# Embryo development is impaired in goats that are treated for hydrometra and subsequently subjected to superovulation

Ana Lucia R S Maia [1], Aline M Arrais [2], Lucia Prellwitz [1], Ribrio I T P Batista [1], Lucas M Figueira [3], Lucas F L Correia [1], Jeferson F Fonseca [4], Joanna M G Souza-Fabjan [1]

## Abstract

**Background** Reproductive efficiency after hydrometra (HD) treatment is usually unsatisfactory.

**Methods** To identify mechanisms involved in low reproductive efficiency of HD-treated goats, pluriparous dairy goats treated for HD (n=10, HD) or with no reproductive disorders (n=11, control: CONT) were induced to oestrus and superovulated. Goats were mated with fertile bucks and seven days after oestrus, non-surgical embryo recovery was performed. Embryos were evaluated and gene expression was performed.

**Results** There were no differences (P>0.05) in sexual behaviour parameters, superovulation response, mean number of retrieved structures and viable embryos between groups; although embryo recovery rate was higher (P=0.01) in CONT group. Structures in delayed stage (8–16 cells) were more frequent (P<0.05) in HD (29 vs 1 per cent) goats, as well as the percentage of advanced embryos was greater (P<0.05) for CONT (59.3 vs 33.3 per cent) goats. However, the expression of genes related to apoptosis (*BAX* and *Bcl-2*), trophectoderm differentiation (*CDX2*) and pluripotency maintenance (*NANOG*) was not affected (P>0.05) in embryos that reached the morulae and blastocyst stages.

**Conclusion** Although the HD embryos that developed to morula and blastocyst stages showed no change in the expression of genes related to their quality and implantation capacity, overall, embryo development was impaired in HD-treated goats.

## Introduction

Since the 1980s, with ultrasound (US) evaluation to pregnancy diagnosis, hydrometra (HD) has been considered one of the most important causes of subfertility or infertility in dairy goats.[1 2] Accurate aetiology of the disease has not been fully discovered,[3 4] but some risk factors were recently revealed.[5 6] Accumulation of fluid in uterus is described as a consequence of the persistence of one or more corpora lutea (CL) in ovary.[7] Thus, HD treatment consists in the drainage of uterine fluid which can be obtained with one or preferentially more administrations of prostaglandin F2α (PGF2α).[3 4 8] The proper return to reproductive activity after treatment may vary from 20 per cent[9] to 55 per cent.[8] Furthermore, goats may not carry out full-term pregnancy and/or show recurrence of the disease.[8 10] Thus, the reproductive efficiency after HD treatment results in an unsatisfactory pregnancy rate.[8] In sheep, Regassa and others[11] reported a strong association between ovarian and uterus abnormalities in slaughtered ewes. For instance, long ovarian cystic condition may change uterus immunity and environment leading to fluid accumulation and eventually bacterial infection. Souza and others[9] and Maia and others[12] revealed respectively the occurrence of ovarian follicular cysts and hydrosalpinx simultaneously to HD. These facts lead us to speculate that HD may be associated with

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[1]Departamento de Patologia e Clínica Veterinária, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil
[2]Departamento de Reprodução e Avaliação Animal, Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Brazil
[3]Departamento de Zootecnia, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil
[4]Embrapa Caprinos e Ovinos, Sobral, Ceará, Brazil

E-mail for correspondence: Dr Ana Lucia R S Maia, Departamento de Patologia e Clínica Veterinária, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil; maia.analucia@gmail.com

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ovarian or oviductal disorders, further increasing the chance of temporary or permanent infertility.

It is well known that in ruminants, the high rates of gestational loss in the early stage of pregnancy reflect on female fertility.[13] Inside the follicles, the oocyte stores specific gene products that will be first expressed by driving follicular growth up to ovulation.[14] A healthy oocyte, equipped with entire maternal molecular machinery, must have the ability to be fertilised and sustain embryonic events[15] until the maternal to zygotic transition, which occurs at 8–16 cells in goats.[16] Activation of zygotic/embryonic genes is accompanied by gradual degradation of maternal RNAs and proteins and gene expression reprogramming, necessary for later developmental events.[17] Inside the oviduct, the fertilisation and first embryonic cleavages occur. Thus, the oviduct must provide an optimal environment to support preimplantation embryonic development.[18] Analysis of RNA expression levels of preimplantation genes of embryos recovered from goats previously affected by HD can be an important tool to assess their response to the oviduct/uterine environment.

