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Ovine placental explants: A new ex vivo model to study host–pathogen interactions in reproductive pathogens

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ABSTRACT

Reproductive failure is one of the main performance constraints in ruminant livestock. Transmissible agents such as *Toxoplasma gondii* and *Neospora caninum* are commonly involved in the occurrence of abortion in ruminants, but little is known about the mechanisms involved. While in vivo models are optimal for the study of abortion pathogenesis, they have a high economic cost and come with ethical concerns. Unfortunately, alternative in vitro models fail to replicate the complex in vivo placental structure. To overcome the limitations of currently available models, we developed an ex vivo model based on the cultivation of fresh and cryopreserved sheep placental explants, enabling the biobanking of tissues. Reproducible and simple markers of tissue integrity (histology, RNA concentrations), viability (resazurin reduction), and functionality (synthesis of steroid hormones) were also investigated, allowing a clear quality assessment of the model. This work shows that, similar to fresh explants, tissues cryopreserved in ethylene glycol using slow freezing rates maintain not only their structure and function but also their receptivity to *T. gondii* and *N. caninum* infection. In addition, the findings demonstrate that explant lifespan is mainly limited by the culture method, with protocols requiring improvements to extend it beyond 2 days. These findings suggest that cryopreserved tissues can be exploited to study the initial host–pathogen interactions taking place in the placenta, thus deepening the knowledge of the specific mechanisms that trigger reproductive failure in sheep. Importantly, this work paves the way for the development of similar models in related species and contributes to the reduction of experimental animal use in the future.

1. Introduction

The performance of sheep flocks is heavily influenced by reproductive failure, which is currently considered one of the main economic burdens of ovine production systems. The aetiology of such failure varies and includes alterations in the oestrous cycle, nutritional deficiencies, environmental toxicity and infections from pathogens, some of which have zoonotic potential. In particular, abortions are one of the major constraints for optimal sheep breeding operations, with a high percentage (~50%) of the abortions being caused by transmissible agents displaying placental tropism. These include the apicomplexan parasites *Toxoplasma gondii* and *Neospora caninum*, the bacteria *Brucella melitensis*, *Chlamydia abortus* and *Coxiella burnetii*, and the Border disease virus, among others [1]. In recent years, extensive efforts have been made to describe the specific mechanisms involved in abortions caused by transmitted pathogens. In most cases, the abortions seem to be a consequence of the placental damage caused by replication of the pathogenic agent, resulting in an alteration of key placental functions (e.g., nutrient and oxygen exchange and hormone synthesis). This is confounded by the ability of some of these agents to modify and evade host immune responses, favouring their replication and allowing the chronification of the infection [2–5]. However, little is known about the specific molecular mechanisms triggering such abortions and, consequently, the means to prevent them.

The placenta is an ephemeral but specialised organ finely tailored to provide continuous support to the foetus during pregnancy in mammals.

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Although its role is similar for all species, covering the function of the respiratory, digestive, excretory, and endocrine systems, it shows incredible morphological and histological diversity among different species [6]. Notably, sheep have chorioallantoic and cotyledonary placentae, in contrast to the haemotrichorial and discoid placentae of mice [7]. Such differences are also reflected in their physiology and immune responses, limiting to some extent the usefulness of mice as animal models for studying the transmissible causes of abortion in ruminants [8]. Considering the higher costs of working with pregnant sheep and also the longer experimental periods and the ethical issues associated with their use, some research groups have opted to develop simplified cell culture models based on the major population of the foetal placenta: the trophoblast. Several studies have demonstrated the usefulness of the ovine trophoblast AH-1 cell line for the study of abortifacient agents such as *C. abortus*, *Waddlia chondrophila*, *N. caninum* and *T. gondii* [9–12]. Nevertheless, similar experiments performed with bovine-derived cells isolated from the foetal and maternal placenta (trophoblasts and caruncular cells, respectively) have failed to mimic foetal–maternal interactions and the local immune modulations described in vivo during experimental *N. caninum* infections. This likely reflects the scarce representation of the placental cell populations in these systems [13–15]. Consequently, the use of placental explants could represent an intermediate approach to overcome the limitations of both in vitro and in vivo models, as these explants retain the placental architecture and functions for a short period of time under artificial culture conditions [16].

Previous works have shown that ex vivo systems based on placental explants are useful for the study of the pathogenesis of *Brucella abortus*, *Listeria monocytogenes* and *Listeria ivanovii* in cattle [17–20] but also for describing the effect of *Trypanosoma cruzi* and *T. gondii* infections in sheep [21,22]. However, a bottleneck for such approaches is the short lifespan and limited availability of tissues suitable for obtaining explants [23]. Explant cryopreservation could represent a convenient tool to circumvent such drawbacks, and has, in fact, proven successful with human and canine placental explants [24,25]. Consequently, the objective of this work was to develop an ex vivo model based on cryopreserved sheep placental explants. Placental tissues from healthy ewes were collected, subjected to different cryopreservation methods, and characterised before and after thawing over different time points. Then, we (i) assessed in detail different markers related to tissue architecture, viability, and functionality to study the effect of culture and cryopreservation on placental explants and (ii) evaluated their suitability to serve as a quality control for the model. We also investigated the susceptibility of fresh and thawed explants to *N. caninum* and *T. gondii* infection. This work paves the way for the generation of tissue biobanks and their exploitation to deepen our understanding of the pathogenic mechanisms of infectious reproductive failure in sheep and the findings reveal promising prospects for related species, such as cattle and goats.

2. Materials and methods

2.1. Ethics approval and animals

All protocols involving animals followed current Spanish and European regulations (R.D. 53/2013; Council Directive, 2010/63/EU) and were approved by the Animal Welfare Committee of the Community of Madrid, Spain (PROEX 65.6/20, 2 ewes). To reduce the number of animals used, most tissues were obtained from ewes that were culled for other purposes in parallel experiments also approved by the Animal Welfare Committee of the Community of Madrid, Spain (PROEX 68.0/20, 8 ewes). All animals ($n = 10$) were strictly handled under good clinical practices to minimise potential suffering.

Animal selection was performed as described in previous research [26]. In brief, Rasa Aragonesa breed ewes were purchased from a commercial flock after verifying their seronegativity for *T. gondii*, *N. caninum*, Border disease virus, Schmallenberg virus, *Coxiella burnetii* and *Chlamydia abortus* by commercial or in-house ELISAs. After oestrus synchronisation using progestogens and pregnant mare serum gonadotrophin, the ewes were mated with rams for 2 days. Pregnancy was confirmed by ultrasound scanning on Day 40 after mating. Subsequently, pregnant ewes were transferred and housed at the Clinical Veterinary Hospital facilities (Complutense University of Madrid, Spain) until the day of culling, which took place around Day 100 of gestation. Foetal viability was confirmed by ultrasound scanning before culling.

2.2. Isolation of ovine placental explants

Pregnant ewes were intravenously sedated with xylazine (Calier laboratories, Spain) and later euthanised by an overdose of embutramide and mebezonium iodide (T61, MSD, Spain). Necropsies were performed at the Clinical Veterinary Hospital facilities (Complutense University of Madrid, Spain) following standard protocols. Immediately after euthanasia, placentomes with diameters between 3 and 6 cm were randomly selected, dissected from the placenta using sterile material, and transported to the laboratory in an ice-cold sterile washing solution (phosphate buffered saline -PBS- supplemented with 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 0.25 μg/ml of fungizone and 100 μg/ml of L-glutamine, all from Gibco, UK). The placentomes were longitudinally cut into 2-3 mm-thick slices using sterile skin graft blades (Swann Morton, UK) inside a laminar flow cabinet. Afterwards, the slices were transversely cut into 60–80 mg sections containing both the maternal and foetal parts of the placentome using switch-blade shears (Feather, Japan) (Fig. 1a). Once obtained, the explants were maintained on ice-cold sterile washing solution for less than 30 min and then washed 10 times with ice-cold washing solution before cultivation or cryopreservation.

2.3. Experimental design

As a first approach, we tested the performance of four different cryopreservation methods by comparing the behaviour of fresh and thawed explants in terms of tissue damage (histology, see Section 2.5) and viability (LDH test, see Section 2.6.1) after 1–3 days in culture (Fig. 1b). For this purpose, a group of freshly obtained explants from two ewes (OV1 and OV2) were cultured in 24-well plates (12 explants/sheep/time-point, see Section 2.4). In parallel, another group of fresh explants was subjected to cryopreservation (Fig. 1b). For this, explants were incubated for 20 min with two different cryoprotectants consisting of foetal bovine serum (FBS, South America origin, Gibco, UK) supplemented with 3 M dimethyl sulfoxide (DMSO, Sigma−Aldrich, MA, USA; OV1) or FBS supplemented with 2 M ethylene glycol (EG, Merck Chemicals, Germany; OV2). In both cases, two different cryopreservation speeds were tested: snap freezing in liquid nitrogen and a slow-controlled freezing rate. For the latter, Mr. Frosty® containers (Thermo Fisher Scientific, MA, USA) were used and maintained at −80 °C for 24 h; subsequently, tubes were transferred to liquid nitrogen. To analyse the effect of the cryopreservant in the tissue, three explants were fixed in 10% buffered formalin immediately after incubation with each of them and before cryopreservation. Four weeks after cryopreservation, the explants were thawed in a 37 °C water bath, washed 10 times with washing solution (see Section 2.2), transferred to 24-well plates and cultured with protocols identical to those used for the fresh explants (12 explants/sheep/time-point, see Section 2.4).

