Inmunofarmacología Tumoral, CEFYBO (CONICET – Facultad de Medicina, UBA), [5]Charité

45 Centrum 12 für Innere Medizin und Dermatologie, Reproductive Medicine Research Group,

46 University Medicine of Berlin, Berlin, Germany, [6]Laboratorio de Biología de la Reproducción

47 en Mamíferos, CEFYBO (CONICET – Facultad de Medicina, UBA), Buenos Aires, Argentina,

48 [7]Laboratorio de Fisiopatología de la Preñez y al Parto, CEFYBO (CONICET – Facultad de

49 Medicina, UBA), Buenos Aires, Argentina.

50 ***Both authors contributed equally to this manuscript.**

51 **Corresponding author:** Micaela S Sordelli, Paraguay 2155, 16th floor, CEFYBO (CONICET-

52 School of Medicine, University of Buenos Aires), Phone Number: 54 11 4962 4431 ext: 113,

53 Fax: 54 11 4508 3680 ext: 106, e-mail: micaelasordelli@yahoo.com.ar.

54 **Running Title:** LPA in decidual and vascular remodeling.

55

56 **Abstract.**

Lysophosphatidic acid (LPA) influences several female reproductive functions through G protein-coupled receptors. LPA contributes to embryo implantation via lysophosphatidic type 3 receptor (LPA3). In this study, we investigated the participation of endogenous LPA signaling through LPA3 in vascularization and decidualization, two crucial events at the maternal-fetal interfase. Pregnant rats were treated with diacylglycerol pyrophosphate (DGPP), a highly selective antagonist of LPA3, in day 5 of gestation. DGPP treatment provoked aberrant embryo spacing and increased embryo resorption. Also, LPA3 antagonist decreased the cross sectional length of the uterine and arcuate arteries and induced histological anomalies in the decidua and placentas. Marked hemorrhagic processes, infiltration of immune cells and tissue disorganization were observed in the decidual and placental tissues from resorpted sites. The mRNA expression of interleukin 10 (*Il-10*), vascular endothelial growth factor (*Vegf-a*) and vascular endothelial growth factor receptor 1 (*Vegf-r1*), three vascularization markers, was reduced in resorpted sites from day 8. Our results show that the disruption of endogenous LPA signaling by blocking LPA3 modified the development of uterine vessels with consequences in the formation of the decidua and placenta and in the growth of the embryos.

74 **Introduction.**

75 The implantation of the blastocyst into the uterus initiates a series of events like
76 decidualization and vascular remodeling, fundamental for placentation and pregnancy
77 success. The coordination of vascular processes at the maternal–fetal interfase ensures an
78 adequate blood flow in response to the increasing metabolic demands of the embryo (Torry
79 *et al*. 2007, Shimizu *et al*. 2012). Blood vessels development is crucial during inflammation
80 under pathological conditions like septic shock (de la Torre *et al*. 2013), and in the
81 physiological step of implantation (Demir *et al*. 2010). This process involves the action of
82 several growth factors, cytokines and chemokines (Blois *et al*. 2011). Decidualization implies
83 proliferation and differentiation of endometrial stromal fibroblasts into decidual cells. The
84 decidua is a transient tissue that sustains embryo development, regulates maternal immune
85 response and controls trophoblast invasion into the uterus (Dey *et al.* 2004, Fonseca *et al.*
86 2012).

87 LPA is a small and bioactive phospholipid that has a range of influences mediated by G
88 protein-coupled receptors in female reproduction (Tokumura *et al.* 2000, Kunikata *et al*. 1999,
89 Kobayashi *et al*. 1994). Through LPA-induced chemokine production, human first-trimester
90 trophoblast cells regulate angiogenesis and innate immune system in early pregnancy (Chen
91 *et al*. 2010). LPA3-deficient female mice showed implantation failure phenotypes including
92 delayed implantation and crowded implantation sites (Ye *et al*. 2005). Also, human
93 pregnancy is associated with elevated production of LPA in serum and it is postulated its
94 placental origin (Tokumura *et al*. 2002). Moreover, patients displaying recurrent implantation
95 failure express reduced levels of LPA3 in the endometrium (Achache *et al*. 2010). Previously,
96 we observed that LPA via LPA3 augments the production of prostaglandins and nitric oxide,

97 mediators of vascular and decidual responses, in an *ex vivo* model of implantation in the rat
98 (Sordelli *et al*. 2012, Beltrame *et al*. 2013). These evidences suggest that LPA signaling
99 participates in the development of the fetal-maternal interfase at implantation and LPA3 is
100 postulated as a new checkpoint during implantation. Thus, the aim of the present study was
101 to investigate the participation of LPA3 in implantation events such as the formation of the
102 uterine blood vessels and the decidua.

103 **Materials and Methods.**

104 *Drugs and chemicals*.

105 DGPP 8:0, Luminol (Fluka), p-coumaric acid (Fluka), western blot detergents and inhibitors
106 (Sigma Chemical Company, Buenos Aires, Argentina and Bio Rad, Tecnolab, Buenos Aires,
107 Argentina), goat anti-rabbit horseradish peroxidase-conjugated IgG (Jackson
108 ImmunoResearch Laboratories, Inc, SERO-IMMUNO DIAGNOSTICS, INC, Tucker, GA,
109 USA), VEGF-A antibody (ab46154, Abcam, Cambridge, United Kingdom), xylazine and
110 ketamine (König and Holliday Scott SA, Buenos Aires, Argentina), trizol reagent (Genbiotech,
111 Buenos Aires, Argentina), RNAse free DNAse I, Moloney Murine Leukemia virus reverse
112 transcriptase (MMLV-RT) and random primers (Invitrogen, Buenos Aires, Argentina),
113 master mix for real time polymerase chain reaction (RT-PCR) (Promega, Biodynamics,
114 Buenos Aires, Argentina), chemiluminescence detection solutions (Romek Laboratories,
115 Buenos Aires, Argentina). All other chemicals were analytical grade.

116 *Animals*.

117 This study was carried out in strict accordance with the recommendations in the Guide for the
118 Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was
119 approved by the Committee on the Ethics of Animal Experiments of the University of Buenos