During in vitro oocyte maturation and early embryo development, the regulation of apoptosis can be evaluated by expression of B-cell lymphoma 2 (*Bcl-2*, anti-apoptotic) and Bcl-2 associated X protein (*BAX*, pro-apoptotic) factors.[19] In addition, specific genes such as caudal type homeobox transcription factor 2 (*CDX2*) play a role in the maintenance of trophectoderm integrity.[20] In goats, the Nanog homeobox (*NANOG*) mRNA can be detected since 8–16 cells. At the blastocyst stage, the *NANOG* transcript is higher expressed in inner cell mass than trophectoderm and acts in the maintaining of pluripotency and proliferation.[21] Therefore, the evaluation of aforementioned gene information can predict the ability of embryos to be implanted in recipients, without any interference of the recipient physiology, endocrinology besides all environmental factors that certainly affect pregnancy rate.

We hypothesised that HD causes reproductive failure even after its treatment due to an effect in any of the stages necessary for pregnancy success: oocyte ovulation, fertilisation, embryo development and its quality. Thus, monitoring all different steps of superovulation (SOV), non-surgical embryo recovery (NSER) and embryo evaluation, we may be able to identify any effect of HD in the oocyte quality, migration of gametes, fertilisation or oviduct/uterine environment for the proper embryonic development. Thus, this study aimed to assess physiological mechanisms related to the reproductive failure in HD-treated goats, by evaluating SOV response and embryo development and quality.

## Materials and methods

### Period, location and experimental conditions

The study was conducted in anoestrus season on a dairy goat farm (21° 21′ S and 43° 14′ W) in Minas Gerais state, Brazil. Goats were managed in an intensive production system, confined in group pens, and fed corn silage. A balanced concentrate supplement was provided on demand.[22] Mineralised salt (Caprinofós Tortuga, São Paulo, Brazil) and drinking water were available ad libitum.

### Animals

For this study, pluriparous goats (n=21) diagnosed by transrectal US (Mindray M5VET, Shenzen, China—8.0 MHz) according to Maia and others[8] with hydrometra (n=10, HD) or not (n=11, control: CONT) were used. The HD goats aged 4.3±0.5 years (mean±SEM), weighed 70.5±1.4 kg and had body condition score (BCS) of 3.0±0.1 (scale 1–5[23]). In order to avoid an imbalance between the groups, goats of CONT had similar age (3.5±0.4 years), body weight (67.3±1.8 kg) and BCS (2.9±0.1) of the goats diagnosed with HD. However, CONT goats had no reproductive disorder in transrectal US assessment as well as in their reproductive history. Goats with HD were treated with three doses of 37.5 µg d-cloprostenol (Prolise; Tecnopec, São Paulo, Brazil) intramuscularly at intervals of 10 days[8] and remained with no liquid in the uterine lumen for two months until the start of the experiment.

### Oestrus induction, superovulation and non-surgical embryo recovery

At the onset of the protocol (D0; 5 pm), controlled internal drug release (CIDR) devices containing 0.33 g progesterone (Eazi-Breed CIDR; Pfizer do Brasil Saúde Animal, São Paulo, Brazil) were inserted and removed six days later (D6; 5 pm). The SOV was performed with 133 mg of porcine follicle stimulating hormone[24] (pFSH—Folltropin-V; Bioniche Animal Health, Belleville, Canada) intramuscularly in six decreasing doses (25, 25, 15, 15, 10 and 10 per cent), every 12 hours, starting 48 hours before device removal (D4). Three intramuscular injections of 37.5 µg d-cloprostenol (Prolise; Tecnopec) simultaneously to fourth and fifth doses of pFSH and 12 hours before the NSER, plus 25 µg gonadotrophin releasing hormone (GnRH—Gestran; Tecnopec) intramuscularly 24 hours after device removal, were given. In addition, three doses of 75 mg flunixin meglumine (Flumax; J.A. Saúde Animal, São Paulo, Brazil) intramuscularly (to prevent premature luteal regression) were administered 36, 60 and 84 hours after GnRH. After device removal, oestrus was monitored twice daily (8 to 9 am and 5 to 6 pm). Goats were mated with fertile bucks with a ratio of 4:1. At the first day of the protocol (D0) and six days after oestrus onset (D13), transrectal US evaluations were done to observe ovarian status and estimate the number of CLs,[25] respectively. Seven days after oestrus onset, NSER was performed[26] (figure 1). Goats with <3 CLs were considered as non-responding to the SOV protocol.