Based on the results obtained in the first test, in consecutive experiments, a similar approach was followed to compare fresh and thawed explants using placentomes from 6 ewes (OV3-OV8) but using the best cryopreservation method, culturing the explants from 5 h to 2 days, and expanding the parameters measured in all cultured explants (12 explants per sheep for each time point and for each condition, Fig. 1c). In this manner, tissue viability (resazurin and XTT tests), integrity (damage scores based on histology, *KRT8* expression) and functionality (*PLAC-1* expression, synthesis of steroid hormones) were studied as indicated in

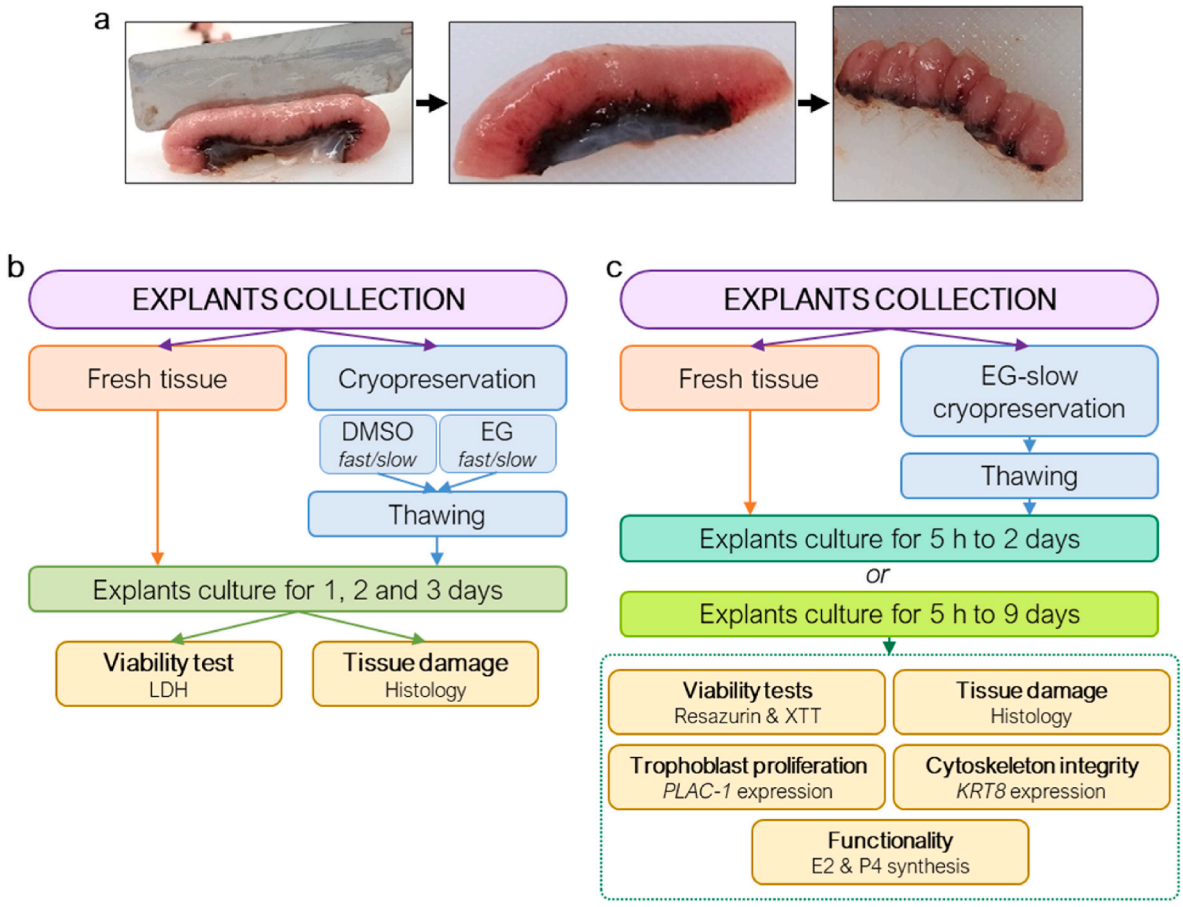

Fig. 1. Collection of ovine placental explants and experimental design. a: Placentomes were cut longitudinally and transversely into 60–80 mg sections containing both the maternal and foetal components. **b:** Workflow diagram followed by explants obtained from pregnant ewes to determine the best cryopreservation method. **c:** Workflow diagram followed by explants obtained from pregnant ewes to further characterise the differences between fresh and EG-slow-cryopreserved explants cultured up to 2 days or up to 9 days. DMSO: dimethyl sulfoxide; EG: ethylene glycol; fast/slow: speed at which freezing was performed.

the sections below.

Based on works that have described the regeneration of placental explants after 2–4 days in culture [27,28], in subsequent trials, we extended the explant culture up to 9 days using placentomes from 2 ewes (OV9 and OV10) to measure tissue viability (resazurin test), integrity, and functionality, as detailed in the sections below (12 explants per sheep for each time point and for each condition, Fig. 1c). To increase the number of replicates and corroborate the obtained results, cryopreserved explants from OV5 to OV9 were thawed, cultured for up to 9 days, and analysed (8–12 explants per sheep for each time point and for each condition).

To ensure the reproducibility of the model, all parameters tested in each experimental design and at each time point were measured in at least two different ewes using triplicate wells whenever possible. The highest number of biological replicates was performed in explants cultured from 5 h to 2 days (6 ewes on average), while the lowest number was performed in explants cultured from 3 to 9 days (2 ewes on average).

2.4. Culture of fresh and thawed placental explants

Placental explants were individually allocated in 24-well plates previously filled with 2 mL of M-199 culture media without phenol red (Gibco, UK) (phenol red stimulates oestrogenic receptors) supplemented with 20 mM HEPES (HyClone, Cytiva, MA, USA), 20% FBS (South America origin, Gibco, UK), and an antibiotic-antimycotic mixture (100 UI/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml Fungizone from Gibco, UK). The explants were then incubated from 5 h to 9 days at 37 °C and 5% CO_2. The culture media was replaced on a daily basis. In the absence of indicators in the media, the pH was regularly measured at each time-point using pH indicator strips (Fisherbrand, Thermo Fisher Scientific, MA, USA).

2.5. Histological analyses, damage scoring, and immunohistochemical staining

Histological studies were considered our gold-standard technique for assessing the extent of tissue damage in all explants. These were conducted in fresh and thawed tissues collected from all ewes at all the time points of the cultures tested (5 h–9 days). For each placental condition (fresh or thawed), triplicate explants were fixed in buffered formalin for 5 days and subsequently processed for paraffin embedding and haematoxylin-eosin staining for histological evaluation.

The tissue sections were examined in a blinded manner, and the damage to the tissue was scored from 0 to 3 according to the following criteria (Fig. 2). A **score of 0** indicated the absence of any evident or significant histological changes. A **score of 1** indicated the presence of a layer of sloughed epithelial cells dispersed among cellular debris, as well as retraction of cotyledonary and caruncular villi, isolated apoptosis of epithelial cells, and/or mild autolysis. A **score of 2** indicated evident loss of the epithelial layer in both cotyledonary and caruncular villi, with the latter being shrunken and eosinophilic; frequent apoptosis of epithelial cells was also noted, along with loss of defined borders in the cells that were still attached to the villi. Finally, a **score of 3** indicated generalised autolysis of tissue with evident apoptosis, lack of nuclear definition in most epithelial cells, and/or indistinguishable cell borders.

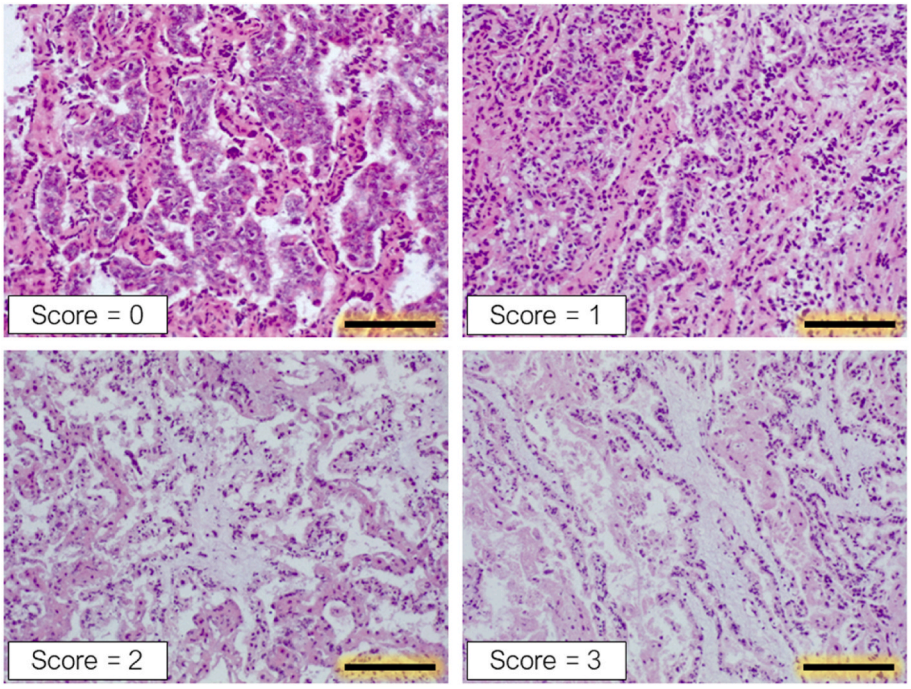

Fig. 2. Representative microscopy images of the tissue damage scores of placental explants cultured ex vivo. Haematoxylin-eosin staining, 10x magnification. Bars: 100 μm.

After evaluation of single sections from three different explants, a single agreement score was assigned for each placental condition tested following the aforementioned criteria (Fig. 2). In general, the appearance of the tissue was generally homogeneous within the same sample and among the replicates from the same animal, although variations were occasionally observed. In cases where differences were present between replicates or within the same sample, the highest score was assigned.

Immunohistochemical labelling of *N. caninum* in tissue sections was also performed as described previously [29].

2.6. Viability assays

2.6.1. Lactate dehydrogenase (LDH) assay

As an abundant intracellular enzyme, extracellular detection of LDH is routinely used as an indirect measure to study the extent of breakage of cellular membranes. To assess the damage to placental explants due to cultivation and/or freezing, we quantified LDH levels in culture supernatants of fresh and thawed explants obtained from two sheep (OV1 and 2). A total of six supernatants per animal and placental condition were collected and kept at −80 °C until further analysis. The LDH levels were quantified using the CyQUANT™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. All samples were diluted 1:20 in fresh culture medium and measured using a Synergy H1 microplate reader (Biotek, 490 nm).