120 Aires (CICUAL, Permit Number: 2550/2010). Animals were provided by the School of
121 Dentistry (University of Buenos Aires).
122 Pregnant Wistar rats were obtained as previously described (Sordelli *et al*. 2012, Beltrame *et*
123 *al*. 2013). Briefly, Virgin female rats were mated with fertile males of the same strain. The
124 morning the spermatozoa were observed in the vaginal fluid was defined as day of
125 pregnancy 1 (Dp1). Under the conditions of our animal facilities, spontaneous term labor
126 occurs on Dp22 of gestation. In rats, blastocysts enter the uterus lumen in the afternoon of
127 Dp4 and implantation begins in the evening of Dp5 post coitus, and is preceded by embryo
128 spacing, uterine edema and luminal closure resulting in an intimate apposition of the
129 blastocyst with the uterine luminal epithelium. Around this precise moment, the endometrium
130 acquires the ability to implant the developing embryo within a specific time window, termed
131 the ''receptive phase'' or ''window of implantation''. During this period, the endometrium
132 undergoes pronounced structural and functional changes induced by the ovarian steroids,
133 estrogen and progesterone, which prepare it to be receptive to invasion by the embryo.
134 Females were anaesthetized (xylazine 6 mg/kg, ketamine 1 mg/kg) and the uterus
135 was surgically exposed. Pregnant rats received single doses of DGPP (0.05, 0.1 or
136 0.2 mg/kg) intra-uterine (i.u.), total volume: 2 μl, in the left horn (treated horn) in the
137 morning of Dp5, during the apposition phase, just before implantation begins. The
138 right horn of the uterus was injected with the same volume of vehicle (control horn).
139 DGPP and vehicle injections were made directly within the uterine lumen (gauge
140 needle: 30G). Once injected, rats were housed in separate cages and continuously
141 monitored until sacrifice on Dp 6, 7, 8, 15 or 21.
142 Implantation sites from Dp 6 were determined by an intravenous injection of 0.1 ml of

143 Evans Blue dye solution in saline 5 minutes before killing. Blue bands around the
144 uterine horns indicated that the implantation process had been initiated. Implantation
145 units from Dp 8 and 15 were weighted and fixed in 4% paraphormaldehyde and
146 stained with hematoxylin-eosin, or homogenized in Trizol for RT-PCR assays. In
147 addition, a group of rats was monitored until delivery. Day of parturition was
148 registered when the first pup was expulsed. Pups were weighted and activity, feeding
149 and general well-being of the mothers and the pups were evaluated for 4 weeks.

150 *Resorption rate.*

151 Uterine horns were examined macroscopically in Dp8 and Dp15. The number of
152 healthy and resorpted implantation units was recorded. Resorpted implantation sites
153 were identified by their reduced size, high haemorrhage and signs of necrosis. The
154 resorption rate was calculated as previously reported: [resorpted embryos/(resorpted
155 + healthy embryos)] x 100 (Aisemberg *et al*. 2007).

156 *Macroscopic vascular analysis.*

157 Uterine horns with their irrigating vessels were exposed and photographed on Dp 8
158 and 15. The vessels that irrigate DGPP treated and control horns were counted.
159 Results were expressed as the number of vessels/implantation site. Images were
160 analyzed using the Image-Pro Plus Program (version 4.5.0.29) to determine cross
161 sectional length of the uterine and arcuate arteries.

162 *RNA isolation and quantitative RT-PCR.*

163 Total RNA isolation, cDNA synthesis and qRT-PCR were performed as previously described
164 (Sordeli *et al*. 2012, Beltrame *et al*. 2013, Freitag *et al*. 2013). Total RNA obtained under different
165 conditions was isolated using Tri Reagent according to the manufacturer's recommendations

166 (Molecular Research Center, Genbiotech, Argentina). RNA was thawed on ice, quantified
167 spectrophotometrically at 260 and 280 nm and RNA quality assessed using gel red nucleic acid
168 stained gels. RNA with a 260:280 ratio of 1.8 and above was further treated with RNase free
169 DNase I to digest contaminating genomic DNA. First strand cDNA was synthesized from total
170 RNA (3 µg) using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and
171 random primers according to the manufacturer's recommendations (Invitrogen, Buenos Aires,
172 Argentina) in the presence of ribonuclease inhibitor. The PCR conditions in all cases started with
173 a denaturation step at 95°C for 30 seg and followed by up to 40 cycles of denaturation, annealing
174 and primer extension (*β-actin*, *il-10* and *vegf-a*: 95°C, 30 seg – 59°C, 30 seg – 72°C, 20 seg;
175 *vegf-r1*: 95°C, 30 seg – 56°C, 30 seg – 72°C, 20 seg). PCR primers are detailed for *β-actin*
176 (Forward 5´-CCATGTACGTAGCCATCC-3´, Reverse 5´-CTCTCAGCTGTGGTGGTGAA-3´), *il-*
177 *10* (Forward 5´-GCCAAGCCTTGTCAGAAATGA-3´, Reverse 5´-TTTCTGGGCCATGGTTCTCT-
178 3´), *vegf-a* (Forward 5´-ATCATGCGGATCAAACCT-3´, Reverse 5´-ATTCACATCTGCTATGCT-
179 3´), *vegf-r1* (Forward 5´-AAGACTCGGGCACCTATG-3´, Reverse 5´-CGGCACCTATAGACACC-
180 3´), *prolactin* (Forward 5´-ATCAATGACTGCCCCACTTC-3´, Reverse 5´-
181 ATTCCAGGAGTGCACCAAAC-3´) and *IGFBP-1* (Forward 5´-GCGGTAGTGCCTAGAACGAG-
182 3´, Reverse 5´-TGGGATTCGATGAGGAAGTC-3´). *il-10*, *Vegf-a* and *vegf-r1* mRNA levels were
183 corrected to the levels of rat β-actin using the $2^{-\Delta\Delta Ct}$ method. β-actin was chosen as the
184 housekeeping gene as it did not alter its expression under our experimental control.
185 *VEGF-A identification by western blotting.*
186 Tissue slices frozen at –70°C were incubated in triple detergent buffer (PBS pH =7.4 with sodium
187 azide 0.02% w/v, SDS 0.1% w/v, nonidet P-40 1% v/v, sodium deoxycholate 0.5% v/v) in buffer
188 inhibitors 1:100. Tissues were homogenized (Ultra Turrax, T25 basic, IKA Labortechnik),

189 sonicated for 30 sec (Ultrasonic Cell Disrupter, Microson, Heat systems Inc.) and centrifuged for
190 30 min at 20000 g. Protein determination was assayed by the Bradford method (Bradford 1976)
191 using bovine serum albumin as standard. Equal amount of proteins (100 µg/lane) were
192 separated in 12.5% w/v SDS-PAGE (15 mA at room temperature) and subsequently transferred
193 to nitrocellulose membranes (30 V at 4°C for 18 h). Non-specific binding sites of the membranes
194 were blocked using dried non-fat milk 5% w/v in PBS pH =7.4. Membranes were incubated with
195 anti VEGF-A (1:1000 in PBS) followed by a goat anti-rabbit horseradish peroxidase conjugated
196 IgG (1:5000 in PBS pH =7.4 containing non-fat milk 5% w/v). Non-specifically bound antibody
197 was removed by washing three times with PBS containing Tween-20 0.1% v/v. Each membrane
198 was exposed to CL- XPosure films for 30 min and photographed. An homogenate from a
199 xenograft induced by human breast cancer cell line MDA-MB-231 was used as positive control
200 (Pontillo et al. 2015). Immunoreactive specificity was assessed by omitting the first antibody.
201 Protein bands were identified by molecular weight markers. GADPH (1:400) was used as loading
202 control. The intensity of bands was determined using the Image J software package (open
203 source). Results were expressed as relative optic density VEGF-A/GADPH.