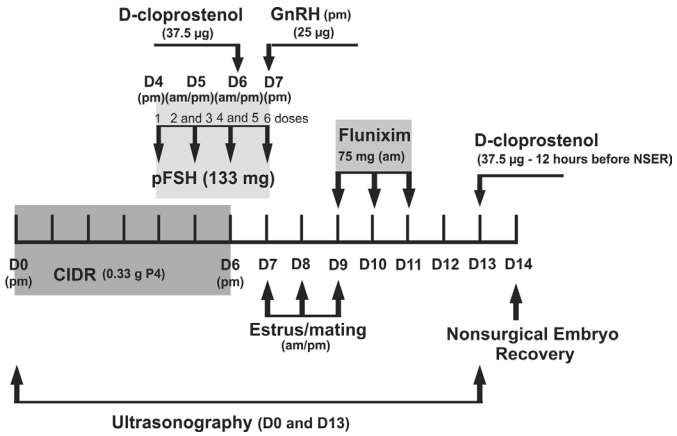


**Figure 1** Schematic presentation of experimental design: detailed protocol induction of oestrus and superovulation for non-surgical embryo recovery in hydrometra-treated (n=10) or control (n=11) dairy goats. CIDR, controlled internal drug release; GnRH, gonadotrophin releasing hormone; NSER, non-surgical embryo recovery; pFSH, porcine follicle stimulating hormone; P4, progesterone.

## Structure evaluation

All recovered structures were enumerated, and embryos were transferred to the holding medium (Holding Plus; Cultilab, Campinas, Brazil). Embryonic evaluation followed the same principles used for cattle, in which Grade I: excellent; Grade II: good; Grade III: bad and Grade IV: degenerated.[27] Morphological evaluations were performed under a stereomicroscope (x 40 magnification). Morulae and blastocysts grades I and II were equally transferred to cryotubes and frozen in liquid nitrogen for subsequent RNA extraction.

## Gene expression analyses

Samples from CONT and HD groups were analysed by quantitative reverse transcription polymerase chain reaction (qRT-PCR), according to Batista and others.[28] Total RNA extraction was performed from three pools of five embryos/group using RNeasy Micro kit (Qiagen, Hilden, Germany) according to manufacturer instructions and treated with DNase. Complementary DNA (cDNA) was synthesised using Superscript III First-strand supermix kit (Invitrogen, Carlsbad, CA, USA) and a random hexamer primer, according to manufacturer instructions. The cDNA quantification from each pool per group was performed using 1 µL of sample in spectrophotometer ND-100 (NanoDrop Products, Wilmington, DE, USA). Relative quantification was performed in triplicate in ABI Prism1 7300 (Applied Biosystems, Foster City, CA, USA) and reactions using a mixture of Power SYBR Green PCR Master Mix (Applied Biosystem), 200 ng cDNA, nuclease-free water and specific primers for each reaction. Template cDNA was denatured at 95°C for 2 min, followed by 45 cycles of 95°C for 15 s; gene-specific primer annealing temperature for 30 s and elongation at 60°C for 30 s. After each PCR run, a melting curve analysis was performed to confirm that a single specific product was generated. Negative controls, composed of PCR reaction mix without nucleic acid, were also run for each group of samples. Amplicon size was confirmed by agarose gel electrophoresis. Primer sequences and sizes of amplified fragments for all transcripts are shown in table 1. Primer efficiency was calculated for each reaction using Lin-RegPCR software.[29] Average efficiency of each set of primers was calculated and considered in all groups. Expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and H2A Histone Family Member Z (*H2AFZ*) genes were used as endogenous reference genes. Relative abundance analyses were performed using *REST* software[30] and based on primer efficiency. Values found in embryos from HD group are shown as n-fold differences relative to control.