2.6.2. XTT assay

XTT assays were used to detect the presence of metabolic activity at different culture time points on fresh and thawed explants in duplicate or triplicate. For this purpose, we used the Cell Proliferation Kit II (Sigma–Aldrich, MA, USA) following the manufacturer's instructions. The explants were incubated with the reagent for 18 h at 37 °C and 5% CO_2, and after incubation, 200 μL from each well was transferred to an ELISA microplate (Maxisorp®, Nunc, Denmark) and measured using a Synergy H1 microplate reader (450 nm, using a wavelength of 603 nm as reference; BioTek, VT, USA). A background control (culture medium) and a negative control (nonviable explants previously fixed in 4% paraformaldehyde for 30 min) were included in all the assays.

2.6.3. Resazurin assay

Resazurin assays were also used to detect the occurrence of metabolic activity at different culture time points on fresh and thawed explants in triplicate. For this purpose, 0.2 μm-filtered resazurin (0.25 mg/mL, Acros Organics, Belgium) was added to each well at the pertinent time points and incubated for 18 h at 37 °C and 5% CO_2. After incubation, 200 μL from each well was transferred to an ELISA microplate (Maxisorp®, Nunc, Denmark), and the optical density was measured using a Synergy H1 microplate reader (570 nm, using a wavelength of 600 nm as a reference; BioTek, VT, USA). Background and negative controls were included in all assays as detailed for the XTT assay (Section 2.6.2).

2.7. Quantification of cell proliferation and cytoskeleton integrity by RT–qPCR

Total RNA was extracted using the Maxwell® 16 LEV simplyRNA Purification Kit (Promega, WI, USA) following the manufacturer's recommendations. For this purpose, 20 mg from each explant (two or three per condition tested) was homogenised in the provided buffer using a Politron® PT1600E homogeniser (Kinematica AG, Switzerland). RNA quality was assessed by the visualisation of the 18S and 28S ribosomal fragments after electrophoresis on bleach agarose gels (0.8% agarose, 0.05% sodium hypochlorite, Sigma–Aldrich, MA, USA) and by spectrophotometry (NanoPhotometer®, Implen, Germany). Reverse transcription to complementary DNA (cDNA) was carried out using the SuperScript® VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA) using a maximum of 2.5 μg of RNA per reaction.

Quantitative reverse transcription PCR (RT–qPCR) was employed to quantify the mRNA expression levels of *PLAC-1*, a marker of trophoblast proliferation, *KRT8*, a keratin protein of the villi cytoskeleton used as a marker of structural integrity, and *β-actin*, the housekeeping gene, using the primers detailed in Table 1 [30]. For each gene, a seven-point standard curve based on tenfold serial dilutions of PCR products was included in each batch of amplifications using glycogen as a carrier (33

Table 1
Primers used for the generation of standard curves and RT−qPCR assays to quantify *PLAC-1* and *KRT8* expression in cultured explants. F: forward, R: reverse.

Gene	GenBank accession number	Primer sequence for standard curves (5′-3′)	Primer sequence for RT-qPCR (5′-3′)
β-actin	NM_001009784.3	F: AGACCGCGTCCGCCCCGCCAG R: GTACATGGCAGGGGTGTTGA	F: ACACCGCAACCAGTTCGCCAT R: GTCAGGATGCCTCTCTTGCT
PLAC-1	XM_012107306.3	F: GCCTGAACTCCTCAAGAGATAGC R: GCTAAGTTCCTGGAGCAGGAC	F: CCGGACAAAATCCAATGACTGT R: AACCCAGGCCCAAGTGTAACTCA
KRT8	XM_012174208.3	F: CTGATGAGATCAACTTCTACAGGC R: CTGCTTGGCATTCCTCAGAG	F: ATGGACAACAACCGCAACCT R: CCAGTGTCTGCAGCTCCTCAT

μg/mL, Thermo Fisher Scientific, MA, USA). The primers used for the generation of standard curves are detailed in Table 1. All cDNA samples were diluted 1:5 in nuclease-free water prior to analysis. The reaction mix contained 12.5 μL of Power SYBR® Green PCR Master Mix (Applied Biosystems, MA, USA), 10 pmol of each primer and 5 μL of the diluted cDNA. The reaction was performed in an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, MA, USA). mRNA expression levels for each target were normalised by the $2^{-\Delta Ct}$ method [31].

2.8. Functionality assays: oestradiol and progesterone synthesis

To assess the functionality of fresh and thawed explants at the different time points studied, the levels of 17-β-oestradiol (E2) and 17-OH-progesterone (P4) released into the medium were quantified in duplicate or triplicate using a commercial competitive ELISA test (E2-ELISA [KAP0621] and 17OH-Progesterone ELISA [KAP1401], DiaSource, Belgium) following the manufacturer's instructions. Blank controls (media supplemented with FBS) were included in all runs to determine the basal levels of both hormones in the media.

2.9. Explant susceptibility to T. gondii or N. caninum infection

Fresh and thawed explants were experimentally infected with *T. gondii* (Tg-PRU isolate) or *N. caninum* (Nc-Spain7 isolate) to assess tissue receptivity to two relevant ruminant reproductive pathogens. The parasites were propagated, harvested, and dosed following previous protocols [32]. Since the culture media used to grow the parasites differed from that used to culture the explants, infected monolayers were washed three times with washing solution and replenished with supplemented M-199 media before harvest. In a first trial, fresh explants were infected in triplicate with 10^5 or 10^6 *T. gondii* or *N. caninum* tachyzoites for 1, 3, and 5 days. In a second trial, EG-thawed explants were infected in triplicate with both parasites at the same time points, but only with the higher tachyzoite dose (10^6). In both trials, media was replaced on a daily basis, and explant viability was recorded at every collection time as detailed in Section 2.6.3. The infected explants were individually collected in microcentrifuge tubes and immediately stored at −80 °C until DNA extraction, which was performed using the DNeasy Blood & Tissue Kit (Qiagen, Germany). For that purpose, the explants were thawed, individually supplemented with 180 μl of ATL buffer (Qiagen, Germany) per 25 mg of tissue and homogenised using a Politron® PT1600E homogeniser (Kinematica AG, Switzerland). Only 25 mg (~180 μl) of tissue was subjected to DNA extraction. The parasite burden was quantified by qPCR using previously described methods [33,34] and the Go Taq® qPCR Master Mix (Promega, WI, USA). In both protocols, *β-actin* was used as a housekeeping gene, while the TgRE (529-bp repeat element) and Nc-5 sequences were targeted for quantification of *T. gondii* and *N. caninum*, respectively. All primers used for qPCR are listed in Table 2.

Table 2
Primers used for qPCR assays to quantify *N. caninum* and *T. gondii* replication in infected explants. F: forward, R: reverse.

Gene	GenBank accession number	Primer sequence for qPCR (5′-3′)
β-actin	NM_001009784.3	F: AGCGCAAGTACTCCGTGTG R: CGGACTCATCGTACTCCTGCTT
Nc-5	X84238.1	F: ACTGGAGGCACGCTGAACAC R: AACAATGCTTCGCAAGAGGAA
TgRE	AF146527.1	F: CACAGAAGGGACAGAAGT R: TCGCCTTCATCTACAGTC

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism software, v.8 (Dotmatics, UK). Data normality was checked using the Shapiro–Wilk test prior to performing any further statistical analyses. Data fitting a normal distribution were analysed with one-way ANOVA coupled to Tukey's multiple comparison test. The rest of the data were analysed using the Kruskal–Wallis test coupled to Dunn's multiple comparison. Statistical significance for all analyses was established at $p < 0.05$.

3. Results

3.1. Selection of optimal cryopreservation methods for ovine placental explants

The performance of four cryopreservation protocols based on the use of DMSO and EG at two different freezing speeds was assessed through LDH-based viability assays and histological studies of fresh and thawed explants cultured from 1 to 3 days (Fig. 1b).

The LDH analyses were performed on supernatants collected from fresh explants (OV1 and 2) and from thawed explants previously subjected to fast and slow freezing rates with DMSO (OV1) or EG (OV2). Overall, enzyme levels remained high under all conditions tested. No significant differences were observed between fresh explants obtained from different animals or between fresh and thawed explants obtained from the same animal, regardless of the speed at which cryopreservation was performed ($p > 0.05$, one-way ANOVA and Tukey's multiple comparison test) (Fig. 3). Under all experimental conditions, we observed a drop in LDH activity at 3 days of culture, but this was only significant for fresh-OV1 (Day 1 *vs.* Day 3; Fig. 2a), DMSO-Fast (Day 1 *vs.* Day 3; Fig. 2b), and EG-Slow (Day 1 *vs.* Day 3; Fig. 2c) ($p < 0.05$, one-way ANOVA and Tukey's multiple comparison test).

Given the absence of obvious differences in the LDH secretion levels between the fresh and thawed explants, the damage score of the tissue was used as a gold-standard criterion to determine the best cryopreservation method to be applied in subsequent trials. The histological analyses revealed that the mere incubation of fresh explants with DMSO induced certain levels of tissue alteration (score = 1), and this was not observed in explants incubated with EG (score = 0) (data not shown). After thawing, DMSO-treated explants showed worse tissue damage scores than those preserved with EG (Fig. 4). In addition, the use of slow freezing rates in EG-thawed explants resulted in lower damage scores (Fig. 4). Therefore, cryopreservation with EG at slow freezing rates was selected for subsequent experiments.

