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Additional Information

Oviductal and endometrial mRNA expression of implantation candidate biomarkers during early pregnancy in rabbit

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Summary

Prenatal losses are a complex problem. Pregnancy requires orchestrated communication between the embryo and the uterus that includes secretions from the embryo to signal pregnancy recognition and secretion and remodelling from the uterine epithelium. Most of these losses are characterized by asynchronization between embryo and uterus. To better understand possible causes, an analysis was conducted of gene expression of a set of transcripts related to maternal recognition and establishment of rabbit pregnancy (uteroglobin, *SCGB1A1*; integrin 1, *ITGA1*; interferon- , *IFNG*; vascular endothelial growth factor, *VEGF*) in oviduct and uterine tissue at 16, 72 or 144 h post-ovulation and insemination. In the oviduct tissue, a significant decrease in the level of *SCGB1A1* mRNA expression was observed from 144 h post-ovulation. In the case of *ITGA1,* the transcript abundance was initially lower, but mRNA expression increased significantly at 72 and 144 h post-ovulation. For *IFNG,* a huge decrease was observed from 16 to 72 h post-ovulation. Finally, no significant differences were observed in the *VEGF* transcript. For the endometrium, the results showed a significant decline in the level of *SCGB1A1* mRNA expression from 16 to 144 h post-ovulation induction. The highest levels of *ITGA1* transcript were detected at 144 h, followed by the 16 h group and lower at 72 h post-ovulation. For *IFNG* there were no significant differences among post-ovulation induction times. Finally, it was possible to observe that *VEGF* mRNA abundance was present at low levels at 16 h post-ovulation and remained low at 72 h, but increased at 144 h. The functional significance of these observations may provide new insights into the maternal role in prenatal losses.

Keywords: Endometrium, Implantation biomarkers, Oviduct, Rabbit.

37 **Introduction**

38

39 Determination of the genetic basis of prenatal survival or the genetic or environmental
40 causes of prenatal losses is a complex problem. Most losses are characterized by
41 asynchronization between embryo and uterus that leads to problems in the process of
42 implantation and/or placentation. The successful establishment and maintenance of
43 pregnancy requires orchestrated communication between embryo and uterus that
44 includes secretions from the embryo to signal pregnancy recognition and secretion and
45 remodelling from the uterine epithelium to support attachment, development, and
46 growth of the embryo.

47

48 Attachment of the embryo to the maternal endomet- rium is considered to be an active
49 process facilitated by the attainment of a period of uterine receptivity. This interval,
50 known as the implantation window, was first suggested by McLaren & Michie (1954).
51 Subsequent comparative studies refined this concept and, in some instances, the
52 differences between species have been very informative (Psychoyos, 1986; Enders,
53 1994; Weitlauf, 1994). Several key maternal factors that may contribute to maximal
54 uterine receptivity have been identified: ultrastructural components such as pinopodes
55 (Psychoyos & Nikas, 1994); steroids or cytokines and growth factors (Pollard et al.,
56 1991; Stewart et al., 1992; Fukuda et al., 1995, Zhu et al., 1998; Hoffman et al., 1998).
57 Rabbits are good experimental models in embryology and developmental biology
58 because of their reproductive characteristics. The precise timing of ovulation (8–10 h
59 after induction) is advantageous for documenting the moment of embryo development,
60 apposition and attachment (Yang & Foote, 1987; Hoffman et al., 1998); several
61 biochemical markers have been described that define the period of receptiv- ity in this