## Statistical analyses

Statistical analyses were performed using SAS software (Statistical Analysis Software, Cary, NC, USA). Results expressed as percentages (goats in oestrus, responsive donors, cervical transposition and uterine flushing, recovery rate, viable rate, young and advanced embryos) were analysed using $\chi^2$ or Fisher exact tests, as appropriate. Quantitative variables were evaluated for residual normality with the univariate procedure and tested for homogeneity of variances with the Bartlett test, using the GLM procedure. Variables that did not meet the analysis of variance (ANOVA) assumptions as interval to oestrus, oestrus duration, number of mating and number of recovered structures were square root transformed

**Table 1** Description of primer sequences used in the analysis of gene expression in embryos recovered by non-surgical technique after induction of oestrus and superovulation in hydrometra-treated or control dairy goats

| Gene | Sequence of primers (5′–3′) | Annealing temperature (°C) | Size (bp) | Reference |
|---|---|---|---|---|
| *BAX* | F, CCT GGG ATC TTG AAA CTC TCC TT<br>R, CTG AGC CAG GCT GAA ATC AAA A | 60 | 566 | Chakravarthi and others[41] |
| *Bcl-2* | F, GCC GAG TGA GCA GGA AGA C<br>R, GTT AGC CAG TGC TTG CTG AGA | 60 | 214 | Chakravarthi and others[41] |
| *CDX2* | F, GCC ACC ATG TAC GTG AGC TAC<br>R, ACA TGG TAT CCG CCG TAG TC | 60 | 140 | Sakurai and others[42] |
| *NANOG* | F, TTC CCT CCT CCA TGG ATC TG<br>R, AGG AGT GGT TGC TCC AAG AC | 53 | 501 | Sanna and others[43] |
| *GAPDH* | F, ATG TTT GTG ATG GGC GTG AA<br>R, ACA GTC TTC TGG GTG GCA GT | 60 | 176 | O'Connor and others[44] |
| *H2AFZ* | F, GTC GTG GCA AGC AAG GAG<br>R, GAT CTC GGC CGT TAG GTA CTC | 57 | 182 | O'Connor and others[44] |

**Table 2** Parameters of healthy (control) or hydrometra-treated goats submitted to oestrus induction and superovulation, followed by non-surgical embryo recovery (mean±SEM)

| Parameters | Control (n=11) | Hydrometra* (n=10) | P value |
|---|---|---|---|
| Goats in oestrus (%) | 100.0 | 100.0 | 1.00 |
| Interval to oestrus (h) | 29.1±3.9 | 31.0±2.3 | 0.55 |
| Duration of oestrus (h) | 21.8±2.0 | 18.2±2.8 | 0.25 |
| Number of mating | 2.8±0.2 | 2.5±0.3 | 0.29 |
| Number of corpora lutea (CL) | 8.5±1.3 (93) | 7.7±0.9 (77) | 0.65 |
| Responsive donors (≥3 CL) (%) | 91.0 (10/11) | 100.0 (10/10) | 1.00 |
| Cervical transposition and uterine flushing (%) | 100.0 | 100.0 | 1.00 |
| Collection duration (min) | 23.2±1.9 | 19.1±0.6 | 0.08 |
| Recovery rate (%)† | 81.7 (76/93) | 58.4 (45/77) | 0.01 |
| Recovered structures per goat | 6.9±1.7 | 4.5±1.2 | 0.37 |
| Viable rate (%) | 71.0 (54/76) | 60.0 (27/45) | 0.23 |
| Viable embryos per goat | 4.9±1.6 | 2.7±0.9 | 0.23 |

() Number of total CL, number of animals or number of structures.
*Animals detected with hydrometra were treated with three doses of prostaglandin F2a and presented no uterine fluid for two months before the start of this experiment.
†Total of structures/CLx100.

before analysis. These variables and the number of CL were analysed using the GLM procedure. The quantitative data were expressed as means±SEM. For all tests, significance level of 5 per cent was used.

