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ELSEVIER

INTERSPECIES PREGNANCY OF SPANISH IBEX (Capra pyrenaica) FETUS IN DOMESTIC GOAT (Capra hircus) RECIPIENTS INDUCES ABNORMALLY HIGH PLASMATIC LEVELS OF PREGNANCY-ASSOCIATED GLYCOPROTEIN

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ABSTRACT

Interspecies embryo transfer could be a valuable tool in preservation programs of endangered species. In this work the results of both interspecific-monospecific (ibex-in-goat) and interspecific-bispecific (mixed-species; ibex+goat-in-goat) embryo transfers in the capra genus are reported. The aim of this work was to compare the PAG plasmatic profiles occurring in these interspecific gestations to those encountered in normal (i.e. intraspecies) pregnancies of Spanish ibex and domestic goat. Spanish Ibex females were superovulated with 9 mg NIADDK-oFSH-17 and embryos were surgically collected 5.5 d after estrus. Two embryos were transferred per recipient. Domestic goat recipients were previously mated either to vasectomized domestic bucks (n=17 females; interspecific-monospecific gestations) or to fertile ones (n=9 females; interspecific-bispecific gestations). Intraspecific pregnancies were obtained by natural mating between males and females of the same species (Spanish ibex: n=6; domestic goat: n=1). Pregnancy rate diagnosed by progesterone was low in both interspecific-monospecific (7/17) and interspecific-bispecific (3/9) transfers. None of the monospecific (0/7) and 2 (2/3) of the bispecific established pregnancies developed to term. Ibex-in-ibex PAG profile showed 2 similar peaks of 60 to 70 ng/mL on Days 34 and 153 of pregnancy, while goat-in-goat had the maximum value (60 to 70 ng/mL) at Day 50, decreasing slightly afterwards until parturition. Mixed-species gestations (ibex+goat in goat) showed a first peak of 500 to 1000 ng/mL on Day 70 and a second one (200 to 500 ng/mL) on Day 140 of pregnancy. Four ibex-in-goat gestations that terminated with the expulsion of dead fetuses at Days 110 to 170 had their maximum PAG values (100 to 700 ng/mL) on Days 60 to 90. We conclude that it is possible to achieve pregnancies after transfer of ibex embryos into domestic goats, but this requires a great change of the PAG profiles, which increase significantly. Live ibex kids can be produced when embryos from both species share the uterus. This is the first report of successful interspecific pregnancies in the capra genus.

Key words: interspecific transfer, xenogenic embryo, PAG, Spanish ibex, domestic goat

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INTRODUCTION

The transfer of embryos of a given species to the uterus of a different species is performed in animal conservation programs where there is a lack of suitable number of recipients. It has been suggested that interspecies embryo transfer could be a key technique in the conservation of endangered species (24). This type of transfer is also done in basic research studies on development of interspecific gestations (1,23).

It is known that compatibility between different species is only partial in most cases. So, oocytes of some species can be matured and fertilized in the oviduct of a different species (11), and the resulting embryos can develop normally once transferred to the reproductive tract of a female of their own species (33). Mammalian embryos are normally tolerant and tolerated in a different species oviduct until they reach the blastocyst stage, the moment at which a high embryo mortality rate is seen (2). The vast majority of interspecies embryo transfers are unsuccessful, and in those cases were a few gestations are achieved most do not develop to term. In the genus Ovis, there has been success in the transfer to domestic sheep (Ovis aries) recipients of embryos from mouflon (Ovis gmellini mussimon) (8) and Armenian red sheep (O. orientalis) (9) but not from Dall's sheep (O. dalli) (7). In the genus Equus, the transfer of horse embryos to donkey females is more successful than that of donkey embryos to mares (3).

The mechanisms that allow for or prevent the successful outcome in an interspecies embryo transfer are not well understood. Immunological rejections and placental incompatibility have been pointed out as 2 primary causes of pregnancy failure in this type of transfer (5).

Pregnancy-associated glycoproteins (PAGs) are members of the aspartic proteinase gene family that are released by trophoblast cells in several species, including goats (38,19). Bovine PSPB is either identical or closely related to bovine PAG1 (25). Although their biological action is not known, it is considered a useful tool for pregnancy diagnosis in those species that secrete it (40,42). Their function could include binding and transport of bioactive peptides (27), acting as hormones that bind to specific aminoacid sequences on receptor molecules (27), luteotropic or luteoprotective agents (10,37), regulators of placental steroidogenesis (35) or immunomodulators (42).