62 species (Denker 1977; Winterhager et al., 1994). In addition, the points of blastocyst
63 attachment to the uterine epithelium are unique structures, known as trophoblastic
64 knobs, and are readily identifiable during early pregnancy (Enders & Schlafke, 1971).
65 This animal model has been studied to examine the expression of several endometrial
66 biomarkers during implantation, such as uteroglobin (Krishnan & Daniel, 1967; Beier
67 1968), MUC-1 (Hoffman et al., 1998), VEGF (Das et al., 1997), integrin (Illera et al.,
68 2003) or cytokines (Muscettola et al., 2003).
69
70 In rabbit, uteroglobin (SCGB1A1) comprises 40– 60% of the total protein from the
71 histotroph uterine secretion on day 5 of pregnancy. These high levels observed close to
72 early events of implantation (Krishnan & Daniel, 1967; Beier 1968) are induced by the
73 progressive increase of progesterone levels together with the decreasing levels of
74 oestrogens in this period (Kopu et al., 1979; Chandra et al., 1980; Muller & Beato,
75 1980; Snead et al., 1981, Shen et al., 1983). Vascular endothelial growth factor (VEGF)
76 is con- sidered to be a potent promoter of vascular endothelial cell proliferation,
77 microvascular endothelial cell pro- liferation and migration associated with neovascu-
78 larization in implantation, embryogenesis, corpus luteus development, ovarian follicle
79 development and tumorigenesis (Chakraborty et al., 1995; Ferrara & Davis-Smyth,
80 1997; Artini et al., 2008). The expression of VEGF in uterine tissue has been detected in
81 many species, including in rabbit at the sixth day of gestation (Llobat et al., 2012a).
82
83 Integrins are a major class of cell adhesion molecules. Both constitutive and cyclical
84 expression of integrins has been observed in the uterus, and they are now considered to
85 be the most decisive criteria for determining uterine receptivity (Lessey et al., 1996).
86 Apical localization of V 3 and V 5 integrins in the mouse, human, baboon,

87 rabbit, pig and sheep luminal epithelium makes these specific integrin pairs appropriate
88 candidates for mediating trophoblast/epithelial interactions (Bowen et al., 1996; Lessey
89 et al., 1996; Fazleabas et al., 1997; Burghardt et al., 2002; Illera et al., 2003). Moreover,
90 the integrin has also been shown on the surface of the blastocyst (Sutherland et al.,
91 1993), so a reciprocal and cooperative role in attachment is suggested. Maternal-
92 embryonic recognition is mainly related to the expression of different cytokines in
93 various species (Sharkey, 1998). Embryos synthesize factors that stimulate the
94 production of cytokines and prevent local activation of cytotoxic cells. Some of these
95 cytokines are interferons (IFN) that have been linked to pregnancy recognition (IFN-
96 in pigs, IFN- and IFN- in humans, 48 and IFN- in rabbits or IFN- in
97 ruminants) (Cross & Roberts, 1989; Aboagye- Mathiesen et al., 1995; Muscettola et al.,
98 2003; Spencer et al., 2004). Moreover, IFNs are involved in the angiogenesis process
99 and the activation of natural killer cells (IFN- in mouse or IFN- in rabbits) (Krusche
100 et al., 2002; Murata et al., 2005; Godornes et al., 2007).
101

102 The aim of the present study was to evaluate the mRNA expression of a set of
103 transcripts related to maternal recognition and the establishment of early rabbit
104 pregnancy (uteroglobin, SCGB1A1; integrin 1 , ITGA1; interferon- , IFNG;
105 vascular endothelial growth factor, VEGF) in oviduct and uterine tissue at 16, 72 or 144
106 h post-ovulation.
107

108 Materials and methods Animals

109 Twenty four nulliparous does belonging to the New Zealand White line from the ICTA
110 at the Polytechnic University of Valencia (UPV, Spain) were used to obtain
111 preimplantation oviduct and uterus tissues. All experimental procedures involving

112 animals were approved by the Research Ethics Committee of the UPV and licensed by
113 the European Community Directive 86/609/EC.
114
115 Donor females were inseminated with 0.5 ml of fresh heterospermic pool semen at a
116 rate of 40 × 106 spermatozoa/ml in Tris–citric–glucose extender (Viudes-De-Castro &
117 Vicente, 1997). Motility was ex- amined at room temperature under a microscope with
118 phase-contrast optics at ×40 magnitude. Only those ejaculates with >70% motile sperm
119 (minimum re- quirements commonly used in artificial insemination) were pooled
120 (Marco-Jiménez et al., 2010). Immediately after insemination, ovulation was induced by
121 an intramuscular injection of 1 g buserelin acetate
122
123 **Oviduct and uterus tissue recovery**
124 Eight samples from both tissues (oviduct and uterus) were recovered for each
125 experimental group. Donor does were slaughtered at 16, 72 or 144 h after insemination
126 and induction of ovulation. Oviduct and uterine samples were obtained by gently
127 scraping from the ampulla section and endometrium and plunged into Trizol reagent
128 (Invitrogen S.A, Barcelona, Spain).
129
130 **RNA extraction and reverse transcription**
131 Total RNA was extracted using the traditional phenol/chloroform extraction method by
132 sonication of samples in Trizol reagent (Invitrogen S.A, Bar- celona, Spain). To prevent
133 DNA contamination, one deoxyribonuclease treatment step (gDNA Wipeout Buffer,
134 Qiagen Iberia S.L., Madrid, Spain) was performed from total RNA (1000 ng).
135 Afterwards, reverse transcription was carried out using a Reverse Transcriptase (RT)
136 Quantitect kit (Qiagen Iberia S.L.) according to the manufacturer's instructions.