204 *Hematoxylin-Eosin staining.*

205 Serial paraffin-embedded tissue sections from Dp 8 or 15 were processed as described (Ribeiro
206 *et al*. 2005, Sordelli *et al*. 2012). Briefly, uteri and placentas from pregnant rats on Dp 8 and 15 of
207 gestation were removed and fixed overnight in paraphormaldehyde 4% in phosphate buffered
208 saline (PBS) 0.1 M (pH 7.4). The tissue sections were dehydrated with 70–100% ethyl alcohol
209 and embedded in paraffin. The paraffin block was orientated to enable the tissues to be
210 sectioned transversally. Sections of 5 µm were made by a microtome (Leica RM 2125, Wetzlar,

211 Germany) and mounted on 2% silane-coated slides. The sections were stained with
212 hematoxylin–eosin, mounted with Permount. and observed by light microscopy (Nikon Eclipse
213 200, NY, USA) to determine general tissue morphology and to identify the different cell types
214 present.

215 *Statistical analysis.*

216 Statistical analysis was performed using the InfoStat Program (Córdoba, Argentina).
217 Comparisons between values of different groups were performed using one way ANOVA
218 (analyze of variance). Significance was determined using Tukey's multiple comparison test for
219 unequal replicates. A number of 4-6 animals were used for each treatment. All values presented
220 in this study represent mean ± S.E.M. Differences between means were considered significant
221 when $p \leq 0.05$.

222 **Results.**

223 *The administration of DGPP produced implantation failure and resorption.*

224 In order to determine optimal treatment conditions we tested three doses of DGPP. Pregnant rats
225 on Dp 5 were injected with 0.05, 0.1 or 0.2 mg/kg DGPP i.u. and sacrificed on Dp 15. While the
226 administration of 0.05 mg/kg DGPP did not exert any effect, the treatment with 0.1 and 0.2 mg/kg
227 DGPP significantly increased the resorption rate (Table 1). In all subsequent experiments rats
228 received 0.1 mg/kg DGPP on Dp 5, as this was the lowest tested dose that produced resorption.
229 Females on Dp 6 did not show differences between the control and the treated horn (Figure 1A).
230 Signs of resorption and aberrant embryo spacing after DGPP administration appeared on Dp 7
231 (Figure 1B) and were clearly denoted on Dp 8 (Figure 1C). The control horn had well-developed
232 implantation sites that left even spaces between them (Figure 1C). The DGPP treated horn
233 presented embryos with signs of resorption and non-resorpted embryos showing a normal

234 appearance (Figure 1C). Bleeding areas suggested the beginning of embryonic resorption
235 (Figure 1D). Also, some implantation sites were unevenly distributed and showed embryo
236 crowding (Figure 1E). On Dp 15, the control horn exhibited fetuses with morphology according to
237 their gestational age (Figure 1F). The DGPP horn presented both resorpted and non-resorpted
238 sites (Figure 1F). In adittion, fetuses' crowding in different uterine segments was observed
239 (Figure 1F). Control embryos presented normal placentas and fetal membranes were intact
240 containing clear amniotic fluid inside them (Figure 1G). While, non-resorpted embryos continued
241 growing and were similar to control embryos (Figure 1H), resorpted fetuses showed high
242 hemorrhage, signs of necrosis and reduced size (Figure 1H and 1I). The number of implantation
243 sites in DGPP and control horns was similar and almost 100% of the rats responded to DGPP
244 (Table 2). The fetal resorption rate increased to approximately 54% in Dp8 and the difference
245 was statistically not significant compared to Dp 15 (Table 2). On Dp 8 control, non-resorpted and
246 resorpted implantation units did not differ in their weights (Table 3). On Dp 15 the weight of the
247 uterus and the placenta did not show differences between control and non-resorpted implantation
248 sites, and as expected it was decreased in the resorptions (Table 3).
249 The effect of DGPP on the resorption rate was also seen late in Dp 21, prior to the onset of
250 labor (Figure 2A). DGPP did not have any effect on the onset of parturition as rats gave birth
251 normally on Dp 22. The mothers fed their pups in the same way as did non-treated rats (rats
252 that did not receive vehicle and DGPP). There were no differences that could denote variations
253 between the pups delivered from the DGPP or the vehicle horns and the offspring was quite
254 regular (Figure 2B). Also, the offspring from treated females showed a similar appearance to that
255 from untreated rats (Figure 2B). The growth rate of the pups born of treated mothers did not differ

256 from pups born of non-treated females (Figure 2C). In all cases, the body weight of the pups
257 significantly increased among weeks (Figure 2C).

258 *DGPP treatment induced alterations in vascularization and decidualization.*

259 DGPP decreased the cross sectional length of the uterine and arcuate arteries on Dp 8 (Figure
260 3A, 3B and 3C). Similar results were observed on Dp 15 (Figure 3D, 3E and 3F). The number of
261 vessels irrigating each implantation site was not affected by DGPP (Table 4).

262 When we analyzed the expression of vascularization markers, we observed that on Dp 8, *Il-10*
263 decreased in resorpted sites (Figure 4A), while *Vegf-a* (Figure 4B) and *Vegf-r1* (Figure 4C)
264 decreased in non-resorpted and resorpted sites. On Dp 15, these markers increased in the
265 placentas obtained from resorpted units (Figure 4D, 4E and 4F). In the uterus, the expression of
266 *Il-10* (Figure 4G), *Vegf-a* (Figure 4H) and *Vegf-r1* (Figure 4I) did not differ between control, non-
267 resorpted and resorpted implantation sites. VEGF-A protein did not differ in any of the tested
268 conditions (Figure 5A, 5B and 5C).

269 Next, we evaluated whether DGPP treatment affected decidualization. While control implantation
270 sites (Figure 6A) and non-resorpted sites (Figure 6B) presented a highly conserved architecture,
271 resorpted units lost part of its normal tissue organization (Figure 6C). Controls showed the
272 characteristic zones of the normal pregnant uterus on Dp 8: the longitudinal and circular muscle
273 layers (Figure 6D), the endometrial glands and vessels (Figure 6E), the primary decidual zone
274 (Figure 6F) and the anti-mesometrial decidua (Figure 6G). Non-resorpted sites did not differ with
275 the control. In the resorptions, the longitudinal and circular muscle layers remained conserved
276 (Figure 6C). Although the stromal cells located around the uterine lumen resembled decidual
277 cells (Figure 6H), the primary decidual zone and the anti-mesometrial decidua were not
278 distinguishable (Figure 6C). Most of the uterine lumen became filled with blood and neutrophils