## Results

### Oestrus induction, superovulation and non-surgical embryo recovery

Reproductive parameters are depicted in table 2. There were no differences (P>0.05) between CONT and HD in the mean (±SEM) values of interval to oestrus (29.1±3.9 and 31.0±2.3 hours), duration of oestrus (21.8±2.0 and 18.2±2.8 hours) and number of mating (2.8±0.2 and 2.5±0.3). Interval to oestrus and duration of oestrus varied from 14 to 62 and 10 to 34 hours, in CONT, and from 24 to 38 and 8 to 34 hours, in HD group, respectively. There were no differences (P>0.05) between mean (±SEM) number of CL between CONT (8.5±1.3) and HD (7.7±0.9). Only one goat (CONT group) was considered as non-responding to the SOV protocol (2 CLs), but took part of the NSER.

All goats had total cervical transposition and the efficiency of flushing medium recovery of 100 per cent (400 ml). To perform all procedures of NSER (from the speculum introduction into the goat's vagina until final uterine flushing), it was necessary per goat the minimum and maximum of 15 and 35 minutes, respectively. There were no differences (P>0.05) between CONT and HD in the mean (±SEM) values of transcervical collection duration (23.2±1.9 and 19.1±0.6 minutes), recovered structures per goat (6.9±1.7 and 4.5±1.2) and viable embryos per goat (4.9±1.6 and 2.7±0.9). There was no difference (P>0.05) in the viable rate of embryos collected, but CONT and HD differed on the recovery rate (P=0.01). Number of collected structures ranged from 0 (CONT and HD) to 11 (HD) and 16 (CONT).

### Structure evaluation

A difference (P<0.05) was detected in the percentage of structures in delayed stage (8–16 cells) between CONT (1 per cent, 1/76) and HD (29 per cent, 13/45). The only structure in delayed stage in CONT group came from a goat that had other five structures retrieved (1 unfertilised oocyte, 2 compact morulae, 1 blastocyst and 1 expanded blastocyst). Thirteen structures in delayed stage in HD group came from six goats that had a total of 29 structures collected (2 unfertilised oocytes, 2 degenerated, 15 compact morulae, 2 early blastocysts, 5 blastocysts and 3 expanded blastocysts).

The CONT group had numerically higher number of unfertilised oocytes (CONT, n=17 compared with HD, n=3; P=0.35) and expanded blastocyst (CONT, n=22 compared with HD, n=3; P=0.08). The high number of unfertilised oocytes in the CONT group was related to only one goat that had 14 structures retrieved, while the expanded blastocysts were obtained in six and three goats from CONT and HD, respectively. When grouping either young (morula+compact morula+early blastocyst) or advanced (blastocyst+expanded blastocyst) embryos, there was a difference (P<0.05) between CONT and HD (figure 2).

### Gene expression analysis

Figure 3 shows gene expression analysis of embryos recovered from CONT and HD-treated goats. There was no difference (P>0.05) in the expression of apoptosis-associated genes (*BAX* and *Bcl-2*), internal cell mass differentiation (*NANOG*) and trophectoderm (*CDX2*) between groups.

## Discussion

To the best of our knowledge, this was the first study comparing the response to induction of oestrus and SOV, followed by NSER and evaluation of embryo development and quality between goats with no reproductive disorder and goats previously affected and treated for HD. In the current study, there were three important aspects to be highlighted. First, HD-treated goats were induced to oestrus and SOV with the same efficiency as CONT goats. Second, impairment in the embryonic development of HD-treated goats has been observed. More embryos blocked at 8–16 cells and even those that developed, did it more slowly since at D7 there were more young embryos in HD than in CONT. Third, well-developed embryos from HD presented no difference if compared with CONT in the expression of genes related to quality and implantation. Altogether, these aspects may help to elucidate why some goats have low reproductive performance while others have a normal pregnancy after HD treatment.[8]

In the present study, all goats showed oestrus. No differences were identified between CONT and HD in the mean interval from device removal to oestrus onset (~30 hours), duration of oestrus (~20 hours)