137 SYBR R Green assay (quantitative real-time polymerase chain reactions)

138 Real-time PCR were conducted in an Applied Biosystems 7500 PCR system (Applied
139 Biosystems, Foster City, CA, USA). Every PCR was performed from 5 l diluted 1:40
140 cDNA template, 250 nM of forward and reverse specific primers (Table 1) and 10 l of
141 PowerSYBR Green PCR Master Mix (Fer- mentas GMBH, Madrid, Spain) in a final
142 volume of 20 l. The PCR protocol included an initial step of 50 °C (2 min), followed
143 by 95 °C (10 min) and 42 cycles of 95 °C (15 s) and 60 °C (60 s). After real- time PCR,
144 a melting curve analysis was performed by slowly increasing the temperature from 65–
145 95 °C, with continuous recording of changes in fluorescent emission intensity. The
146 amplification products were confirmed by SYBR Green-stained 2% agarose gel
147 electrophoresis in 1× bionic buffer. Serial dilutions of cDNA pool made from several
148 samples were done to assess PCR efficiency. A Ct method adjusted for PCR
149 efficiency was used (Weltzien et al. 2005), employing the geometric average of H2AFZ
150 (H2A histone family member Z) and GAPDH (glyceraldehyde-3-phosphate
151 dehydrogenase) as housekeeping normalization factor. Target and reference genes in
152 unknown samples were run in duplicate. The expression of a cDNA pool from various
153 samples was used as a calibrator to normalize all samples within one PCR run or
154 between several runs.

155

156 **Statistical analysis**

157 After data normalization by logarithm transformation, the differences in mRNA
158 expression among different post-ovulation induction times in both tissues (oviduct or
159 uterus) were analysed by one-way analysis of variance (ANOVA), using the General
160 Linear Models (GLM) procedure of Statgraphics Plus 5.1. Significance was taken as a
161 P-value <0.05.

162 **Results**

163 The relative transcript abundance of SCGB1A1, VEGF, ITGA1, and IFNG for oviduct
164 and uterine tissues among different post-ovulation induction times are shown in Figs 1
165 and 2, respectively.

166

167 In the oviduct tissue, a significant decrease in the level of SCGB1A1 mRNA expression
168 was observed from 72 to 144 h post-ovulation. In the case of ITGA1, the transcript
169 abundance was lowest at 16 h post-ovulation, but the mRNA expression increased
170 significantly at 72 and 144 h. For IFNG, a huge decrease was observed from 16 to 72 h
171 post-ovulation, but this mRNA expression did not remain low and increased at 144 h.
172 Finally, no significant differences were observed in VEGF transcript abundance
173 between experimental days (Fig. 1).

174

175 For uterine tissue, the current results showed a significant decline in the level of
176 SCGB1A1 mRNA expression from 16 to 72 h post-ovulation induction. The highest
177 levels of ITGA1 transcript were detected at 144 h, followed by 72 h. In the case of
178 IFNG, the mRNA expression pattern was similar to oviduct tissue, and a decrease was
179 observed from 16 to 72 h post-ovulation followed by an increase at 144 h. Finally, it
180 was possible to observe that VEGF mRNA abundance was present at low levels at 16 h
181 post-ovulation and remained low at 72 h, but the level increased at 144 h (Fig. 2).