The aim if this study was to compare the PAG plasmatic profiles that appear during the interspecific gestations of Spanish ibex fetus in domestic goat recipients to those encountered in normal (i.e., intraspecies) pregnancies of Spanish ibex and domestic goat.

MATERIALS AND METHODS

Animals and Experimental Design

Interspecific monospecific pregnancies (ibex-in-goat) were achieved by transfer of 2 Spanish ibex embryos (Capra pyrenaica hispanica) to each of 17 domestic goats (Capra hircus; mixed breeds, 1.4 kids born/pregnant goat in our conditions) mounted by vasectomized domestic bucks.

Interspecific bispecific pregnancies (ibex+goat-in-goat) were attained by transfer of 2 Spanish ibex embryos to each of 9 pregnant domestic goats that had been previously mated to fertile

domestic bucks. This experimental design was similar to a previous work, where mixed-species Bos Taurus-Bos Indicus twin calves were obtained by reciprocal transfers of embryos to females that had been previously inseminated with semen from the same species (31).

Intraspecific pregnancies (ibex-in-ibex or goat-in-goat) were achieved by natural mating between males and females of the same species (Spanish ibex: n=6 and domestic goat: n=1).

Estrus Synchronization and Superovulation of Spanish Ibexes (Embryo Donors)

Estrous cycles were synchronized with 45 mg FGA sponges[a] for 17 d (n=29 females). Superovulation was achieved with 9 mg NIADDK-oFSH-17[b] equivalents (14). Estrus was assessed with the aid of harnessed fertile ibex males.

Synchronization and Detection of Estrus in Domestic Goats (recipients)

Recipients were synchronized with the same progestagen treatment as donors. A single dose of PMSG[c] (375 IU, im) was given at sponge withdrawal. Estrus was checked with either vasectomized (to achieve monospecific pregnancies) or fertile domestic bucks (to achieve bispecific pregnancies).

Embryo Recovery and Transfer

Animals were anesthetized with 250 mg of a mixture 1:1 w/w of tiletamine and zolazepam[d] injected in the jugular vein. Embryos were surgically flushed (32) 5.5 d after estrus.

Embryo quality was morphologically assessed (30,12). Only Grades 1 and 2 embryos were transferred. Fifty-two Spanish ibex embryos were transferred: 40 (76.9%) were Grade 1 and 12 (23.1%) were Grade 2.

The highest asynchrony between the estrus onset of donor and recipient was 18 h. Embryos were transferred to recipients showing one corpus luteum considered normal by endoscopy. Embryos were placed into the uterine horn ipsilateral to an ovulated ovary with the aid of a tomcat catheter.

Blood Sampling

The evolution of the plasmatic PAG levels was studied in 6 Spanish ibex that kidded viable kids and also in 7 domestic goats. From these domestic goats, 1 carried a normal pregnancy (its own domestic kid; goat-in-goat), and served as a control animal. Four of the remaining 6 domestic goats carried Spanish ibex fetuses (monospecific pregnancy: ibex-in-goat), and the remaining 2

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[a] Chrono-gest; Intervet, Salamanca, Spain.
[b] Ovagen; ICP, New Zealand.
[c] Foligon; Intervet, Salamanca, Spain.
[d] Zoletil 100, Virbac, France.

carried a Spanish ibex fetus and a domestic kid fetus (bispecific pregnancy: ibex+goat-in-goat).

Females diagnosed as pregnant by progesterone[e] and/or real-time transabdominal echography[f] were bled weekly until about 1 month after the termination of pregnancy. Blood samples were taken from the jugular vein with heparinised evacuated blood collection tubes.[g] The samples were immediately centrifuged and the plasma stored at -20 °C until PAG was assayed by RIA.

PAG Radioimmunoassay (RIA)

PAG analysis was performed by means of a heterologous RIA, because purified caprine PAG was not yet available. Purified bovine PAG (42) was used for standards and tracer (125-iodine labeled bovine PAG; bPAG). The antibody was raised in rabbits immunized against ovine PAG (batch # 495; 39). The suitability of another heterologous assay, using an antibody anti-bPAG, was previously proved in sheep (17, 18) and domestic goats (16). The second antibody (sheep anti-rabbit gamma-globulin) was raised in rams and was covalently linked to cellulose in order to facilitate the separation of the bound and free fractions by centrifugation.