3.2. Histological analyses and tissue damage scoring

Further histological analyses were performed to assess the effect of cultivation and EG-slow cryopreservation on the explants' tissue

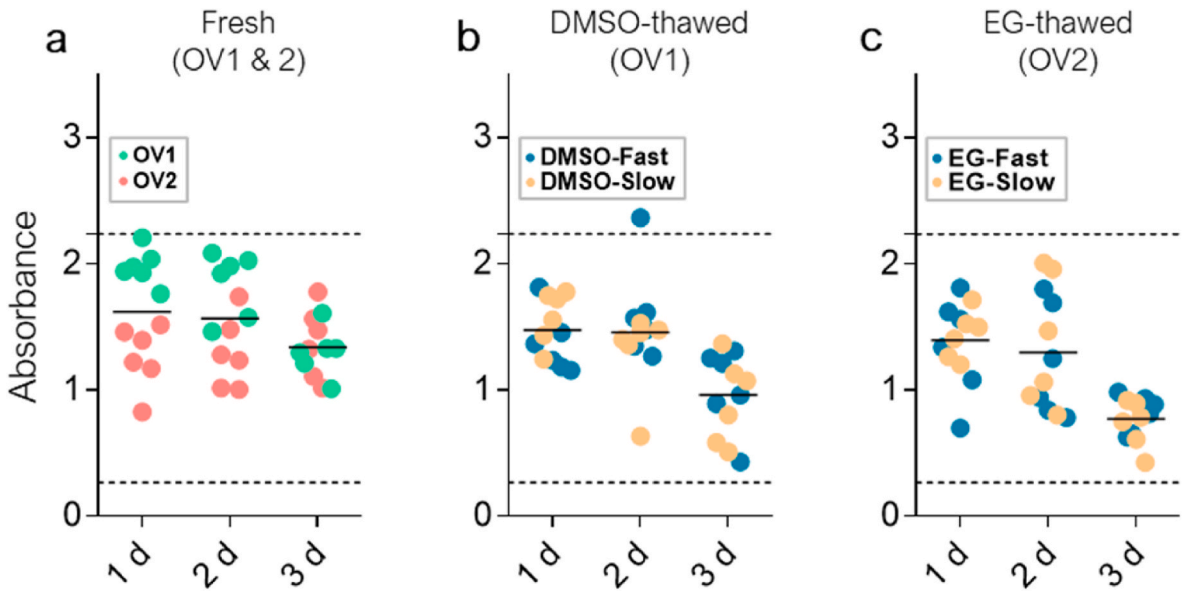

Fig. 3. LDH levels in culture supernatants of fresh and thawed explants. a: Fresh explants; **b:** DMSO-thawed explants; **c:** EG-thawed explants. In all graphs, the top dotted line represents the absorbance levels of the kit's positive control, while the bottom dotted line represents the LDH levels present in fresh culture media without explants. Each dot represents individual explants, and bars represent grand mean values.

integrity in eight additional sheep using the tissue damage score defined in Section 2.5. In fresh explants, the structural integrity was intact during the first day of culture and displayed progressive degradation with time (Fig. 5a). In the EG-thawed explants, we observed a pattern of tissue degradation comparable to that observed in the fresh explants (Fig. 5a). When comparing the fresh and EG-thawed explants, the latter tended to display earlier signs of tissue degradation, although no significant differences were found between them ($p > 0.05$, Kruskal–Wallis test and Dunn's multiple comparison). These results suggest that both fresh and EG-thawed explants might be suitable for performing ex vivo experiments lasting up to 1–2 days. Nevertheless, the lack of signs of severe autolysis and loss of membrane integrity (score 3) until Days 3–7 of culture may suggest that these assays could last for longer periods.

3.3. RNA integrity

Considering that RNA abundance can be exploited as an indirect marker of the transcriptomic activity of the explants, we took advantage of the extractions performed for RT–qPCR assays to analyse the effect that culture of fresh and EG-thawed explants had on the isolated concentrations of RNA (Fig. 5b). Under both conditions, and especially during the first 2 days of culture, we observed a significant progressive decrease in RNA concentrations as the culture time increased ($p < 0.05$, one-way ANOVA and Tukey's multiple comparison test). This decrease was more marked in EG-thawed explants, whose RNA concentrations were lower at 5 h of culture compared to those of fresh explants ($p < 0.05$, one-way ANOVA and Tukey's multiple comparison test). These findings mirrored the behaviour observed in terms of tissue damage, since RNA concentration decreased as tissue degradation increased (Fig. 5).

3.4. Viability assays

Since histology is a time-consuming approach, we explored alternative tests readily accessible in most labs. Considering the limitations of LDH assays in discerning the impact of cryopreservation on explants, in consecutive trials, we evaluated alternative methods for assessing explant viability. In this sense, we used XTT and resazurin assays to measure the metabolic activity of fresh and thawed explants cultured for different durations.

The XTT assays were run in duplicate or triplicate samples using explants obtained from four sheep. These tests corroborated the occurrence of metabolic activity from 5 h to 2 days of culture (Fig. 6, top panel). Experimental reproducibility between biological replicates was confirmed, as no significant differences were found between animals within each time point under all conditions tested ($p > 0.05$, one-way ANOVA and Tukey's multiple comparison test). Grouped analysis showed that the viability of fresh explants was significantly lower after 2 days of culture than after 5 h and 1 day of culture ($p < 0.05$, one-way ANOVA and Tukey's multiple comparison test). These differences were not observed in thawed explants ($p > 0.05$, one-way ANOVA and Tukey's multiple comparison test). The viability of EG-thawed explants at 5 h and 1 day of culture was significantly lower than that observed for fresh explants at the same time points ($p < 0.05$, one-way ANOVA and Tukey's multiple comparison test). However, the assay's threshold between fully viable (fresh explants cultured for up to 5 h) and nonviable explants (paraformaldehyde-fixed explants) was very tight, preventing us from drawing clear conclusions.

Considering the low sensitivity of the XTT assays, we focused our efforts on the resazurin tests, which were run in triplicate using explants obtained from seven sheep and cultured for 5 h, 1 day, or 2 days. All fresh and EG-thawed samples showed metabolic activity from 5 h to 2 days of culture (Fig. 6, bottom panel). We also confirmed the experimental reproducibility between biological replicates, as no significant differences were found between animals when they were compared within every time-point and placental condition (fresh or EG-thawed) ($p > 0.05$, one-way ANOVA and Tukey's multiple comparison test). In addition, no significant differences were observed between the tested time points in fresh or EG-thawed explants ($p > 0.05$, one-way ANOVA and Tukey's multiple comparison test).

Given the good results obtained with the resazurin tests, which largely correlated with what we described using histology, in consecutive experiments, we decided to assess explant viability after 9 days of ex vivo culture. All fresh and EG-thawed samples showed metabolic activity from 3 to 9 days of culture (Fig. 6, bottom panel). In addition, no significant differences were found between animals when they were compared within every time point and placental condition (fresh or EG-thawed) ($p > 0.05$, one-way ANOVA and Tukey's multiple comparison test). The grouped analysis of fresh explants cultured for 3–9 days did not show differences between the time points tested ($p > 0.05$, one-way

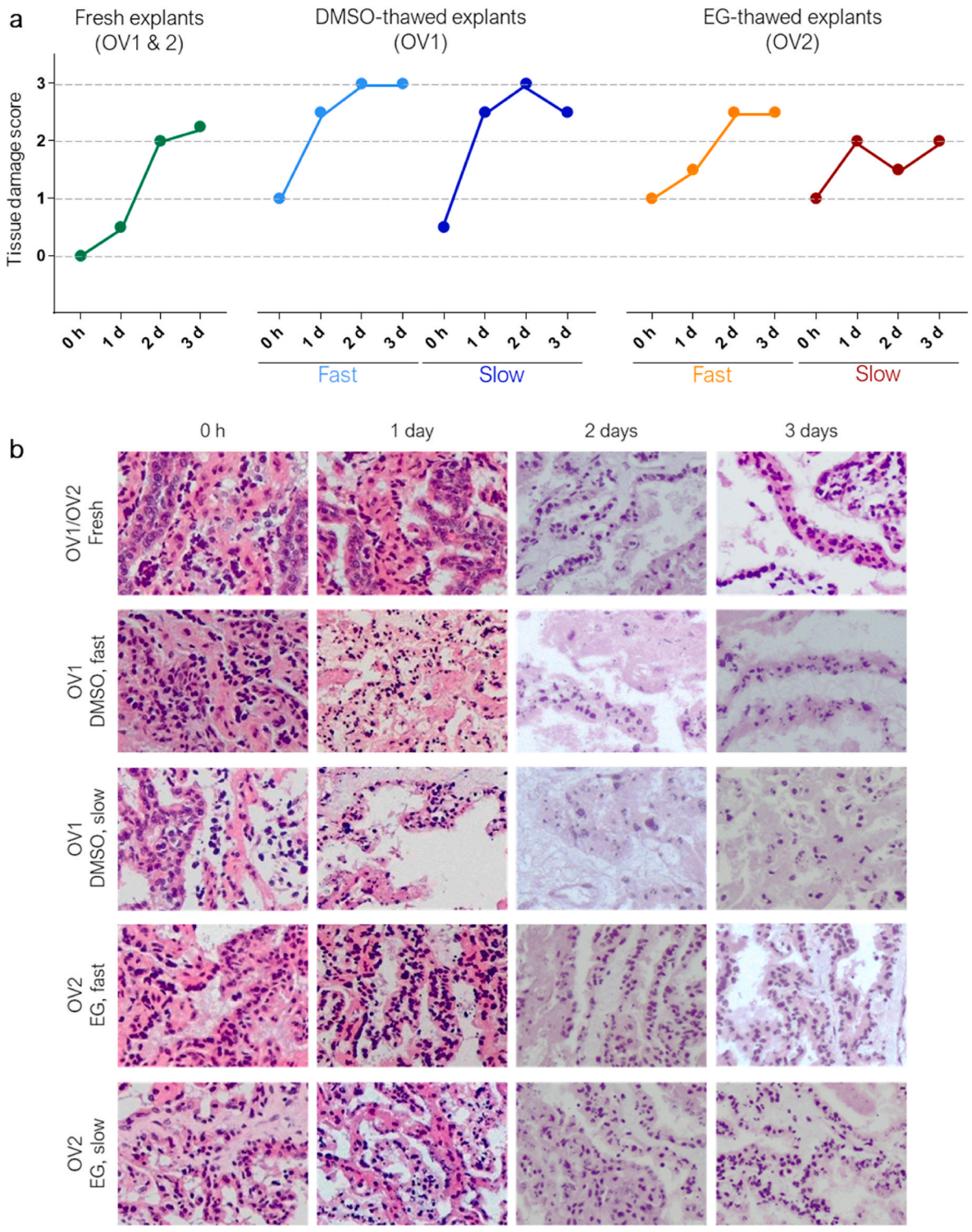

Fig. 4. Tissue damage of fresh, DMSO-thawed and EG-thawed explants cultured for 1–3 days. a: Graphical representation of the tissue damage scores of fresh and thawed explants. Each dot represents the average score of three different explants cultured under the same conditions. **b:** Representative histological sections obtained from fresh, DMSO-thawed and EG-thawed explants. OV1/OV2: sheep identification; Fast/Slow: speed at which freezing was performed.