182

183 **Discussion**

184

185 In rabbits, losses from ovulation to days 6 to 7 post- insemination have been estimated
186 at 8–14% (Adams, 1960; Mocé et al., 2002; Llobat et al., 2012b). From fertilization to

187 implantation, embryonic development is influenced during its migration by the maternal
188 environment (Fleming et al., 2004). As the current results show, the oviduct exhibits a
189 spatial-temporal pattern of transcripts involved in peri-implantation events. In rabbits,
190 the embryo remains in the oviduct from fertilization until days 3 to 4 of development.
191 During these days, the zygote should be converted into a competent embryo for
192 implantation, requiring several changes such as cell cleavage divisions, activation of the
193 embryonic genome, segmentation and compaction of the morula and blastocyst
194 formation (Lonergan et al., 2003). Carney et al. (1990) found that co-culture of rabbit
195 zygotes with rabbit oviduct epithelial cells increased blastocyst formation. Ovarian
196 steroids, growth factors, glucose, lactate, pyruvate, proteins, cholesterol, phospholipids
197 and ions as sodium, potassium, chloride and calcium have been found in oviduct fluid
198 (Leese, 1988; Henault & Killian, 1993; Grippo et al., 1994; Killian, 2004; Aviles et al.,
199 2010; Vecchio et al., 2010) and several reports have confirmed that this support of
200 oviduct secretions to embryo development are not species specific (Minami et al., 1994;
201 Lai et al., 1996; Yadav et al., 1998; Lloyd et al., 2009). In the current experiment, the
202 mRNA expression of a set of genes (SCGB1A1, ITGA1, IFNG and VEGF) associated
203 with maternal recognition and establishment of rabbit pregnancy was examined. The
204 specific hours (16, 72 and 144 h post-induction of ovulation) were selected because at
205 16 h the zygotes are in the oviduct, at 72 h the morulae or early blastocysts are exiting
206 the oviduct and entering the uterus, and finally at 144 h the late blastocyst are in the
207 uterus before the onset of gastrulation and adhesion to endometrium. As expected, the
208 gene expression pattern of the oviduct changed from 16 to 144 h post-ovulation
209 induction. It seemed that after ovulation the oviduct started to prepare the best case
210 scenario to carry out the first steps of preimplantation development, by maintaining or
211 increasing the quantity of crucial molecules such as uteroglobin, integrins or growth

212 factors. Although the uteroglobin gene (SCGB1A1) was first identified in rabbit as a
213 specific uterus protein, previous studies have detected mRNA expression in the oviduct
214 (Kay & Feigelson, 1972). As stated previously, studies that focussed on regulation of
215 uteroglobin in the uterus have identified that progesterone had the ability to induce it
216 and oestrogen to repress it.

217 However, in the case of the oviduct, it has been detected that SCGB1A1 expression was
218 induced by oestrogen, not progesterone (Mukherjee et al., 2007), a finding that could
219 explain why its expression is higher at 16 h than at 144 h post-ovulation induction.
220 Integrins comprise a large family of heterodimeric transmembrane receptors linked with
221 a great variety of extracellular matrix ligands. Regulation of the transport and stability
222 of gametes and early embryos in the oviduct requires the support of cell adhesion
223 molecules and, for this reason, it was possible to observe an increase in mRNA
224 expression of ITGA1 from 72 h. It is well known that interferons have a multipotential
225 role in the immune response through- out pregnancy. In particular, successful pregnancy
226 requires a protective immunomodulatory mechanism, including a reduction in
227 inflammatory and cytotoxic reactions mainly carried out via IFNG, IL-2 and TNF
228 (Druckmann & Druckmann, 2005). As the expression of IFNG is considered an
229 immunoreaction related to pregnancy failure, it could be posited that transcript
230 abundance was reduced significantly at 72 h in order to avoid embryo abortion.
231 Moreover, it has been suggested that IFN- is also enhanced by oestrogens (Platt &
232 Hunt, 1998), a suggestion that would correlate with the high transcript abundance
233 observed after ovulation. Regarding VEGF mRNA expression, no differences were
234 found in the oviduct tissue between post-ovulation and preimplantation stage. The
235 current results complemented the observations by Wijayagunawardane et al. (2005),
236 which showed that, after ovulation, the elevated VEGF mRNA expression is