The assay protocol is a modification of that described in cows (42) and sheep (26). Briefly, plasma samples and standards (100 µL) were incubated overnight at room temperature with the first antibody in TRIS-BSA buffer, pH=7.5 (total volume: 400 µL). Next morning, 100 µL of radiolabeled PAG (about 26,000 CPM) were added. The purpose of this delayed addition of the tracer is to enhance assay sensitivity. After 4 h of incubation, 1 mL of a suspension of the second antibody was added. Two hours later, the tubes were centrifuged for 10 min at 2000 g. The supernatant was discarded and the precipitate washed with 2 mL of TRIS-BSA buffer followed by a second centrifugation and elimination of the supernatant. Radioactivity in tubes (bound phase) was measured in a gamma counter.[h]

All samples were analyzed in duplicate. Those with a concentration higher than 100 ng/mL (the highest standard) were re-assayed after appropriate dilution with lamb plasma (PAG free). Intra- and inter-assay coefficients of variation were 5.4 and 14.4 %, respectively. Sensitivity was 1.25 ng/mL.

RESULTS

Superovulation and Embryo Yield

The percentage of ovulated ibexes was 62.1%. Ovulation rate in these females averaged 5.9 corpora lutea per ibex. Recovery rate (embryos and unfertilized oocytes/corpora lutea) was 69.8%, of which 70.3% were considered transferable.

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[e] ^{125}I-Progesterone Coatria®, bioMérieux sa, France.

[f] Sonolayer Sal-32B, 5 MHz, Toshiba, Japan.

[g] Venoject, Terumo Europe N. V., Belgium.

[h] CompuGamma 1282, LKB Wallac, Turku, Finland.

Interspecies Pregnancies Success

Results of the transfer of Spanish ibex embryos to domestic goats are shown in Table 1. Thirty-eight percent of the recipients were diagnosed as pregnant by progesterone on Day 21 after estrus. Eighty percent of these gestations terminated expelling a dead fetus.

Table 1. Results of the transfer of Spanish ibex embryos to domestic goats

Type of interspecific gestation	Number of domestic goat recipients	Number of domestic goat recipients carrying Spanish ibex fetus(es)	Number of domestic goat recipients that aborted	Domestic goats kidding live Spanish ibex kids
Monospecific[a]	17	7	7	0
Bispecific[b]	9	3	1[c]	2
TOTAL	26	10	8	2

[a] Monospecific interspecific gestation: Domestic goat recipient carrying only Spanish ibex fetuses (ibex-in-goat).

[b] Bispecific gestation: Domestic goat recipient carrying Spanish ibex and domestic goat fetuses.

[c] The goat died of uterine prolapse at d 140 of pregnancy.

Ibex embryo survival was null in interspecific monospecific gestations (domestic goat carrying only ibex embryos; ibex-in-goat). In the 9 domestic goat recipients mounted by fertile domestic bucks and receiving 2 ibex embryos, 4 did not become pregnant. Of the remaining 5 females, 2 delivered only their own domestic kids (goat-in-goat). The remaining 3 goats had bispecific gestations (domestic goat carrying an ibex fetus and its own domestic goat fetus simultaneously; ibex+goat-in-goat). One of these domestic goats died of uterine prolapse before parturition (Day 140), and each of the remaining 2 goats kidded their own domestic kid and a transferred ibex kid. In this pair of twin gestations, kidding took place at 150 d (i.e., the normal gestation length of a domestic goat). Since the gestation length of the ibex is 162.4 ± 2.2 d (15) (161.8 ± 1.9 in the present work), ibex kids were premature, presented respiratory distress, and needed intensive care to survive.

PAG Levels in Normal (Intraspecific) Pregnancies (Ibex-in-Ibex or Goat-in-Goat)

Figures 1A and 1C show the mean profile of plasmatic PAG throughout normal (intraspecific) gestations of 6 Spanish ibexes and 1 domestic goat, respectively. Spanish ibex gestations presented 2 PAG peaks at 34 ± 5.1 and 153 ± 7.3 d of gestation (mean ± SD). In contrast, the domestic goat presented a PAG peak at Day 50, decreasing slowly until parturition.