279 were observed along the lumen border (Figure 6H). The stroma located near the muscle layers
280 presented cells and uterine vessels highly disorganized that seemed to be broken (Figure 6I).
281 Some endometrial vessels had extravasated neutrophils near the sites of tissue injury (Figure 6I).
282 The expression of prolactin (Figure 7A) and IGFBP-1 (Figure 7B), two markers of decidualization,
283 did not change between control, non-resorpted and resorpted implantation sites.
284 Histological studies of the uteri from Dp 15 showed no morphological differences between control
285 (Figure 8A), non-resorpted (Figure 8B) and resorpted (Figure 8C) implantation sites. The
286 endometrial epithelium and the circular and longitudinal muscle layers were present. Control
287 (Figure 8D) and non-resorpted (Figure 8E) placentas presented the typical histological structure
288 with the basal zone, the labyrinth and the basal decidua. The resorpted placentas showed a
289 completely disorganized architecture and the basal decidua, the basal zone and the labyrinth
290 were not distinguishable (Figure 8F). In the control, the labyrinth showed trophoblast cells, fetal
291 erythrocytes and maternal sinusoids filled with maternal blood (Figure 8G). In the basal zone we
292 observed spongiotrophoblasts and glycogen cells (Figure 8H). Also, the giant cells appeared
293 adjacent to the basal decidua (Figure 8I). In the non-resorpted placentas, although most of the
294 tissue appeared as the control, some red blood cells and leukocytes were denoted in the basal
295 decidua (Figure 8J). In the resorpted placentas we observed high tissue disorganization,
296 extensive vacuolation and cellular degeneration (Figure 8K). Trophoblasts appeared disrupted
297 with red blood cells in between them (Figure 8L). Also, we observed zones with extensive
298 fibrin deposition and high neutrophils infiltration (Figure 8M).

299 **Discussion.**

300 Here we observed that endogenous LPA regulates vascularization and decidualization at the
301 maternal-fetal interfase in the rat. The pharmacological blockade of LPA3 causes vascular and

302 decidual damage that affects placental and embryo development. Most of the studies on LPA
303 reflect its importance as a signaling molecule during migration, proliferation, differentiation and
304 cell survival (Ishii *et al*. 2004, Ye *et al*. 2005, Kano *et al*. 2008, Skoura and Hla 2009), which are
305 essential functions during vascularization and decidualization at the implantation sites.
306 As accumulating evidences suggest that LPA3 is the LPA receptor subtype with major
307 participation during embryo implantation (Wei *et al*. 2009, Achache *et al*. 2010, Ye *et al*. 2005),
308 we decided to block LPA3 by injecting DGPP, an LPA3 antagonist. It has been published that the
309 expression of LPA3 is decreased in the endometrium of women with endometriosis (Wei *et al*.
310 2009) and with multiple implantation failures (Achache *et al*. 2010). The phenotype described for
311 LPA3$^{-/-}$ females is not observed in mice lacking LPA1 or LPA2 (Ye *et al*. 2005). Deletion of LPA3
312 disrupts fine local balance of progesterone and estrogen signaling in mouse uterus during
313 implantation (Diao *et al*. 2015). Moreover, only LPA3 is found exclusively expressed in the
314 epithelial endometrium of mice (Ye *et al*. 2011) and rat (Sordelli *et al*. 2012) just before
315 implantation begins. Based on binding and functional data described in the data sheet and on a
316 previous work from our laboratory (Sordelli *et al.* 2012, Beltrame *et al.* 2013), DGPP at the
317 selected doses used here is a highly potent and selective antagonist for LPA3. Thus, taking
318 these data into account we hypothesize that the effects described here elicited by endogenous
319 LPA are mediated by LPA3.
320 We postulate that the attachment phase during the implantation process does not depend
321 exclusively on LPA in the rat since attachment of the blastocyst to the luminal epithelium is
322 normal after DGPP administration. However, LPA3 blockade produces significant post-
323 implantation defects (embryo crowding and fetal resorption) that are irreversible and stable
324 throughout pregnancy, denoting that LPA3 might be involved in the development of the embryo

325 and its supporting tissues. LPA3 *knockout* mice also present clear phenotypic changes as altered
326 positioning/crowding of embryos (Ye *et al.* 2005). These observations are in accordance with the
327 fact that LPA3 is involved in the generation of the contractions that allow the accurate location
328 of the embryos along the uterine horn (Hama *et al*. 2007, Ye *et al*. 2005).
329 The proper coordination of several vascular processes at the maternal-fetal interfase ensures an
330 adequate blood flow in response to the increasing metabolic demands of the embryo (Torry *et al*.
331 2007). Our results demonstrate that endogenous LPA via LPA3 regulates the diameter of the
332 main vessels that irrigate the uterus suggesting that a decline in the supply of nutrients and
333 oxygen to the developing embryos might occur after DGPP administration, and therefore some of
334 the embryos survive (non-resorpted) while others are resorpted. Early in gestation, the
335 attachment reaction coincides with increased stromal vascular permeability. Rat implantation
336 sites show significant increase in localized vessel permeability and vessel density. It has been
337 demonstrated that LPA could induce *Vegf-c* mRNA expression in human umbilical vein
338 endothelial cells and subsequent endothelial cell tube formation (Lin *et al*. 2008). Further, LPA-
339 induced IL-8 of human first-trimester trophoblast cells functionally enhances multistep processes
340 of angiogenesis including permeability, migration, proliferation, and capillary tube formation of
341 endothelial cells (Chen *et al*. 2010). The enhancement effects are dependent on LPA1 and LPA3
342 activation (Lin *et al*. 2008, Chen *et al*.2010). All together, these results suggest that LPA may
343 play a role in angiogenesis of endometrium and placenta. In fact, spatiotemporal and reciprocal
344 interactions between the trophoblast and the endothelium are required for normal angiogenesis
345 during early gestation. Thus, abnormal LPA signaling at the initiation of implantation may result in
346 aberrant regulation of endothelial adaptations, trophoblast functions and angiogenesis. Disruption
347 of these pathways collectively could lead to placental abruption, fetal demise and pregnancy

348 loss.

349 Molecular studies on the mRNA expression of *Il-10*, *Vegf-a* and *Vegf-r1*, three vascularization
350 markers, reflect the macroscopic vascular changes observed in the resorpted sites on Dp 8.
351 Previously, we reported that LPA augments the expression of *Il-10* through the activation of LPA3
352 in the rat pregnant uterus (Sordelli *et al*. 2012), and this mechanism is also involved in the
353 stimulation of nitric oxide and prostaglandin E2 production, which are key mediators of
354 vascularization (Beltrame *et al*. 2013). Other authors have also informed that LPA promotes
355 angiogenesis in different biological systems inducing the formation of endothelial capillaries and
356 the secretion of different vascular cytokines (Chen *et al*. 2008, 2010, Hu *et al*. 2001, So *et al*.
357 2004, Chou *et al*. 2005, Lin *et al*. 2008, Zhou *et al.* 2010). However, on Dp 15, the expression of
358 these markers is increased in the placenta obtained from resorpted sites. We speculate that in
359 Dp 8, the process of vascularization (at the macroscopic and molecular levels) is diminished due
360 to LPA3 antagonism. May be the placenta attempts to compensate the lack of nutrients due to a
361 non-functional decidual vasculature, by increasing the production of those molecules directly
362 involved in angiogenesis. This regulation may be directed by the decidual cells specifically, as in
363 Dp15 the uterine tissue does not show any difference in the markers analyzed. Unfortunately, we
364 were not able to detect differences in the expression of VEGF-A protein. We postulate that as
365 real time RT-PCR technique is more sensible than western blot analyses, modulation in VEGF-A
366 expression could not be detected at the protein level.