ANOVA and Tukey's multiple comparison test). Further analyses performed from 5 h to 9 days of culture demonstrated a slight decrease in explant viability after 2 days of culture compared to that observed at 3, 5 and 7 days ($p < 0.05$, one-way ANOVA and Tukey's multiple comparison test) (Fig. 6, bottom panel). In EG-thawed explants, a marked drop in viability was observed at 3 days of culture compared to that measured at 5 h and 1, 2, 5, 7, and 9 days of culture ($p < 0.05$, one-way ANOVA and Tukey's multiple comparison test). Remarkably, explant viability was markedly recovered from 5 days of culture onwards (Fig. 6, bottom panel). Apart from the drop in viability of EG-thawed explants on Day 3, no differences were found in the viability of fresh and thawed explants at equivalent culture durations ($p > 0.05$, one-way ANOVA and Tukey's multiple comparison test).

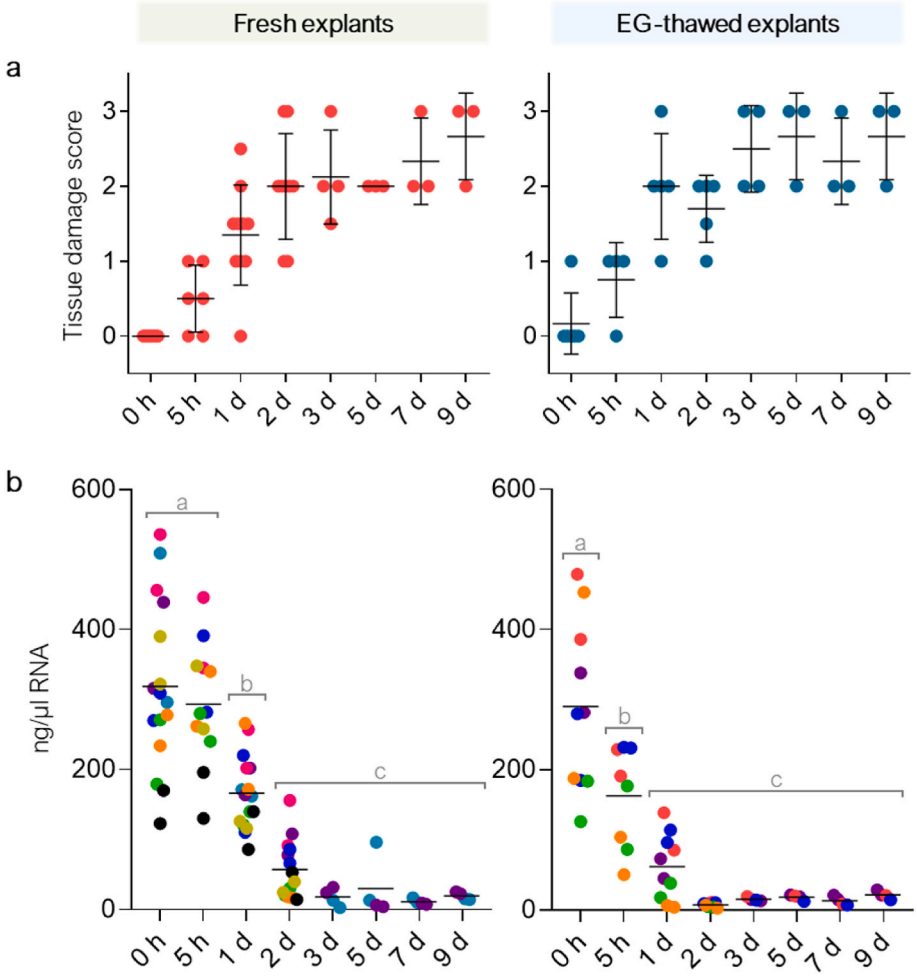

Fig. 5. Evolution of the tissue damage and RNA concentration of fresh and EG-thawed explants throughout the culture time. a: Tissue damage scores recorded from fresh (red dots) and thawed (blue dots) explants obtained from different sheep and cultured in vitro from 0 h to 9 days. The scores were analysed from an average of six (5 h–2 days) or two (3–9 days) different ewes at each time point. Each dot represents the average score of three different explants obtained from the same sheep and cultured under the same conditions; bars represent the mean value and the standard deviation. **b:** Evolution of RNA concentrations extracted from fresh (left graph) and EG-thawed (right graph) explants from different sheep and cultured in vitro from 0 h (h) to 9 days (d). Each dot represents the RNA concentration of an individual explant; each colour represents different ewes; bars represent grand mean values; groups marked with different letters were significantly different ($p < 0.05$, one-way ANOVA and Tukey's multiple comparison test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. PLAC-1 and KRT8 mRNA expression

To further characterise fresh and thawed explants, we quantified the expression of *PLAC-1*, regarded as a marker of trophoblast proliferation, and *KRT8*, a cytoskeletal protein related to the structural integrity of placental villi. Due to the low amounts of RNA extracted from the explants, we only assessed the expression of both genes from samples obtained from 0 h to 2 days of culture (Fig. 7).

In fresh explants, *PLAC*-1 mRNA expression decreased gradually with the time of culture, being significantly low after 1 and 2 days ($p < 0.05$, Kruskal–Wallis test and Dunn's multiple comparison). In EG-thawed explants, the expression of *PLAC-1* transcripts was similar from 0 h to 2 days of culture ($p > 0.05$, Kruskal–Wallis test and Dunn's multiple comparison). When comparing equal time points, no differences in *PLAC-1* expression were found between fresh and EG-thawed explants ($p > 0.05$, Kruskal–Wallis test and Dunn's multiple comparison).

KRT8 expression in fresh explants increased significantly after 2 days of culture ($p < 0.05$, Kruskal–Wallis test and Dunn's multiple comparison). This contrasts with what was observed in EG-thawed explants, where a gradual decrease in *KRT8* expression was observed over time, with significantly low expression on Day 2 of culture ($p < 0.05$, Kruskal–Wallis test and Dunn's multiple comparison). Comparison between fresh and EG-thawed explants at equivalent time points demonstrated that *KRT8* expression was significantly higher in fresh explants at Days 1 and 2 of culture ($p < 0.05$, Kruskal–Wallis test and Dunn's multiple comparison).

3.6. Steroid hormone biosynthesis

Under physiological conditions, the placenta synthesises steroid hormones such as 17-β-oestradiol (E2) and 17-OH-progesterone (P4). Consequently, we quantified the explants' production of both hormones at different culture time points as an indirect measure of their functionality.

Both fresh and EG-thawed explants actively secreted E2 throughout the culture time (5 h–9 days) (Fig. 8, top panel). Statistical analyses were focused on the experiments with the highest number of replicates (5 h–2 days) and evidenced no significant differences between biological replicates or between the different time points tested ($p > 0.05$, one-way ANOVA and Tukey's multiple comparison test). Similarly, no differences were observed between fresh and EG-thawed explants when we compared equal time points ($p > 0.05$, one-way ANOVA and Tukey's multiple comparison test).

Regarding P4, variable levels of detection were found in both fresh and EG-thawed explants. Explants obtained from the same individuals and analysed at identical time points displayed uneven P4 levels (Fig. 8, bottom panel). As detailed for E2 levels, statistical analyses were focused on the 5-h to 2-day time points. Overall, no significant differences were found between biological replicates or between the time points tested. Similarly, we found no differences between fresh and EG-thawed explants compared at equal time points ($p > 0.05$, one-way ANOVA and Tukey's multiple comparison test). The exception was observed in P4 levels in fresh explants, which were significantly higher at 5 h than at later time points ($p < 0.05$, one-way ANOVA and Tukey's multiple comparison test), although the number of available samples was lower.

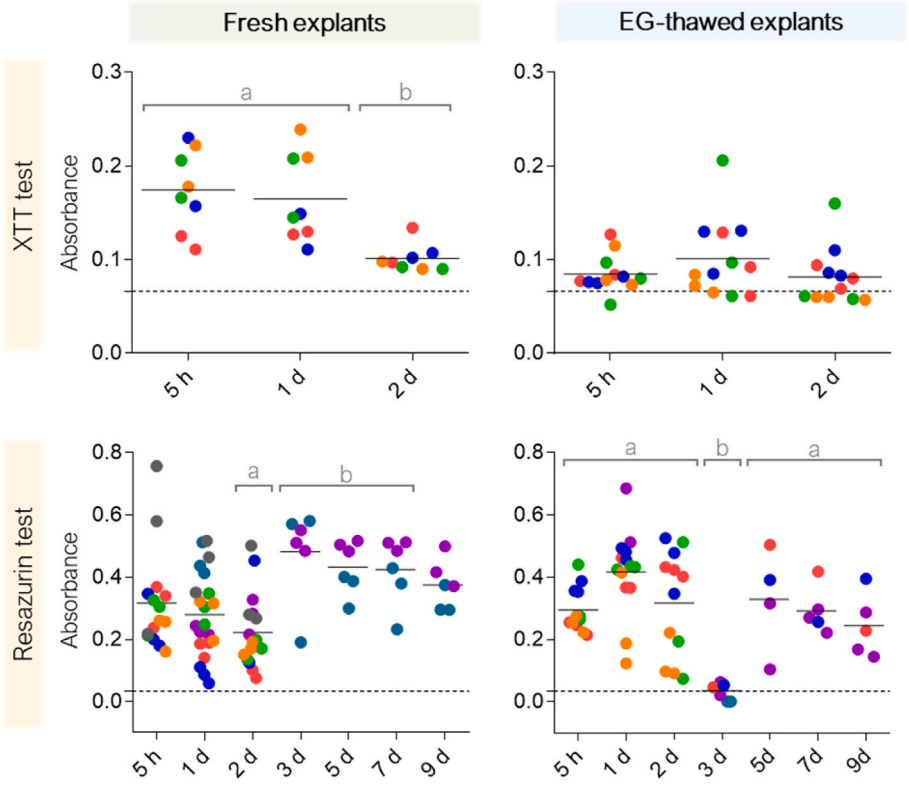

Fig. 6. Quantification of the metabolic activity of fresh and thawed explants using the XTT assay (top panel) and the resazurin assay (bottom panel) over different incubation times. The bottom dotted line represents the average absorbance of explants fixed with 4% paraformaldehyde and used as controls of nonviability. Each dot represents individual explants, each colour represents different ewes, and bars represent grand mean values. Groups marked with different letters were significantly different ($p < 0.05$, one-way ANOVA and Tukey's multiple comparison test). h: hour; d: day(s). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.7. Receptivity to *T. gondii* or *N. caninum* infection

To assess the suitability of this system for studying the interactions between the sheep and two relevant reproductive pathogens, we experimentally infected fresh and EG-thawed explants (from three ewes and one ewe, respectively) with *T. gondii* or *N. caninum* tachyzoites. These parasites display an extraordinary tropism for placental tissues. In a first assay, we infected fresh explants with 5×10^4 tachyzoites of both parasites, but the parasite burden achieved was extremely low (data not shown). Consequently, in successive trials, we opted to increase the challenge dose to 10^5 and 10^6 tachyzoites.