PAG Levels in Interspecific Bispecific Gestations (Domestic Goats Carrying both Ibex and Domestic Fetuses: Ibex+Goat-in-Goat)

PAG profiles of the 3 studied successful interspecific-bispecific gestations are represented in Figure 1B. Similarly to the PAG profiles of normal (ibex-in-ibex) gestations in the Spanish

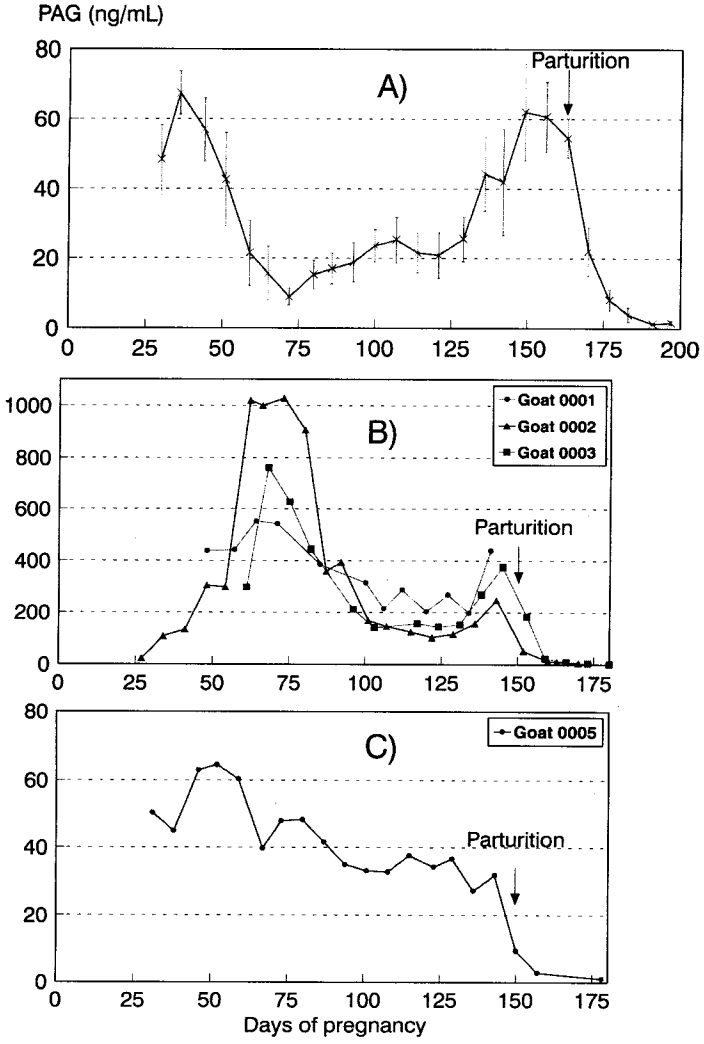

Figure 1. A) Plasmatic PAG levels (means ± SEM) throughout the natural gestation (intraspecific: ibex-in-ibex) of Spanish ibex (n=6). B) PAG profiles of 3 domestic goats that were carrying both a Spanish ibex and a domestic goat fetuses (bispecific pregnancy: ibex+goat-in-goat). Goat 0001 died of uterine prolapse at Day 140. Four kids (2 ibexes and 2 domestic kids) from Goats 0002 and 0003 were born live. C) Plasmatic PAG levels throughout the natural (intraspecific: goat-in-goat) gestation of a domestic goat (n=1).