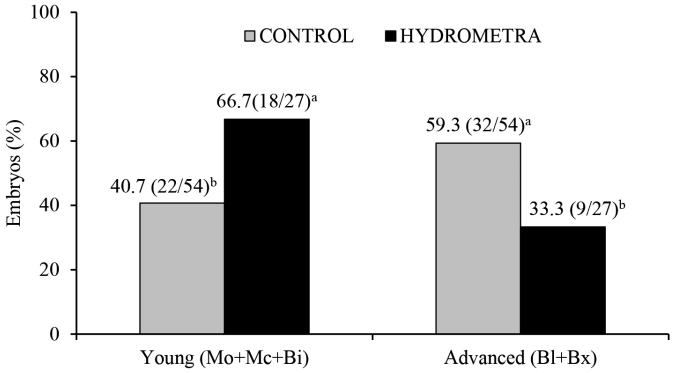


**Figure 2** Percentage of young and advanced stage of development of embryos retrieved from dairy goats (Control, n=11; Hydrometra-treated, n=10) submitted to induction of oestrus, superovulation with 133 mg porcine follicle stimulating hormone (pFSH) and non-surgical embryo recovery seven days after natural mating. [a,b]Different superscripts indicate difference by Fisher's exact test groups (P<0.05). Bi, early blastocyst; Bl, blastocyst; Bx, expanded blastocyst; Mc, compact morula; Mo, morula.

and the number of mating (~2.7), respectively. These values were similar to a previous study from our group with dairy goats submitted to SOV and NSER during the non-breeding season.[31] Moreover, Motlomelo and others[32] observed a mean oestrus interval and duration of 27.2 and 35.2 hours using 16-day CIDR during the breeding season. Although there was a similarity between results of the interval to oestrus, the duration of oestrus presented by CONT and HD seemed to be inferior, probably because our study was done during the anestrous season. It is relevant to highlight that our results showed that two months after HD treatment, the goats were able to resume normal ovarian activity and respond to the protocols.

In the US evaluation performed at the day before NSER, the mean number of CLs (~8.1) counted in CONT and HD were similar to 8.6 observed during the winter by Sánchez-Dávila and others[33] and below to 12.8 found by Batista and others.[24] In the present study, the lack of difference between groups in the number of CLs demonstrates the ovarian ability of the HD-treated goats in responding to pFSH stimulation and GnRH action. Thus, NSER was efficiently performed in 100 per cent of the goats with total flushing medium recovery. Currently in Brazil, NSER is the technique of choice[26] and HD-treated goats presented no anatomical

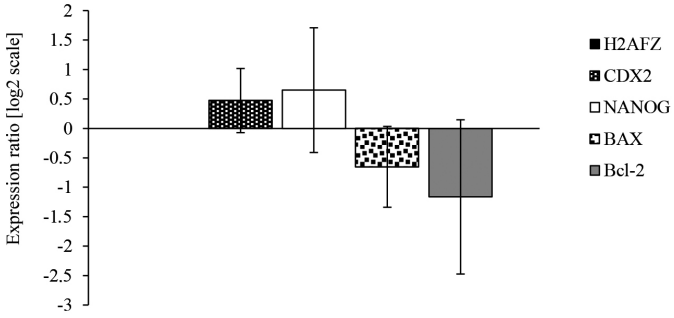


**Figure 3** Expression profile of apoptosis-associated genes (*BAX* and *Bcl-2*) and embryonic differentiation (*CDX2* and *NANOG*) in embryos recovered from hydrometra-treated goats. Data show mean±SD fold changes relative to the calibrator (=1), which was the control data. As reference genes, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and H2A Histone Family Member Z (*H2AFZ*) were used.

alteration that has made it impossible or difficult to transpose the cervical ostium. The mean number of structures (~5.8) and viable embryos (~3.9) recovered in CONT and HD goats were similar to 4.0 and 3.8 found during the winter by Sánchez-Dávila and others[33] and below to 8.2 and 5.2 observed by Batista and others,[24] respectively. In the present study, as there was no difference in total structures and viable embryos between groups, HD-treated goats demonstrate to have no impairment in steroidogenesis and folliculogenesis. These facts suggest that the low efficiency observed in goats after HD treatment is not related to oocyte growth or ovulation failure.