367 The decidua supports embryo growth until the placenta is entirely formed (Gellersen and Brosens
368 2014) and secrets molecules that participate in neovascularization (Lim and Wang 2010).
369 Consequently, failures in vascular and decidual formation seriously compromise the success of
370 implantation and pregnancy. We hypothesized that the damage described in the uterine and

371 arcuate arteries affects decidualization and placentation. The expression of prolactin and IGFBP-
372 1, which are considered biochemical markers of the decidualization process, is not modified after
373 LPA3 blockade. Interestingly, the decidual cells as well as the placenta from resorpted sites
374 present important alterations in their structure when compared to the control and non-resorpted
375 embryos. Thus, we speculate that changes in the architecture of the decidua are responsible for
376 the embryo resorptions, despite the secretion of prolactin and IGFBP-1. This data support the
377 idea that non-resorpted embryos survive at least in part due to the maintenance of their decidual
378 and placental structure, as these are conserved and similar to control sites. All together, these
379 data suggest that endogenous LPA participates in the formation of the decidua by binding to
380 LPA3. Previously, we observed that LPA augments the expression of IGFBP-1 through LPA3 in
381 Dp 5 rat uterus (Sordelli *et al*. 2012). We postulate that the difference with the results reported
382 here could be attributed to the experimental models used (*in vitro* vs *in vivo*) and/or to the
383 pharmacodynamics of LPA and DGPP.
384 We observed that decidual and placental damage are accompanied by cellular disorganization,
385 hemorrhage, fibrin deposition and infiltration of neutrophils, which are typical events associated
386 with embryo resorption (Ogando *et al*. 2003, Sacerdoti *et al*. 2015). Despite the differences
387 observed between the control and non-resorpted sites and placentas, these embryos are able to
388 overpass the defects triggered by DGPP administration and grow and develop normally. More
389 studies are being carried out in order to elucidate the mechanisms by which non-resorpted
390 embryos survive.
391 Interestingly, the uterus seems to retain its tissue architecture and vascular function after LPA3
392 antagonism, as the histological characteristics and the expression of the vascular markers do not
393 differ between control, non-resorpted and resorpted sites in Dp 15. We postulate that the loss of

394 the embryos and their extra-embryonic tissues with the recovery of the maternal tissues and
395 function is related to the preservation of the mother for future pregnancies. Other authors
396 described similar results after Escherichia Coli infections in pregnant mice and rats (Ogando *et*
397 *al*. 2003, Burdet *et al*. 2009).

398 The results presented here are supported by previous evidence from our laboratory in which we
399 described that LPA via LPA3 increases the production of nitric oxide and prostaglandin E2, two
400 main mediators in the formation of new uterine vessels and the transformation of the decidua
401 (Sordelli *et al*. 2012, Beltrame *et al*. 2013). Also, both cyclooxygenase-2 and inducible nitric oxide
402 synthase mediated LPA effect on IGFBP-1 and IL-10 expression, suggesting the participation of
403 LPA/LPA3 in the production of crucial molecules involved in decidualization and vascularization,
404 respectively. LPA3 knockout results in reproductive problems in female mice due to difficulty in
405 embryonic implantation (Ye *et al*. 2005). Cyclooxygenase 2 is down-regulated in LPA3 knockout
406 mice, leading to a reduction of prostaglandins E2 and I2, both of which are important for
407 implantation. As a lipid mediator, LPA is unique in that it is produced and rapidly degraded by
408 specific routes and that its actions are evoked by six cognate G protein-coupled receptors
409 (Fukushima *et al*. 2015, Aikawa *et al*. 2015). Activation of these G-proteins through LPA-receptor
410 binding mediates the activity of many important ubiquitous signaling pathways thereby
411 modulating the downstream activity of signaling molecules and effectors such as adenylyl
412 cyclase, cAMP, intracellular Ca^{2+}, mitogen-activated protein kinases, phospholipase C, PI3K/Akt,
413 and small GTPases as Ras, Rho and Rac (Ishii *et al*. 2004). Thus, LPA/LPA3 downstream
414 signaling pathways could interact with the production of pivotal mediators, as prostaglandins
415 and nitric oxide, that regulate implantation (Ye *et al*. 2008).

416 In conclusion, this study reveals that the disruption of endogenous LPA signaling by blocking
417 LPA3 alters the development of the uterine vasculature with consequences in decidualization, the
418 formation of the placenta and the growth of the embryos.
419

420 **Acknowledgements.**
421 We thank Enzo Cubas and Marcela Marquez (CEFYBO´s Animal Facility), Ricardo
422 Orzuza (Facultad de Odontología) and P. Moschansky (Blois's lab) for their excellent
423 technical assistance in generating this work. This work was supported by research grants
424 from FONCyT (PICT 2010 N°1974 and PICT 2013 N°0285) to M.L.R., CONICET (PIP 2012
425 Nº0496) to S.P.M and the Fritz Thyssen Stiftung (Az. 10.10.2.125) and Charité Research
426 Funding (2013) to S.M.B. Financial sources had no involvement in study design, in the
427 collection, analysis and interpretation of the data, in the writing of the report, and in the
428 decision to submit the article for publication.
429

430 **References.**
431 Achache, H., Tsafrir, A., Prus, D., Reich, R. and Revel, A. (2010). Defective endometrial
432 prostaglandin synthesis identified in patients with repeated implantation failure undergoing in
433 vitro fertilization. *Fertil. Steril.* **94,** 1271-1278.
434 Aikawa, S., Hashimoto, T., Kano, K. and Aoki, J. (2015). Lysophosphatidic acid as a lipid
435 mediator with multiple biological actions. *J. Biochem.* **157**, 81–89.
436 Aisemberg, J., Vercelli, C., Billi, S., Ribeiro, M.L., Ogando, D., Meiss, R., McCann, S.M.,
437 Rettori, V. and Franchi, A.M. (2007). Nitric oxide mediates prostaglandins' deleterious effect
438 on lipopolysaccharide-triggered murine fetal resorption, *Proc. Natl. Acad. Sci*. **104**, 7534-9.