Infection of fresh explants with *T. gondii* tachyzoites was successful, resulting in active replication of the parasite from 1 to 5 days post-infection (dpi) (Fig. 9a, top panel). This increase in the parasite burden was significantly higher from 3 dpi in explants infected with 10^5 and 10^6 tachyzoites ($p < 0.05$, Kruskal–Wallis test and Dunn's multiple comparison). Since we did not find differences between the two challenge doses used ($p > 0.05$, Kruskal–Wallis test and Dunn's multiple comparison), EG-thawed explants were only infected with 10^6 *T. gondii* tachyzoites. These explants behaved similarly to the fresh ones, with parasite burdens tending to be higher from 3 dpi onwards. However, no significant differences were found between the time points assayed ($p > 0.05$, Kruskal–Wallis test and Dunn's multiple comparison). The resazurin tests that were run in parallel with infected and uninfected fresh and EG-thawed explants demonstrated that all tissues remained viable during the experiments, and in fact, comparable viability scores were observed under all conditions (Fig. 9b).

Fresh explants were also successfully infected by *N. caninum* tachyzoites at 1 dpi, although the replication levels quantified at 3 and 5 dpi were not significantly increased ($p > 0.05$, Kruskal–Wallis test and Dunn's multiple comparison) (Fig. 9a, bottom panel). No differences were observed between the two challenge doses employed ($p > 0.05$, Kruskal–Wallis test and Dunn's multiple comparison). Since *T. gondii* replication appeared to occur more actively than that of *N. caninum*, we corroborated that the latter was able to form parasitophorous vacuoles in fresh explants through immunohistochemical studies (Fig. 9c). Based on these results, we subsequently infected EG-thawed explants only with 10^6 *N. caninum* tachyzoites and observed parasite kinetics similar to those found in fresh explants. No differences were found between fresh and EG-thawed explants ($p > 0.05$, Kruskal–Wallis test and Dunn's multiple comparison) (Fig. 9a, bottom panel). We also carried out parallel resazurin tests under all conditions and these tests demonstrated that all the tissues remained viable during the experiments. In addition, we did not observe differences in viability among all the conditions tested (Fig. 9b).

4. Discussion

The availability of useful and ethically acceptable models for the study of reproductive failure-causing agents at the foetal–maternal interface is an ongoing effort in veterinary medicine. In the case of apicomplexan parasites, multiple animal models developed in the target ruminant species are suitable to carry out experimental trials that mirror a natural scenario of infection. Nevertheless, their use is constrained by the availability of adequate facilities with trained staff, by the higher costs and longer experimental periods of the animal models, and, increasingly, by ethical concerns. Alternative in vitro systems have brought a cost-effective and animal-free source of experimental material, although the use of these alternative systems cannot replicate the close interaction between the various cell populations locally present in the placenta [23]. Organoids and 'organs-on-chip' are increasingly being proposed as alternatives to the use of animals, but these approaches require technical resources that are still difficult to access for most laboratories and that rely on the use of pluripotent or immortalised cell lines [35]. Therefore, interest in the development of ex vivo systems that are able to reproduce what happens in naturally infected animals is increasing [23]. The use of placental explants for research in human medicine has been extensively reported on thus far. This has been facilitated by the fact that deliveries and voluntary pregnancy interruptions take place under specialised medical care and near research

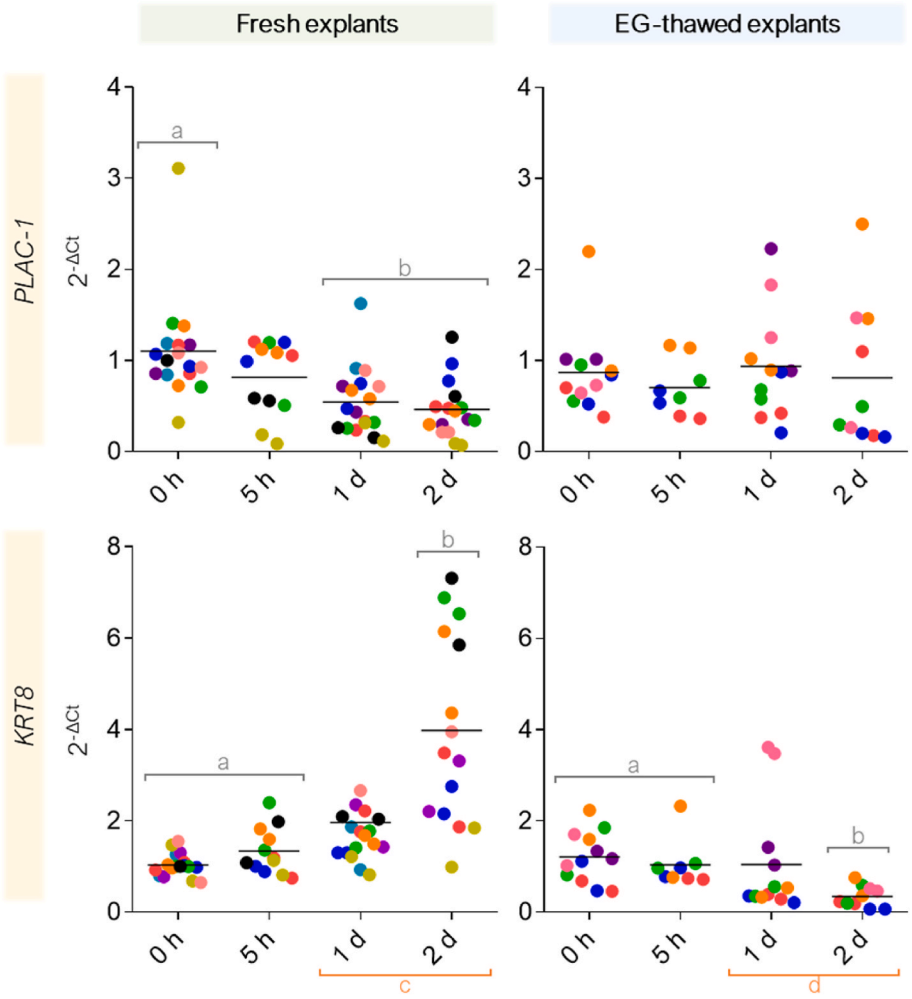

Fig. 7. *PLAC-1* and *KRT8* mRNA expression. Evolution of the abundance of *PLAC-1* and *KRT8* transcripts in fresh (left graph) and EG-thawed (right graph) explants cultured from 0 h (h) to 2 days (d). Each dot represents the normalised expression value of an individual explant; each colour represents different ewes; bars represent grand mean values; groups marked with different letters were significantly different ($p < 0.05$, Kruskal–Wallis test and Dunn's multiple comparison) within the same condition (grey letters) or between different conditions (orange letters). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

facilities [16,36]. This contrasts with the scenario found in veterinary medicine, where the birth of livestock animals occurs on farms and the slaughter of pregnant females is rare, thus limiting the availability of suitable tissues [23]. Recent publications have reported the collection of placentomes from heifers through caesarean section, but this requires skilled surgeons [37]. Other studies have entailed obtaining explants from sheep full-term placentas and using them to assess the effect of infection with *T. cruzi* and *T. gondii* [21,22,38]. However, these explants lacked the caruncles, which are regarded as the main actors in the local control of parasitic infections [23]. In addition, naturally expelled placentas display extensive disintegration of the chorionic epithelium [39] and possibly modified MHC I expression, which triggers alloimmune rejection [40]. Hence, these tissues would not represent the environment found during gestation. In fact, we decided against the use of full-term placenta explants in experiments performed with three different healthy ewes that delivered lambs in our teaching facilities, as these displayed high tissue damage scores and extremely low RNA concentrations at the time of collection (data not shown).

Based on what is described in the literature, we took advantage of ongoing experiments using healthy pregnant ewes to create a tissue biobank. Sheep have been shown to be receptive to infection with both *T. gondii* and *N. caninum* [41] and therefore represent a good starting point to standardise ex vivo models for the study of both agents. To make the most of the collected tissue and basing our approach on previous works that successfully cryopreserved human and canine placental explants, we followed similar methods to develop cryogenic protocols suitable for sheep explants. Using human explants cultured up to 2 days, Huppertz et al. (2011) demonstrated that cryopreservation with DMSO did not impair tissue morphology, viability, proliferation, or functionality after thawing [24]. Following similar protocols, Sahlfeld et al. (2012) isolated proliferative trophoblasts from canine placentas cryopreserved with DMSO, showing the same morphological features observed in fresh cells [25]. In the present study, four different cryopreservation methods were assessed, and these were based on the use of DMSO or EG as cryoprotectants under two different freezing rates. We used the tissue damage scores as a gold-standard criterion to determine the best freezing method. Unfortunately, these results were not supported by our LDH secretion findings, which were inconclusive, as discussed below. In contrast with the findings reported in the aforementioned studies, we observed that pretreatment of fresh explants with DMSO had a negative effect on their tissue integrity but also that DMSO-thawed explants tended to display worse tissue integrity scores. The histological architecture was better preserved in EG-thawed explants, especially in those cryopreserved with slow freezing rates. Hence, we focused on our slow EG cryopreservation protocol to create a tissue biobank for the subsequent experiments.