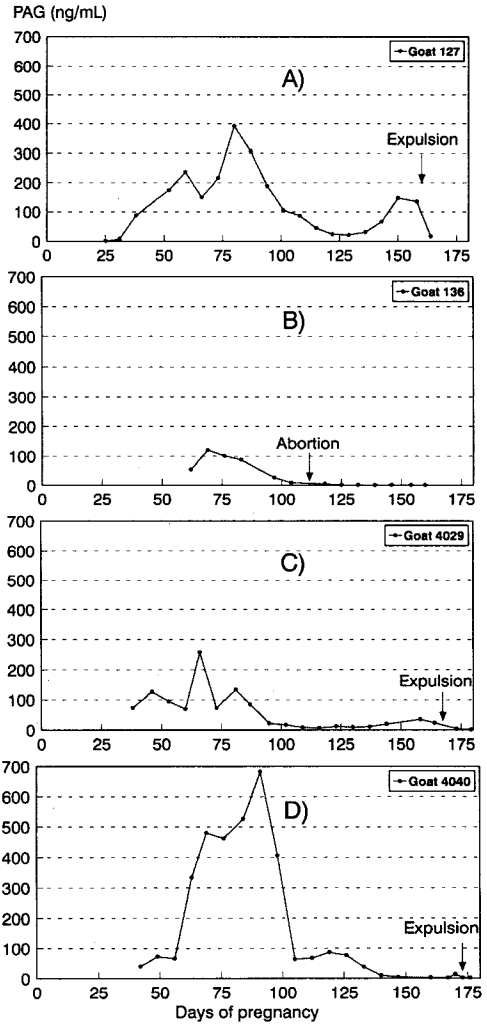

Figure 2. PAG levels throughout gestation in 4 domestic goats carrying only ibex fetuses (ibex-in-goat): A) Goat 127 delivered at Day 160 of pregnancy a well-formed fetus without any sign of autolysis but that did not come to breathe as evidenced at necropsy. B) Goat 136 aborted 2 autolytic fetuses with fetal malnutrition signs at Day 112 of pregnancy. C) Goat 4029 expelled 2 autolytic fetuses with fetal malnutrition signs at Day 167 of pregnancy. D) Goat 4040 expelled 1 autolytic fetus with fetal malnutrition signs at Day 173 of pregnancy.

ibex (Figure 1A), they each had 2 defined peaks and these profiles were markedly different from that of the domestic goat (Figure 1C). The most striking difference is that the PAG levels observed in these 3 interspecific-bispecific gestations were about 10 times higher than those from intraspecific gestations (either ibex-in-ibex or goat-in-goat). Another difference is that the first PAG peak was delayed 40 d in the 3 successful interspecific-bispecific gestations when compared with that of an intraspecific (i.e., normal) Spanish ibex gestation (ibex-in-ibex).

PAG Levels in Interspecific Monospecific Gestations (Domestic Goats Carrying Only Spanish Ibex Fetuses: Ibex-in-Goat)

None of the 7 domestic goats diagnosed as pregnant resulted in live offspring. One goat (1/7) delivered at Day 160 of pregnancy a well-formed fetus without any sign of autolysis but that did not come to breathe as evidenced at necropsy. The remaining goats (6/7) either aborted or expelled uterine contents and autolytic fetuses with fetal malnutrition signs at the expected time of parturition. Cotiledonary villi of placentas proceeding from interspecific gestations were longer in comparison to villi of placentas from intraspecific (normal) gestations. In 4 of these females PAG levels throughout gestation were studied, and the values are represented in Figure 2. These levels were greater than those from intraspecific (ibex-in-ibex or domestic-in-domestic) pregnancies.

DISCUSSION

In this study the success in the interspecies embryo transfer of Spanish ibex embryos to domestic goats has been low, but we have demonstrated that these pregnancies can be achieved and lead to live offspring at least when a domestic fetus is also present in the uterus.

Only one third of the domestic goat recipients became pregnant, and most of these gestations did not result in live offspring. Similarly, gaur (Bos gaurus) embryos transferred to domestic cows (Bos taurus) established pregnancies (6 out of 9), most of which (4 out of 6) were aborted (28,29).

Ibex embryo survival was null in monospecific interspecific gestations (domestic goat carrying only ibex fetuses: ibex-in-goat). The same occurred in the transfer of Dall's sheep embryos to domestic sheep, in which 37% of the recipients became pregnant but all the females aborted (7). It has been suggested that chances of success for an interspecies embryo transfer are maximized when the 2 species involved hybridize (13). We had no success in spite of we have proved that hybrids (Spanish Ibex x domestic goat) can be obtained, either by artificial insemination or natural mating to domestic goats, and the resulting hybrid females are fertile when mated to Spanish Ibex males (data not shown; preliminary results). At present, we have not proven whether the hybrid males are fertile or not.

Conversely, the 2 established bispecific gestations (domestic goat carrying ibex and domestic goat fetuses simultaneously: ibex+goat-in-goat) in which the recipient goat survived produced live ibex and domestic kids. It seems that the domestic embryo acted as a probable "helper embryo" in the survival of the ibex embryo, but the numbers were too small to conclude this point. To our knowledge, the unique precedent in which embryos of the same species as the mother helped in the establishment of "non allowed by natural law" pregnancies is the work by Bratanov and Dikov (6). In their work, (sheep x goat) hybrids survived to term only when twinned with embryos of the maternal species and dams were pretreated with blood and semen from the heterologous genus.

Other authors were unable to achieve similar results by transfer of hybrid embryos to previously mated ewes or goats, although they did not precede the transfer procedure by any pretreatment of the dams with heterologous blood or semen (21).

Other bispecific gestations have been achieved as are the cases of domestic cows carrying both Bos taurus and Bos indicus fetuses (31) and jennies carrying both donkey and horse fetuses (4). Nevertheless, it should be remarked that in these two cases interspecific monospecific gestations are possible (31,3).