439 Beltrame, J.S., Sordelli, M.S., Cella, M., Perez Martinez, S., Franchi, A.M. and Ribeiro, M.L.
440 (2013). Lysophosphatidic acid increases the production of pivotal mediators of
441 decidualization and vascularization in the rat uterus. *Placenta* **34,** 751-756.
442 Blois, S.M., Klapp, B.F. and Barrientos, G. (2011). Decidualization and angiogenesis in early
443 pregnancy: unravelling the functions of DC and NK cells. *J. Reprod. Immunol.* **88,** 86-92.
444 Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram
445 quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72,** 248–
446 254.
447 Burdet, J., Zotta, E., Franchi, A.M. and Ibarra, C. (2009). Intraperitoneal administration of
448 shiga toxin type 2 in rats in the late stage of pregnancy produces premature delivery of dead
449 fetuses. *Placenta* **30,** 491-496.
450 Chen, S.U., Chou, C.H., Chao, K.H., Lee, H., Lin, C.W., Lu, H.F. and Yang, Y.S. (2010).
451 Lysophosphatidic acid up-regulates expression of growth-regulated oncogene-alpha,
452 interleukin-8, and monocyte chemoattractant protein-1 in human first-trimester trophoblasts:
453 possible roles in angiogenesis and immune regulation. *Endocrinology* **151,** 369-379.
454 Chen, S.U., Lee, H., Chang, D.Y., Chou, C.H., Chang, C.Y., Chao, K.H., Lin, C.W. and Yang,
455 Y.S. (2008). Lysophosphatidic acid mediates interleukin-8 expression in human endometrial
456 stromal cells through its receptor and nuclear factor-kappaB-dependent pathway: a possible
457 role in angiogenesis of endometrium and placenta. *Endocrinology* **149,** 5888-5896.
458 Chou, C.H., Wei, L.H., Kuo, M.L., Huang, Y.L., Lai, K.P., Chen, C.A. and Hsieh, C.Y. (2005)
459 Up-regulation of interleukin-6 in human ovarian cancer cell via a Gi/PI3K–Akt/NF-_B pathway
460 by lysophosphatidic acid, an ovarian cancer-activating factor. *Carcinogenesis* **26,** 45–52.
461 de la Torre, E., Hovsepian, E., Penas, F.N., Dmytrenko, G., Castro, M.E., Goren, N.B. and

462 Sales, M.E. (2013). Macrophages derived from septic mice modulate nitric oxide synthase
463 and angiogenic mediators in the heart. *J. Cell Physiol.* **228,** 584-1593.
464 Demir, R., Yaba, A. and Huppertz, B. (2010). Vasculogenesis and angiogenesis in the
465 endometrium during menstrual cycle and implantation. *Acta. Histochem.* **112,** 203-214.
466 Dey, S.K., Lim, H., Das, S.K., Reese, J., Paria, B.C., Daikoku, T. and Wang, H. (2004).
467 Molecular cues to implantation. *Endocr. Rev.* **25,** 341-373.
468 Diao, H., Li, R., El Zowalaty, A.E., Xiao, S., Zhao, F., Dudley, E.A. and Ye, X. (2015).
469 Deletion of lysophosphatidic acid receptor 3 (Lpar3) disrupts fine local balance of
470 progesterone and estrogen signaling in mouse uterus during implantation. *Biol. Reprod*.
471 **93**,123.
472 Fonseca, B.M., Correia-da-Silva, G. and Teixeira, N.A. (2012). The rat as an animal model
473 for fetoplacental development: a reappraisal of the post-implantation period. *Reprod. Biol.* **12,**
474 97-118.
475 Fukushima, N., Ishii, S., Tsujiuchi, T., Kagawa, N. and Katoh, K. Comparative analyses of
476 lysophosphatidic acid receptor-mediated signaling. (2015). *Cell. Mol. Life Sci*. **72**, 2377-2394.
477 Gellersen, B. and Brosens, J.J. (2014). Cyclic Decidualization of the Human Endometrium in
478 Reproductive Health and Failure. *Endocr. Rev.* **35**, 851-905.
479 Freitag, N., Tirado-González, I., Barrientos, G., Herse, F., Thijssen, V.L., Weedon-Fekjær,
480 S.M., Schulz, H., Wallukat, G., Klapp, B.F., Nevers, T., Sharma, S., Staff, A.C., Dechend, R.
481 and Blois, S.M. (2013). Interfering with Gal-1-mediated angiogenesis contributes to the
482 pathogenesis of preeclampsia. *Proc. Natl. Acad. Sci.* **110**, 11451-6.
483 Hama, K., Aoki, J., Inoue, A., Endo, T., Amano, T., Motoki, R., Kanai, M., Ye, X., Chun, J.,
484 Matsuki, N., Shibasaki, M. and Arai, H. (2007). Embryo spacing and implantation timing are

485 differentially regulated by LPA3-mediated lysophosphatidic acid signaling in mice. *Biol.*
486 *Reprod.* **77,** 954-959.
487 Hu, Y.L., Tee, M.K., Goetzl, E.J., Auersperg, N., Mills, G.B., Ferrara, N. and Jaffe, R.B.
488 (2001). Lysophosphatidic acid induction of vascular endothelial growth factor expression in
489 human ovarian cancer cells. *J. Natl. Cancer Inst.* **93,** 762–768.
490 Ishii, I., Fukushima, N., Ye, X. and Chun, J. (2004). Lysophosphatidic receptors: Signaling
491 and Biology. *Annu. Rev. Biochem.* **73,** 321–354.
492 Kano, K., Arima, N., Ohgami, M. and Aoki, J. (2008). LPA and its analogs-attractive tools for
493 elucidation of LPA biology and drug development. *Curr. Med. Chem.* **15,** 2122-2131.
494 Kobayashi, T., Yamano, S., Tokumura, A. and Aono, T. (1994). Effect of lysophosphatidic
495 acid on the preimplantation development of mouse embryos. *FEBS Lett.* **351,** 38–40.
496 Kunikata, K., Yamano, S., Tokumura, A. and Aono, T. (1999). Effect of lysophosphatidic acid
497 on the ovum transport in mouse oviducts. *Life Sci.* **65,** 833–840.
498 Lim, H.J. and Wang, H. (2010). Uterine disorders and pregnancy complications: insights from
499 mouse models. *J. Clin. Invest.* **120,** 1004-1015.
500 Lin, C.I., Chen, C.N., Huang, M.T., Lee, S.J., Lin, C.H., Chang, C.C. and Lee, H. (2008).
501 Lysophosphatidic acid upregulates vascular endothelial growth factor-C and tube formation in
502 human endothelial cells through LPA1/3, COX-2, and NF-κB activation- and EGFR
503 transactivation-dependent mechanisms. *Cellular Signalling* **20,** 1804-1014.
504 Ogando, D.G., Paz, D., Cella, M. and Franchi, A.M. (2003). The fundamental role of
505 increased production of nitric oxide in lipopolysaccharide-induced embryonic resorption in
506 mice. *Reproduction* **125,** 95-110.
507 Pontillo, C., Español, A., Chiappini, F., Miret, N., Cocca, C., Alvarez, L., Kleiman de Pisarev,