In this study, we also aimed to optimise culture conditions to extend the lifespan of fresh and EG-thawed explants. In parallel experiments, we observed no apparent differences between explants from the same donor incubated with different culture media (M-199, DMEM/F12, RPMI 1640) in terms of media pH, tissue damage scores and viability (data not shown). Similar results have been reported in studies performed with human explants [42]. We opted to use M-199 media based on the report of Wojciechowska et al. (2015), which demonstrated its

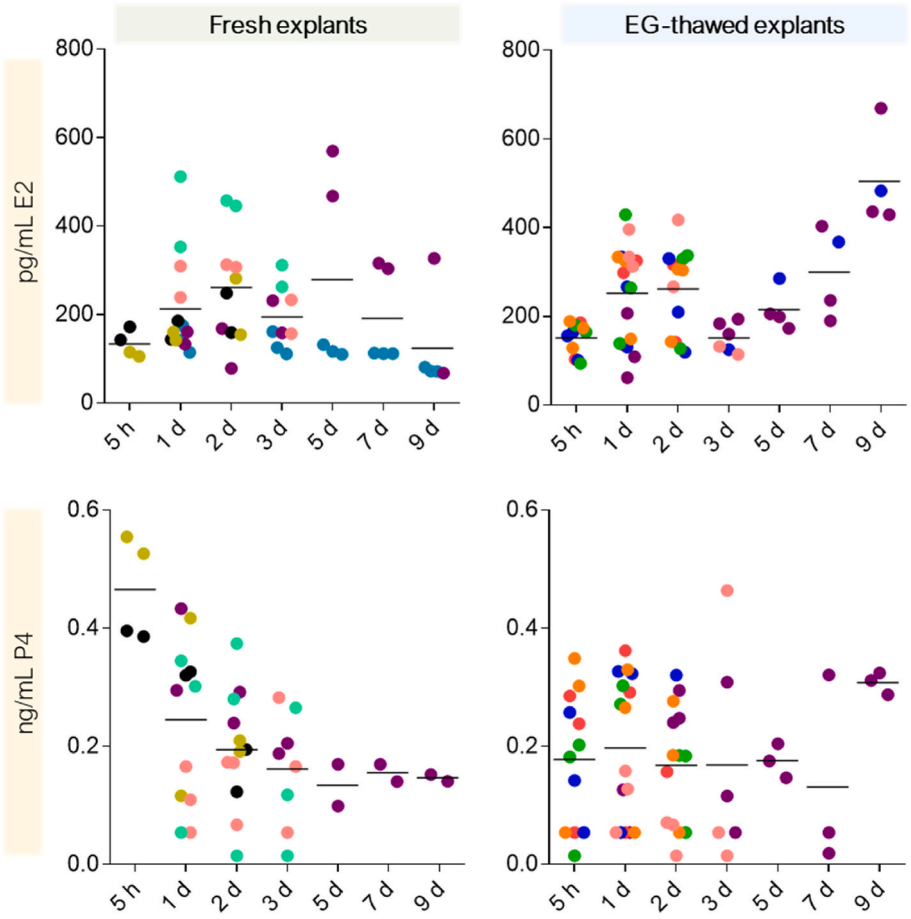

Fig. 8. Secretion of 17-β-oestradiol (E2, top panel) and 17-OH-progesterone (P4, bottom panel) in the supernatants of fresh (left panel) and EG-thawed (right panel) explants throughout the culture time (5 h–9 days). Each dot represents the hormone concentration of an individual explant; bars represent grand mean values.

improved capacity to maintain tissue integrity and hormone secretion in fresh placental explants obtained from cows [30].

Once culture conditions were established, we next studied the impact of ex vivo culture (and cryopreservation) on their structural integrity. In general, both types of explants preserved their tissue architecture and displayed progressive degradation with time, which was more evident from Day 2 onwards. EG-thawed explants showed earlier signs of tissue degradation, but compared to fresh explants, no significant differences were found. This indicates that cryopreservation has no effect on tissue degradation and that this is inherent to ex vivo culture. The results also demonstrated that fresh and EG-thawed explants could be suitable for ex vivo studies lasting up to 1–2 days. Similar findings have been described for fresh explants obtained from human placentas both at microscopic and ultrastructural levels as early as at 8 h of culture [27,43,44]. Interestingly, (i) culture of human explants under static conditions has been shown to negatively affect tissue structure and biochemical integrity; (ii) nonperfused explants display higher levels of apoptotic cells, especially trophoblasts [42,43]; and (iii) the oxygen tension present in the culture can induce changes in the synthesis of cell adhesion markers, cytokines, and hormones [45]. Altogether, these works indicate that the use of flow culture protocols and oxygen-controlled atmospheres might significantly improve our sheep explant culture model.

On the other hand, previous works carried out with human samples have described a marked degeneration of the syncytiotrophoblast after 24 h of culture; this degeneration is followed by an active generation of a new syncytiotrophoblast layer after 2–4 days in culture, which maintains the natural connections with the extracellular matrix and remains morphologically intact for up to 11 days [27,28]. Based on that, we extended the explant culture time in some samples up to 9 days and observed that the average tissue damage score of both fresh and EG-thawed explants was close to 2 until the seventh day of culture, when the expectation was to find autolytic tissue (score 3). In fact, explants cultured up to 9 days displayed metabolic activity and were able to synthesise steroid hormones. This suggests that our ex vivo model could be used to perform assays lasting beyond 2 days of culture. Nevertheless, we did not identify signs of active regeneration of the chorionic epithelium using classic histological approaches. Hence, additional studies focused on the identification of cytoskeleton and apoptosis markers through immunohistochemistry are needed to draw more precise conclusions in this regard [43]. In addition, previous studies have shown that human placental explants can retain their intact tissue structure for up to 10 days when cultured on collagen sponges [46–48]. This indicates that the application of collagen sponges to sheep explants may be advantageous, allowing experiments lasting more than 2 days.

To identify whether explant cultures remained metabolically active, we also performed different viability assays. The quantification of LDH levels in culture supernatants is a method broadly employed for human explants [24]. However, its usefulness was limited under the conditions in our study, since enzyme levels remained high under all conditions tested. This was likely a consequence of the placentome slicing, which resulted in a massive rupture of the cells present in the explant edges. A similar effect has been described in experiments carried out with placental explants from different species [49,50]. Based on these results, we opted to use two alternative assays based on XTT and resazurin. Overall, both tests demonstrated that fresh and EG-thawed explants remained metabolically active during all the time points tested, although the XTT assays were less sensitive. Previous studies have shown that the XTT assay itself can be toxic to cells when used for an

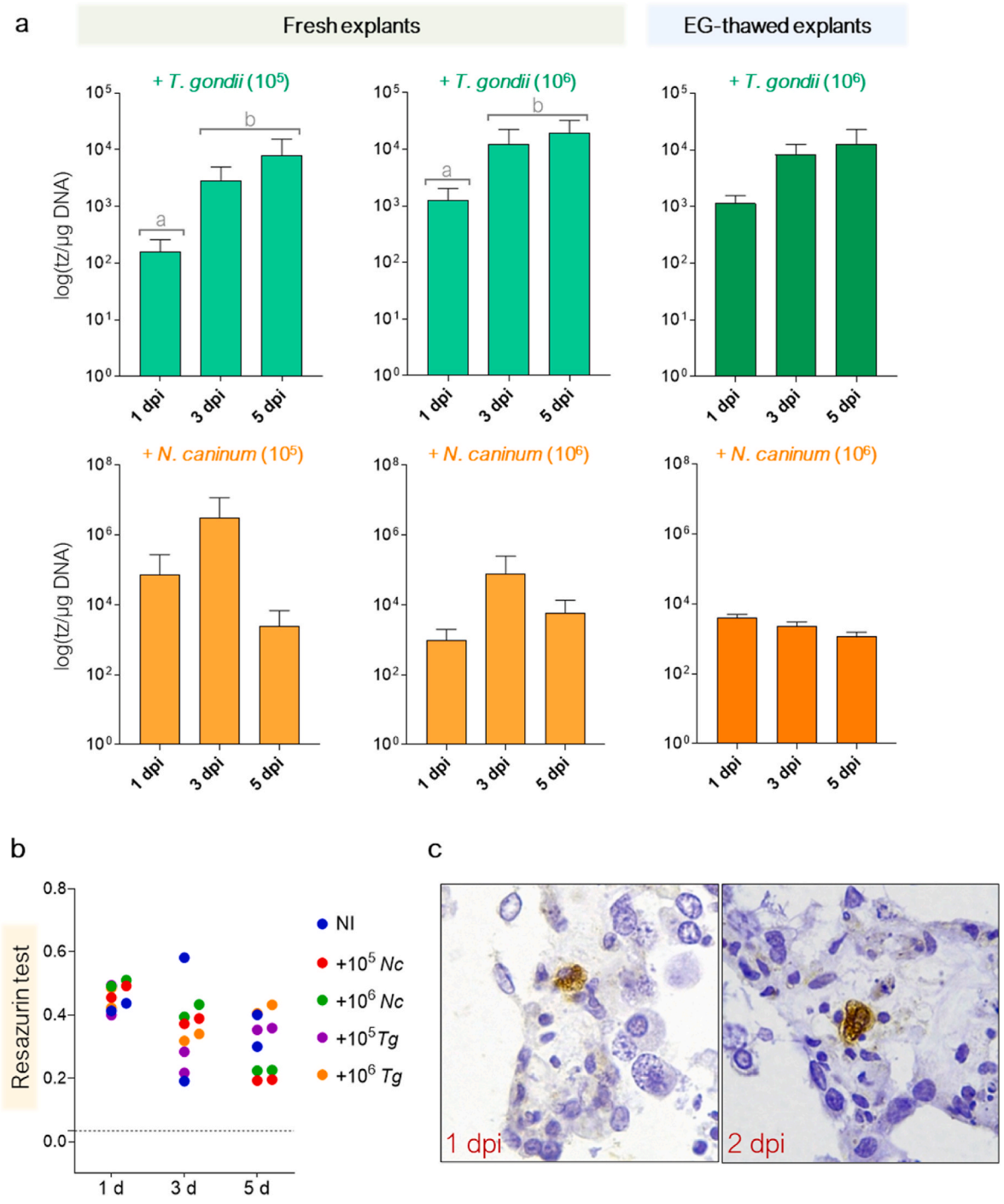

Fig. 9. a: Parasite burdens of fresh (left and centre graphs) and EG-thawed explants (right graphs) experimentally infected with *T. gondii* (top panel) or *N. caninum* (bottom panel). Column bar graph representing the mean and SD values of each condition. The parasite and challenge dose employed for each experiment are indicated on the top of every graph. Groups marked with different letters were significantly different ($p < 0.05$, Kruskal–Wallis test and Dunn's multiple comparison). **b:** Representative resazurin assay of infected and noninfected fresh explants. The bottom dotted line represents the average absorbance of explants fixed with 4% paraformaldehyde and used as controls of nonviability. Each dot represents individual explants. NI: noninfected; 10^5 and 10^6: challenge dose; *Nc*: *N. caninum*; *Tg*: *T. gondii*. **c:** Positive IHC labelling of *N. caninum* antigen within the cytoplasm of cells in experimentally infected fresh placental explants at 1 and 2 dpi. 60x.