In this study, we have found that the shape of the PAG curve from interspecific gestations resembled that of the normal pregnancy of the Spanish ibex, showing 2 peaks separated by an intermediate zone of lower values. As PAG is secreted by cells of the fetal trophoblast (41) it would be reasonable to think that the PAG plasmatic profile of interspecific pregnancies should resemble that of the fetus species.

In the bispecific gestations (ibex+goat-in-goat) studied in this work, the measured PAG is a mixture of domestic and ibex PAGs. As PAG levels in domestic natural pregnancy are about 10 times lower than those from interspecific monospecific pregnancies (ibex-in-goat), it can be supposed that the profile observed in bispecific pregnancies is mainly due to PAG from the ibex fetus.

In the interspecific monospecific gestations (ibex-in-goat) achieved in this study the expelled fetuses were more developed in those cases in which the PAG curve resembled that of the ibex (presenting 2 different peaks) and had higher values.

Granulated binucleate cells account for 15-20% of cells in the trophectodermal epithelium of the ruminant placenta (36). Binucleate cells differentiate in trophectoderm just prior to the onset of implantation, and are directly involved in nidation and subsequent formation of the placenta (22). In goats, these cells first appear 18 d after mating, migrating to the microvillar junction at Day 19 where fuse with individual uterine epithelial cells to form hybrid feto-maternal trinucleate cells and syncytial plaques. This fusion is suggested to be important in facilitating the delivery of characteristic granules of the trophectodermal binucleate cells to the base of the uterine epithelial layer for subsequent exocytosis of their contents into maternal tissue (34). The elevated levels of PAG observed in all interspecific pregnancies observed in this study may reveal that this mechanism is intact, or perhaps enhanced, even in those gestations that failed.

The high levels of PAG observed in the interspecific gestations could be due either to increased numbers of binucleate cells and/or to increased PAG production of such cells. Bovine PAG is localized mainly in the cytoplasm of large binucleate cells present predominantly in fetal cotyledonary tissue (villi) (41). As we have observed that villi were longer in placentas from interspecific gestations than in those from intraspecific (i.e., normal) ones, we hypothetize that such elevated levels of PAG were caused by an increased amount of binucleate cells, but this must be taken with caution. Intervention during pregnancies to obtain placental and uterine tissues to examine the status of binucleate cells could have put some light into this subject, with the drawback of disturbing these rare and valuable gestations.

On the other hand, it is not known whether there is any target organ/cell in the mother for PAG or not, nor whether the secretion (synthesis/release) of PAG is regulated in any way. Assuming that PAG secretion was regulated by feed-back mechanisms between the mother and the fetus, the high levels of PAG observed here could also be likely due to a weak recognition of the ibex PAG by the recipient domestic goat. A longer half-life of plasmatic PAG in these gestations cannot be discarded.

As the biological function of PAG remains still unknown, the significance of the high levels of PAG in the interspecific gestations observed in this study can not be explained. It might be a reflect of the hard efforts made by the xenogenic fetus to survive in a hostile medium, perhaps suffering a severe fetal distress. Similarly to our work, recipient cows carrying female fetuses of a different breed had PAG values about 3 times higher than those carrying fetuses of its own breed (42). In that work, it was postulated that PAG constitutes a protective mechanism that either masks the trophoblast antigens from maternal immune recognition or suppresses or neutralizes any maternal reaction that would otherwise be stimulated in later pregnancies (3). Our results reinforce this hypothesis. From these evidences we believe that PAG could be involved in the maternal immunotolerance of the embryo. On the other hand, as it has also been observed that prepartum increase in oestrone concentrations was faster in Ayrshire heifers bearing Limousin fetuses (a different breed) than in those bearing Ayrshire fetuses (20), all these evidences may likely reflect a higher placental activity in allogenic pregnancies. Anyway, from the data of this work it seems that normal fetal development in these interspecific gestations (ibex-in-goat or ibex+goat-in-goat) is correlated with the production of high levels of PAG with a similar pattern to that seen in an ibex-in-ibex pregnancy.

In conclusion, our results show that it is possible to achieve pregnancies after transfer of ibex embryos into domestic goats, but this requires a great change of the PAG profiles, which increase significantly. Live ibex kids can also be produced when a domestic goat embryo shares the uterus with an ibex embryo. This is the first report of successful interspecific pregnancies in the capra genus.

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