508 D., Sales, M.E. and Randi, A.S. (2015). Hexachlorobenzene promotes angiogenesis in vivo,
509 in a breast cancer model and neovasculogenesis in vitro, in the human microvascular
510 endothelial cell line HMEC-1. *Toxicol. Lett.* **239**, 53-64.
511 Ribeiro, M.L., Aisemberg, J., Billi, S., Farina, M.G., Meiss, R., McCann, S., Rettori, V.,
512 Villalón, M., and Franchi, A.M. (2005). Epidermal growth factor prevents prepartum luteolysis
513 in the rat. *Proc. Natl. Acad. Sci.* **102**, 8048-53.
514 Sacerdoti, F., Amaral, M.M., Aisemberg, J., Cymeryng, C.B., Franchi, A.M., and Ibarra, C.
515 (2015). Involvement of hypoxia and inflammation in early pregnancy loss mediated by Shiga
516 toxin type 2. *Biol. Reprod.* **93**, 1–9.
517 Shimizu, T., Hoshino, Y., Miyazaki, H. and Sato, E. (2012) Angiogenesis and
518 microvasculature in the female reproductive organs: physiological and pathological
519 implications. *Curr. Pharm. Des.* **18,** 303-309.
520 Skoura, A. and Hla, T. (2009). Lysophospholipid receptors in vertebrate development,
521 physiology, and pathology. *J. Lipid Res.* **50,** S293-S298.
522 So, J., Navari, J., Wang, F.Q. and Fishman, D.A. (2004). Lysophosphatidic acid enhances
523 epithelial ovarian carcinoma invasion through the increased expression of interleukin-8.
524 *Gynecol. Oncol.* **95,** 314–322.
525 Sordelli, M.S., Beltrame, J.S., Cella, M., Gervasi, M.G., Perez Martinez, S., Burdet, J., Zotta,
526 E., Franchi, A.M. and Ribeiro, M.L. (2012). Interaction between lysophosphatidic acid,
527 prostaglandins and the endocannabinoid system during the window of implantation in the rat
528 uterus. *PLoS One* **7,** e46059.
529 Tokumura, A., Kanaya, Y., Miyake, M., Yamano, S., Irahara, M. and Fukuzawa, K. (2002).
530 Increased Production of Bioactive Lysophosphatidic Acid by Serum Lysophospholipase D in

531 Human Pregnancy. *Biol. Reprod.* **67,** 1386–1392.

532 Tokumura, A., Yamano, S., Aono, T. and Fukuzawa, K. (2000). Lysophosphatidic acids
533 produced by lysophospholipase D in mammalian serum and body fluid. *Ann. N. Y. Acad. Sci.*
534 **905,** 347–350.

535 Torry, D.S., Leavenworth, J., Chang, M., Maheshwari, V., Groesch, K., Ball, E.R. and Torry,
536 R.J. (2007). Angiogenesis in implantation. *J. Assist. Reprod. Genet.* **24,** 303-315.

537 Wei, Q., St Clair, J.B., Fu, T., Stratton, P. and Nieman, L.K. (2009). Reduced expression of
538 biomarkers associated with the implantation window in women with endometriosis. *Fertil.*
539 *Steril.* **91,** 1686-1691.

540 Ye, X., Hama, K., Contos, J.J., Anliker, B., Inoue, A., Skinner, M.K., Suzuki, H., Amano, T.,
541 Kennedy, G., Arai, H., Aoki, J. and Chun, J. (2005). LPA3-mediated lysophosphatidic acid
542 signalling in implantation and embryo spacing. *Nature* **435,** 104-108.

543 Ye, X. (2008). Lysophospholipid signaling in the function and pathology of the reproductive
544 system. *Hum Reprod Update* **14**, 519–536.

545 Ye, X., Herr, D.R., Diao, H., Rivera, R. and Chun, J. (2011). Unique uterine localization and
546 regulation may differentiate LPA3 from other lysophospholipid receptors for its role in embryo
547 implantation. *Fertil. Steril.* **95,** 2107-2113.

548 Zhou, Z., Niu, J. and Zhang, Z. (2010). The role of lysophosphatidic acid receptors in
549 phenotypic modulation of vascular smooth muscle cells. *Mol. Biol. Rep.* **37,** 2675-2686.

550

551 **Legends.**

552 **Figure 1. DGPP treatment produced aberrant embryo spacing and embryo resorption.**

553 Females on Dp 5 were injected i.u. with 0.1 mg/kg DGPP (treated horn) and with vehicle

554 (control horn) and were sacrificed on Dp 6, 7, 8 and 15. (A, B, C and F) Panoramic view of
555 DGPP treated and control horns on Dp 6, 7, 8 and 15 respectively. Dp 8: detailed view of the
556 crowded embryos unevenly distributed (D) and zones of hemorrhage denoting possible
557 embryo resorption (E). Dp 15: uterus, placenta and fetus from a control implantation site (G),
558 detailed view of DGPP treated horn showing non-resorpted and resorpted embryos (H),
559 uterus and a resorpted feto-placental unit from the DGPP treated horn (I). Left horn: injected
560 with DGPP, right horn: control. Black arrows indicate areas with signs of hemorrhage (Dp 7
561 and 8) and resorpted sites (Dp 15). White arrows denote the equidistant embryo spacing.
562 Open arrows point normal embryos in the control horn and non-resorpted embryos in the
563 DGPP horn. Asterisk indicates a spontaneous resorption in the control horn. N=4-6/group.
564 Dp: day of pregnancy, UT: uterus, PLA: placenta, FPU: feto-placental unit.

565 **Figure 2. Effect of DGPP administration on Dp 21 pregnant rats.** Females were injected
566 i.u. with 0.1 mg/kg DGPP i.u. (treated horn) and with vehicle (control horn) on Dp 5 and were
567 sacrificed on Dp 21. (A) Panoramic view of DGPP treated and control horns on Dp 21. Left
568 horn: treated, right horn: control. Open arrows point healthy embryos. Black arrows indicate
569 resorpted sites. (B) Litters born from non-treated dams and from mothers treated with DGPP
570 and vehicle. (C) Body weight curve of pups born from non-treated dams and from mothers
571 treated with DGPP and vehicle. The weight of the pups is expressed in mg. Each point
572 represents the mean ± S.E.M. Different letters indicate statistical difference ($p<0.05$) using
573 ANOVA test (Tukey). N=4-6/group. Dp: day of pregnancy.

574 **Figure 3. DGPP treatment reduced the cross sectional length of the uterine and**
575 **arcuate arteries in pregnant rats on Dp 8 and 15.** Pregnant female rats were injected i.u.
576 with 0.1 mg/kg DGPP (treated horn) and with vehicle (control horn) on Dp 5. Representative

577 images of the main vessels irrigating the DGPP and vehicle injected horns are shown on Dp
578 8 (A) and Dp 15 (D). The cross sectional length of the uterine and arcuate arteries was
579 determined on Dp 8 (B, C) and 15 (E, F). Bars represent the mean ± S.E.M. Different letters
580 indicate statistical difference ($p<0.01$) using Student T test. N= 4-6/group. Dp: day of
581 pregnancy.

582 **Figure 4. DGPP administration regulated the mRNA expression of vascularization markers**
583 **in the implantation sites and placentas of pregnant rats on Dp 8 and 15**. Females were
584 injected i.u. with 0.1 mg/kg DGPP (treated horn) and with vehicle (control horn) on Dp 5 and
585 were sacrificed on Dp 8 and 15. The expression of *Il-10, Vegf-a* and *Vegf-r1* was assessed
586 by real time RT-PCR in the implantation units from Dp 8 (A, B, C) and in the placentas (D, E,
587 F) and uteri (G, H, I) from Dp 15. Bars represent the mean ± S.E.M. Different letters indicate
588 statistical difference ($p<0.05$) using ANOVA test (Tukey). N=4-6/group. Dp: day of pregnancy,
589 C: control, NR: non-resorpted, R: resorpted.