extended period (>4 h) and highly inaccurate when culture conditions promote superoxide formation [51,52]. For this reason, we focused our efforts on the resazurin assays, which are more sensitive, especially when interpreted through fluorescence emission readings [52]. In the absence of specialised equipment, the reduction of resazurin (dark blue) to resorufin (pink) by metabolically active cells can be qualitatively interpreted with the naked eye, providing an easy and relatively inexpensive indicator of tissue viability. Based on the results from this test, we observed a marked reduction in the viability of EG-thawed explants after 3 days of culture, but this was seemingly recovered from Day 5 onwards. This could reflect the activation of tissue recovery mechanisms, but we did not find evidence of that in any of the experiments included here. For this reason, future efforts should target the identification of specific proliferation markers through immunohistochemistry

(e.g., Ki-67 protein) [43]. It is important to note that viability values can be affected by the weight of each explant, which reflects the number of cells present per well. This was not considered in our experimental design to reduce the risk of bacterial contamination due to excessive handling and to prevent a delay in explant plating that could have a negative impact on the explant viability. This drawback could be prevented in future experiments using an automated tissue chopper as described elsewhere [50]. In any case, we have been able to demonstrate, at least qualitatively, the occurrence of metabolic activity in both fresh and EG-thawed explants cultured up to 9 days despite the extent of tissue damage observed through histology. Additionally, we used the amount of RNA isolated from all the cultured explants as a marker of the transcriptomic integrity. Here, we observed that RNA concentrations decreased as tissue degradation increased. Therefore, RNA concentrations could be exploited as an indirect marker of tissue damage when a histological approach is not readily accessible.

Despite being sparse, the isolated RNA allowed us to perform RT–qPCR and quantify *PLAC-1* and *KRT8* expression until the second day of ex vivo culture. PLAC-1 is a cell surface protein whose expression is restricted to trophoblastic cells, determining the proliferative activity of the trophoblast and hence the growth of the placenta [53]. In addition, this gene is involved in the progression of certain types of cancer and in the occurrence of preeclampsia in humans [54]. In our study, only fresh explants displayed a decrease in *PLAC-1* expression after 1–2 days of culture, but this was not observed in EG-thawed explants. This could indicate a decrease in the proliferative activity, but only in fresh explants. In line with this, Wojciechowska et al. (2017) demonstrated in a series of studies that *PLAC-1* expression is downregulated upon treatment of fresh bovine placental explants with pesticides (DDT and DDE) and polychlorinated biphenyls (PCB 153, 126, and 77), and this could be potentially associated with an impairment of the proliferative capacities of the placenta [55,56]. However, considering the low quality of the RNA used for the assays in the present study, the interpretation of our results is difficult. KRT8 is an intermediate filament protein involved in placental villi formation by chorionic epithelial cells and is considered a marker of normal placental barrier function [57]. In our experiments, a progressive upregulation of *KRT8* expression was observed in fresh explants, with levels peaking on Day 2 of culture. In contrast, EG-thawed explants displayed decreasing *KRT8* transcript levels over the culture duration. This could indicate that only fresh explants retain the ability to repair the placental barrier, as demonstrated in fresh bovine placental explants [30,56]. Nevertheless, the interpretation of these findings is challenging considering the low quality of the RNA extracted. Altogether, our results show that the usefulness of *PLAC-1* and *KRT8* as markers of placental proliferative activity and normal placental barrier function, respectively, is limited for this model. Future efforts should aim to detect specific proliferation and cytoskeleton markers through immunohistochemistry to obtain better conclusions [43]. In this sense, we believe that the use of culture systems based on collagen sponges could also improve explant RNA quality. This would enable a comprehensive study of explant modulation through transcriptomic approaches.

Once we described the viability, structural integrity, and transcriptomic activity of fresh and EG-thawed explants, we went one step further and tested their functionality in terms of steroid hormone synthesis. As an endocrine organ, the sheep placenta synthetizes E2 and P4 during gestation to regulate blood flow to the pregnant uterus and maintain pregnancy, respectively [58,59]. Overall, the fresh and EG-thawed explants actively secreted E2 and P4 at all the time points tested (0 h–9 days), suggesting that they were still functional. Importantly, the tissue damage observed over the culture time did not seem to impair the explants' ability to synthesise both hormones. In line with this, previous works have described a significant increase in E2 and P4 levels in bovine placental explants stimulated with pesticides and polychlorinated biphenyls [55,56]. Similarly, human villous explants can secrete hCG and hPL for 5–7 days [60]. In our experiments, secretion of P4 was more variable, and we hypothesise that this response might be normal. Under physiological conditions, the corpus luteum supports most of the P4 synthesis needed to maintain pregnancy in ewes, and it is only at late gestation when the placenta overtakes P4 secretion [58]. Thus, considering that all our placentomes were collected at the end of the second term of gestation, this pattern of P4 synthesis could be expected. It is important to highlight that E2 and P4 secretion values were not normalised and thus could be affected by the weight of each explant, as discussed for the resazurin assays. In addition, the commercial tests employed are only validated for human samples and could yield inaccurate results. Nevertheless, recent reports have demonstrated that human-intended kits are suitable for ovine E2 and P4 analyses [61,62]. In any case, and from a qualitative perspective, our results confirm that cultured explants can produce both hormones, confirming their functionality to an extent.

Since the explants were demonstrated to be viable and functional, we then infected fresh and EG-thawed explants with *T. gondii* or *N. caninum* tachyzoites and quantified the tissue parasite burdens by qPCR. All the explants were receptive to infection by the two parasites, and no differences were recorded in the parasite burdens present in fresh and EG-thawed explants. Direct evidence of effective *N. caninum* infection was also obtained through immunohistochemistry. Compared to *N. caninum*, *T. gondii* tachyzoites showed a higher proliferation capacity, with significant increases in their growth after 3 and 5 days of infection. These findings are aligned with those of previous studies in which explants obtained from ovine term placentas supported *T. gondii* replication [21,22,38]. The results obtained in the explants infected with *N. caninum* were surprising, as pregnant sheep are especially susceptible to experimental infection with this parasite [26]. However, considering that the Nc-Spain7 isolate has a controlled number of passages in cell culture, we hypothesise that the laboratory adaptation of the *T. gondii* strain used for infections (Tg-PRU) might have influenced our results [63]. We also hypothesise that *N. caninum* would need more time than *T. gondii* to achieve its exponential growth. In an attempt to assess whether the receptivity of the explants decreased with their time in culture, a batch of fresh explants was cultured for 6 days in the absence of parasites and then infected with *T. gondii* or *N. caninum* for an additional 1, 3 and 5 days. These explants remained viable at all times in culture and displayed a similar receptivity to freshly obtained/thawed explants (data not shown). Successful infection also supports the occurrence of metabolic activity in the explants, since *T. gondii* and *N. caninum* growth rely on the recruitment of the host organelles in the surroundings of their parasitophorous vacuoles to scavenge or salvage metabolites synthesised in these organelles [64]. It is worth highlighting that the comparison of infected fresh and EG-thawed explants was performed with tissues obtained from a single animal, and this may not be sufficiently representative. However, considering the small variation observed between all animals and conditions in all the parameters measured here (viability, structural integrity, transcriptional activity, and functionality), similar results would be expected in terms of parasite replication. Nevertheless, the susceptibility of EG-thawed tissues to parasitic infection will need to be further characterised in future studies.

5. Conclusions

In summary, we have developed and characterised an ovine placental ex vivo model suitable for the study of the early interactions between *T. gondii* or *N. caninum* and pregnant sheep. This model is based on the collection of placentomes from healthy ewes, the cryopreservation of placental explants using EG under slow freezing rates, and the use of M-199-based culture media. We have also defined reproducible and simple markers of tissue integrity (damage scores, RNA concentrations), viability (resazurin assays), and functionality (synthesis of E2 and P4) that can easily be exploited to assess the quality of the model. Regarding tissue integrity, RNA quality, and *T. gondii* and *N. caninum* receptivity, explants might be useful for the study of host–parasite interactions for

periods of up to 2 days. However, viability and functionality assays suggest that these periods might be longer, up to 9 days. Our findings indicate that explant lifespan is mainly limited by the culture technique employed, and current evidence supports that there is still room for improvement of such methods using flow culture approaches, oxygen-controlled atmospheres, and/or collagen-based culture inserts. Optimisation of explant culture protocols could extend tissue integrity and RNA quality for longer periods, thus boosting their usefulness. This model also has the potential to be applied to other reproductive pathogens affecting sheep (e.g., *Brucella mellitensis*, *Chlamydia abortus*, *Coxiella burnetii*, Border disease virus) and could be easily adapted to other species (e.g., cattle, goats) or even other target tissues (e.g., intestine). While a thorough optimisation and characterisation of this model remains to be achieved, it is expected to have a significant impact on the study of transmissible diseases leading to reproductive failure. This would also contribute to reducing the number of animals used for experimental purposes.

CRediT authorship contribution statement

Pilar Horcajo: Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – review & editing. **Luis Miguel Ortega-Mora:** Conceptualization, Data curation, Funding acquisition, Resources, Supervision, roles/writing - original draft. **Julio Benavides:** Investigation, Methodology, Validation, Writing – review & editing. **Roberto Sánchez-Sánchez:** Investigation, Methodology, Validation, Writing – review & editing. **Rafael Amieva:** Investigation, Methodology, Validation, Writing – review & editing. **Esther Collantes-Fernández:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, roles/writing - original draft. **Iván Pastor-Fernández:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, roles/writing - original draft.

Declaration of competing interest

The authors declare that they have no competing interests.

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