237 immediately downregulated by negative feedback regulation; the current results suggest
238 that this expression remains constant in the days before implantation.
239 To establish embryo–uterine cross-talk and begin the implantation process, the uterus
240 must differentiate into a receptive state (Paria et al., 2001). This change means that the
241 endometrial epithelium is functionally and structurally ready to accept the embryo for
242 implant- ation (Salilew-Wondim et al., 2012). The importance of progesterone in
243 pregnancy recognition and uterine receptivity has been studied widely in many species;
244 inadequate progesterone levels could reduce the ability of the uterus to support embryo
245 development (Rizos et al., 2010; Salilew-Wondim et al., 2012). Apart from ovarian
246 hormones, there are other components such as growth factors, cytokines, chemokines
247 and adhesion molecules, among others, that participate in this dialogue between
248 endometrium and embryo (van Mourik et al., 2009); any modification or absence of
249 these molecules may hinder the implantation process. The results of the present research
250 agree with previous studies that reported the presence of uteroglobin in the uterus
251 during early pregnancy (Peri et al., 1995). In particular, this uteroglobin has been
252 associated with cell proliferation and stimulation of blastocyst growth (Beier, 2000;
253 Riffo et al., 2007; Mukherjee et al., 2007). Previous studies have detected mRNA
254 expression in rabbit blastocysts embryos (Saenz-de- Juano et al., 2012; Naturil-Alfonso
255 et al., 2013), so the synthesis of this protein by the embryo itself could explain the
256 decrease in mRNA expression observed from 72 to 144 h in the uterine tissue. Integrins
257 are considered to be immunohistochemical markers of uterine receptivity (Lessey,
258 1998), and it has been observed that they could be expressed in the endometrium either
259 constitutively or in a cycle- dependent manner. Recently, Tesfaye et al. (2011) analysed
260 the endometrial gene expression of heifers that eventually resulted in calf delivery and
261 those that resulted in no pregnancy, and observed that expression of integrins was up-

regulated in successfully pregnant heifers. Interferons have a crucial role in the uterine immune system and make both implantation and maintenance of pregnancy possible (Szekeres-Bartho, 2002). In the current experiment, as occurs in the oviduct tissue, a significant decrease was observed in IFNG gene expression from 16 to 72 h or 144 h post-ovulation induction; this decrease was also correlated with high progesterone levels at these stages. Finally, in the case of VEGF, up-regulation in expression of this transcript was found in uterus tissue at 144 h. VEGF has been associated with the process of de novo angiogenesis (Lee & DeMayo, 2004); its expression and function has been regarded as ensuring a suitable vasculogenesis during implantation and early placentation (Torry et al., 2007). So, as occurs for the ITGA1 gene, its importance grows as the implantation window approaches.

To understand why prenatal mortality continues to occur, it is important to characterize the causes from a biological point of view. The examination of biochemical changes and gene expression patterns of the oviduct and uterus in the presence of gametes or embryos could help us understand the molecular mechanisms of oviduct–oocyte, oviduct–embryo and uterus–embryo interactions.