Regarding morphological assessment of embryo development, the highest number of embryos in delayed stage in HD group (29 per cent compared with 1 per cent) was probably the main finding in this study. Curiously, most of (6/10) HD-treated goats that had embryos in delayed stage recovered, had also viable embryos at different stages of development. According to Bó and Mapletoft[34] in superovulated cows, it is common to find embryos in various stages of development. In the present study, 29 per cent of HD embryos reached only 8–16 cells stage. In this case, we may speculate two different causes. First, they could be a result of poor-quality oocytes. According to Sirard and others,[35] the developmental ability showed in the first week after fertilisation is inherent of certain oocytes, and it is influenced by the follicular status from which they came from. Embryos that blocked at maternal to zygotic transition period, which in goats occurs at 8–16 cells,[16] may be associated to oocytes that fail to activate properly the embryonic genome.[35 36] Second, it could be related to the occurrence of chromosomal abnormalities, which in bovine embryos is characterised as a delay in the early stage of embryonic development. In vitro, the chromosomal abnormalities affect growth by delaying cleavage, leading to a low number of cells at specific stages of development.[37]

In fact, the percentage of advanced embryos was significantly greater for CONT (59.3 per cent) compared with HD (33.3 per cent). By grouping and classifying embryos in these two categories, we were able to demonstrate that seven days after oestrus/mating, most of the CONT embryos reached advanced stages (Bl and Bx) when compared with HD embryos. It is well known that in in vitro produced embryos, early cleavage presents higher probability to develop to the blastocyst stage and the embryo kinetics may be criteria to estimate embryo quality.[38] Interestingly, the importance of co-culturing early embryos with oviductal cells on blastocyst rate/quality and mRNA abundance of genes associated to embryo development was highlighted.[39] Thus, according to our results, CONT embryos started cleaving earlier probably due to optimal physicochemical environment of the oviduct. Maillo and others[40] reported that early embryos (day 3) were able to avoid maternal immune

response due to a crosstalk between them and the oviduct. Moreover, goats affected by hydrosalpinx presented histological alterations in oviduct and also in endometrial epithelium and glands of uterine horns.[12] Altogether, these data lead us to reflect that goats affected by HD may have some disturbance in embryo–oviduct crosstalk, impairing embryo development. Probably this alteration affected the oviductal capacity of embryo transportation since there was also a difference between HD and CONT in the recovery rate.

Lastly, gene expression analysis reinforces the quality of morulae and blastocysts evaluated, as there was no difference between CONT and HD. These data proved that despite compromising the developmental dynamics of some structures, the embryos from HD-treated goats that reached to the morula and blastocyst stages of grades I and II had no alteration in their quality compared with the ones from healthy goats, regarding the expression profile of genes associated with apoptosis (*BAX* and *Blc-2*), trophectoderm (*CDX2*) and internal cell mass (*NANOG*) differentiation.

## Conclusions

In conclusion, induction of oestrus, SOV response and NSER technique were not impaired in HD-treated goats compared with CONT ones. Although the grade I and II HD embryos that developed to morula and blastocyst stages showed no change in the expression of genes related to their quality and implantation capacity, overall, embryo development was impaired in HD-treated goats. Thus, SOV may not be indicated as an efficient way to return to the reproductive activity in HD-treated goats. The use of this biotechnology should be restricted only to goats of high genetic merit that need to be kept in the herd.

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**Competing interests** None declared.

**Patient consent for publication** Not required.

**Ethics approval** The Animal Care Committee of the Universidade Federal Fluminense approved the study design (protocol no. 5366140319), and it was conducted under the principles of the Brazilian Society of Laboratory Animal Science, which regulates conditions for experiments involving animals.

**Data availability statement** All data relevant to the study are included in the article or uploaded as online supplementary information. The data that support the findings of this study are available from the corresponding author on reasonable request.



**ORCID iDs**
Ana Lucia R S Maia http://orcid.org/0000-0001-6875-1730
Aline M Arrais http://orcid.org/0000-0002-8150-8441
Lucia Prellwitz http://orcid.org/0000-0003-2835-9990
Ribrio I T P Batista http://orcid.org/0000-0003-4938-2655
Lucas M Figueira http://orcid.org/0000-0003-3242-2294
Lucas F L Correia http://orcid.org/0000-0003-1990-3325
Jeferson F Fonseca http://orcid.org/0000-0001-8028-3984
Joanna M G Souza-Fabjan http://orcid.org/0000-0002-4872-1718

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