590 **Figure 5. DGPP administration did not modify the expression of VEGF protein in the**
591 **implantation sites and placentas of pregnant rats on Dp 8 and 15**. Females were injected i.u.
592 with 0.1 mg/kg DGPP (treated horn) and with vehicle (control horn) on Dp 5 and were sacrificed
593 on Dp 8 and 15. The expression of VEGF-A was assessed by western blot in the implantation
594 units from Dp 8 (A) and in the uteri (B) and placentas (C) from Dp 15. Bars represent the mean ±
595 S.E.M. using ANOVA test (Tukey). N=4-6/group. Dp: day of pregnancy, C: control, NR: non-
596 resorpted, R: resorpted.

597 **Figure 6. The administration of DGPP produced changes in the decidua of pregnant**
598 **rats on Dp 8.** Females were injected i.u. with 0.1 mg/kg DGPP (treated horn) and with
599 vehicle (control horn) on Dp 5 and were sacrificed on Dp 8. Control (A, D, E, F, G), non-

600 resorpted (B) and resorpted (C, H, I, J) implantation sites were stained with hematoxylin and
601 eosin. The upper panel has spiked in the area that is amplified. Scale bars represent 500 μM
602 (A, B, C), 100 μM (D), 50 μM (E, H, I) and 10 μM (F, G, J). Dp: day of pregnancy, PDZ:
603 primary decidual zone, AMD: anti-mesometrial decidua, LM: longitudinal muscle, CM: circular
604 muscle, EV: endometrial vessel, EG: endometrial gland, UL: uterine lumen.
605 **Figure 7. DGPP administration did not modify the expression of decidualization**
606 **markers in the implantation sites on Dp 8**. Females were injected i.u. with 0.1 mg/kg
607 DGPP (treated horn) and with vehicle (control horn) on Dp 5 and were sacrificed on Dp 8.
608 The expression of prolactin (A) and IGFBP-1 (B) was assessed by real time RT-PCR in the
609 implantation units from Dp 8. Bars represent the mean ± S.E.M. There was no statistical
610 difference using ANOVA test (Tukey). N=4-6/group. Dp: day of pregnancy, C: control, NR:
611 non-resorpted, R: resorpted.
612 **Figure 8. The injection of DGPP did not affect the uterine histology but produced**
613 **changes in the placentas of pregnant rats on Dp 15.** Animals were injected i.u. with 0.1
614 mg/kg DGPP (treated horn) and with vehicle (control horn) on Dp 5 and were sacrificed on
615 Dp 15. The uteri (A to C) and placentas (G to M) from control (uterus: A, placentas: D, G, H,
616 I), non-resorpted (uterus: B, placenta: E and J) and resorpted (uterus: C, placentas: F, K, L,
617 M) implantation sites were stained with hematoxylin and eosin. Scale bars represent 500 μM
618 (A to F), 50 μM (G to K) and 10 μM (L and M). Dp: day of pregnancy, E: endometrium, CM:
619 circular muscle, LM: longitudinal muscle, L: labyrinth, BZ: basal zone, BD: basal decidua, T:
620 trophoblasts, MS: maternal sinusoids, ME: maternal erythrocytes, FE: fetal erythrocytes, GC:
621 giant cells, GlyC: glycogen cells, ST: spongiotrophoblasts, N: neutrophils, H: hemorrhage,
622 FD: fibrin deposits.
623

Table 1.

Effect of DGPP administration on the resorption rate.

Treatment	Resorption rate (%)
Control (vehicle)	± 0.1[a]
DGPP 0.05 mg/kg	20.1 ± 4.9[a]
DGPP 0.1 mg/kg	64.0 ± 4.8[b]
DGPP 0.2 mg/kg	75.8 ± 5.5[b]

Pregnant rats were injected on Dp 5 with different doses of DGPP i.u. (treated horn) or with vehicle (control horn). Animals were sacrificed on Dp 15 and the resorption rate was calculated. Data are shown as mean ± S.E.M. Different letters indicate statistical significance ($p<0.01$) using ANOVA test (Tukey). N=4-6/group.

632 **Table 2.**

633 Effect of DGPP (0.1 mg/kg) on implantation and resorption rate.

634

Day of gestation	% of animals with resorptions	Number of implantation sites		Resorption rate (%)	
		Control horn	DGPP horn	Control horn	DGPP horn
Dp 8	93	8 ± 2	7 ± 2	1 ± 0.1[a]	53.9 ± 4.2[b]
Dp 15	100	6 ± 1	7 ± 2	1 ± 0.2[a]	64.0 ± 4.8[b]

635

636 Pregnant rats were treated with 0.1 mg/kg DGPP on Dp 5 i.u. (DGPP horn) or with vehicle
637 (control horn). Animals were sacrificed on Dp 8 and 15. Data are presented as mean ± S.E.M.
638 Different letters indicate significant difference between treated groups ($p<0.05$) using Student T
639 test. N = 4-6/group.

640

641 **Table 3.**

642 Effect of DGPP (0.1 mg/kg) administration on the weight of the implantation units on Dp 8 and

643 the uterus and placenta on Dp 15.

644

Weight (mg)			
	Control	Non-resorpted	Resorpted
Implantation sites Dp 8	42,7 ± 5,1	47,5 ± 5,2	50,0 ± 4,9[a]
Uterus Dp 15	18,5 ± 1,4	21,0 ± 1,5	14,7 ± 1,5[b]
Placenta Dp 15	21,6 ± 1,6	19,7 ± 1,5	9,9 ± 1,7[b]

645

646 Females on Dp 5 were injected with 0.1 mg/kg DGPP i.u. (treated horn) and with vehicle i.u.

647 (control horn) and were sacrificed on Dp 8 and 15. Tissues´ weight is expressed in mg. Data are

648 shown as mean ± S.E.M. Different letters indicate significant difference between treated groups

649 using ANOVA test (Tukey). N=4-6/group.

650

651 **Table 4.**

652 Effect of DGPP (0.1 mg/kg) on the number of vessels irrigating implantation sites.

653

	Dp 8		Dp 15	
	Control horn	DGPP horn	Control horn	DGPP horn
N° of implantation sites	6 ± 1	7 ± 1	6 ± 1	7 ± 1
N° of vessels	12 ± 1	14 ± 2	12 ± 1	14 ± 2
N° of vessels/implantation site	2 ± 1	2 ± 1	2 ± 1	2 ± 1

654

655 Pregnant rats on Dp 5 were injected i.u. with DGPP (0.1 mg/kg, treated horn) or with vehicle
656 (control horn). Animals were sacrificed on Dp 8 and 15. Data are represented as mean ± S.E.M.
657 No statistical difference was observed using Student T test. N= 4-6/group.

658

659

660

661

662

663