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288

289 **Conflict of interest**

290 There are no conflicts of interest.

291

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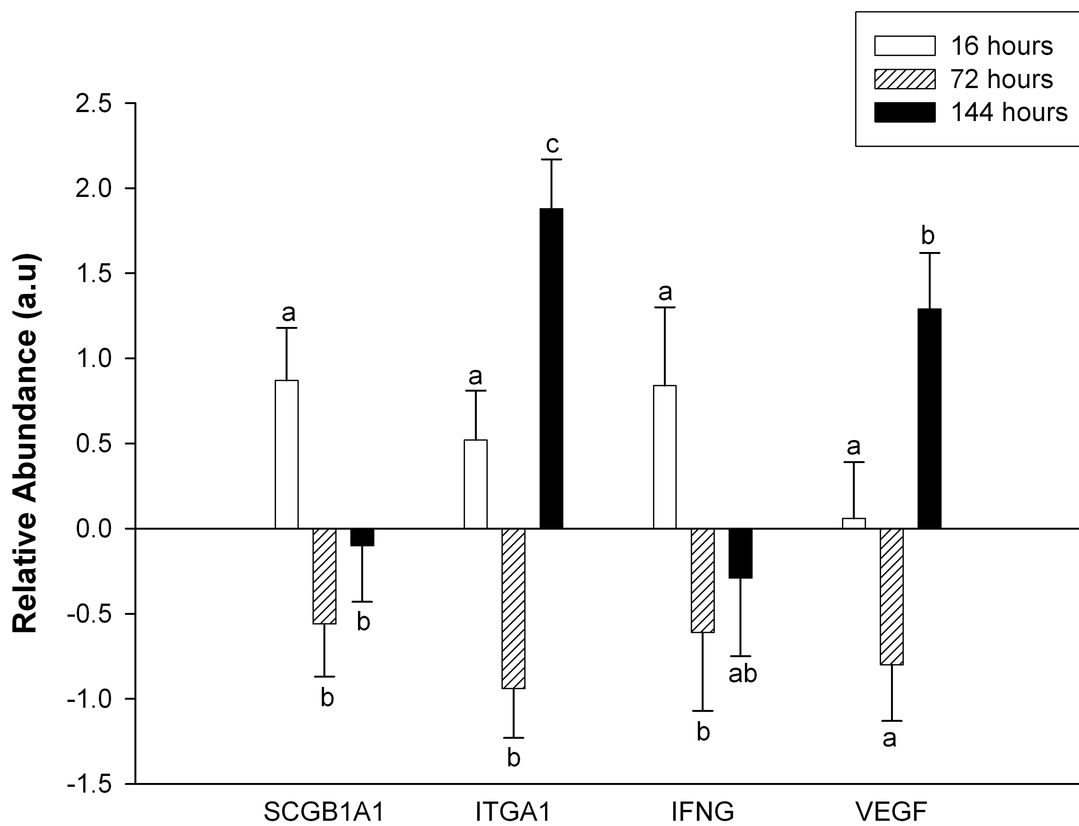
16 hours
72 hours
144 hours
Relative Abundance (a.u)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
a
b
b
a
b
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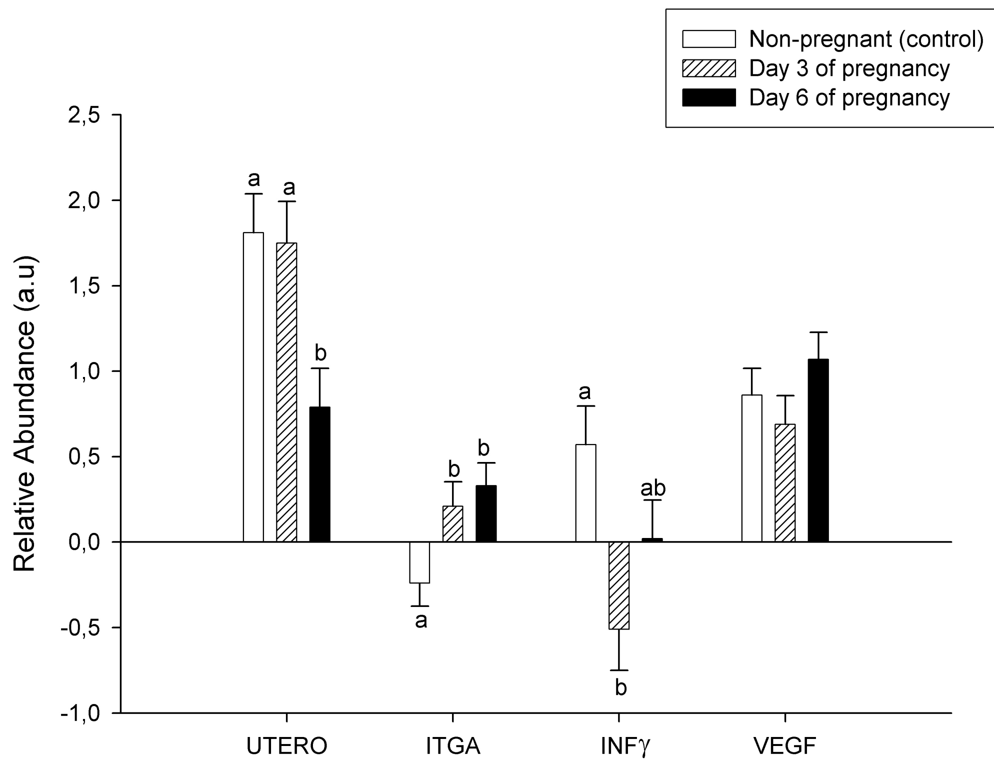
SCGB1A1
ITGA1
IFNG
VEGF


Non-pregnant (control)
Day 3 of pregnancy
Day 6 of pregnancy
Relative Abundance (a.u)
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
a
a
b
b
b
a
a
ab
b
UTERO
ITGA
INFγ
VEGF