The Hyaluronan and its eraser, which is better for embryo development?

W. F.A. Marei[A,B], M. Salavati[A], T. Tremaine[A], K.A. Raheem[A,B], R. Chavoshinejad[A], M. Khalid[A], and A. A. Fouladi-Nashta[A]*

[A]Reproduction and Development Research Group, Department of Comparative Biomedical Sciences, The Royal Veterinary College, Hawkshead Lane, Hatfield, Herts AL9 7TA, UK. [B]Department of Theriogenology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt. [C]Department of Veterinary Surgery and Theriogenology, Michael Okpara University of Agriculture, Umudike, Nigeria.

Short title: Hyaluronan vs. Hyaluronidase on embryo development

Summary sentence: Presence of Hyaluronan (500-750 kDa) in the embryo development environment does not appear to enhance embryo development, while HA fragments created by by Hyal2 is beneficial. This is supported by some transcriptomic changes in oviductal epithelial cells.

Key words: *Blastocyst, Hyaluronidase-2, IGF, HSP70, IL1α*

*Correspondence: Ali A. Fouladi-Nashta, Department of Comparative Biomedical Sciences, The Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Herts AL9 7TA, UK. Fax: 44-1707-666371; e-mail: afouladi@rvc.ac.uk

Abstract

Our aim was to examine size-specific effects of Hyaluronan (HA) on preimplantation embryo development. We investigated the effects of Hyalovet (HA, 500–750 kDa; the size produced by HA synthase-3, which is abundant in the oviduct), or HA treated with Hyaluronidase-2 (Hyal2; also expressed in the oviduct that breaks down HA into 20 kDa fragments). In experiment 1 (in vivo), oviducts of synchronized and superovulated ewes (n = 20) were surgically exposed on Day 2 post-mating, ligated, and infused with either Hyalovet, Hyalovet + Hyal2, Hyal2, or PBS (control). Ewes were killed 5 days later for recovery of embryos and oviductal epithelial cells (OEC). Blastocyst rates were significantly higher in Hyal2 and Hyalovet + Hyal2 oviducts. Hyaluronidase-2 infusion resulted in higher blastocyst cell numbers and hatching rates. This was associated with increased HSP70 expression in OEC. In contrast, Hyalovet resulted in the lowest development to blastocyst stage and lowest hatching rates, and decreased IGF2 and IGFBP2 expression in OEC. IGF1 and IL1α expression were not affected. In experiment 2, to rule out indirect effects of oviductal factors, ovine embryos were produced and cultured with the same treatments in vitro from Day 2 to 8. Hyaluronidase-2, but not Hyalovet, enhanced blastocyst formation and reduced inner cell mass apoptosis. Hyalovet inhibited hatching. In conclusion, the presence of large-size HA (500–750 kDa) in the vicinity of developing embryos appears to disturb the oviductal environment and embryo development in vivo and in vitro. In contrast, we show evidence that breakdown of HA into smaller fragments is required to maximize embryo development and blastocyst quality.

Introduction

The oviduct provides the appropriate conditions required for the process of zona maturation, capacitation, fertilization and early stages of early embryo development. Distinctive differences in the oviductal transcriptome (Hess, et al. 2013) and proteome (Seytanoglu, et al. 2008) between follicular and luteal phases of the reproductive cycle in human and animal models are regulated by the hormonal environment of the oviduct. This in turn interacts with and influences gametes and embryos

during early development. The secretory proteome of the oviduct was also shown to change in response to gametes (Georgiou, et al. 2005) illustrating bidirectional cross-talk. A vast number of oviductal factors that support oocyte and embryo development have been described (Aviles, et al. 2010). Among those factors, hyaluronic acid (Hyaluronan, HA) is of special interest.

Hyaluronan is a major component of the extracellular matrix. It is synthesized by hyaluronic acid-synthase (HAS) enzymes (HAS1, HAS2 and HAS3) (Weigel, et al. 1997) in the form of linear polymer that extrudes through the membrane during its synthesis to the outside of the cell (Laurent 1998). The molecular weight of HA is determined by the isoform of HAS which also determines its functions (for review, see Dicker, et al. 2014, Stern, et al. 2006). HAS1 and HAS2 produce large-sized HA (up to 2000 kDa) while HAS3 produce a lower molecular-weight HA (100–1000 kDa). The functions of low molecular weight HA are mainly mediated through HA receptors, most commonly, CD44 (Aruffo, et al. 1990) and is reported to be involved in cell proliferation, survival, and differentiation, as well as cell-cell and cell-matrix interaction (Dicker, et al. 2014, Lesley, et al. 1993).

Ovulated cumulus oocyte complexes (COCs) reach the oviduct embedded in a large-sized HA-rich matrix of expanded cumulus cells (Russell and Salustri 2006). In addition, HA is synthesized by the oviduct and embryos; HA could be detected in the oviductal fluid of the cyclic heifers and cows and has been shown to be at highest concentration on the day of ovulation (Bergqvist, et al. 2005). Transcripts for HAS2 and HAS3 have been found in the oviduct of several animal species (Mohey-Elsaeed, et al. 2015, Tienthai, et al. 2003, Ulbrich, et al. 2004). It was noticed that HAS3 expression was higher in the isthmus compared to ampulla (Mohey-Elsaeed, et al. 2015, Ulbrich, et al. 2004) suggesting that a gradient decrease in the molecular size of HA is required during embryo development in the oviduct. In addition, *HAS2* messenger RNA (mRNA) has been detected in all stages of preimplantation human embryos (Choudhary, et al. 2007). In cattle, we have demonstrated that *HAS2* and *HAS3* are expressed at all stages of early embryo development (from 2-cell to blastocyst

stage). We have found that *HAS2* mRNA expression tends to decrease with the progression to the blastocyst stage, whereas level of HAS3 expression did not change (Marei, et al. 2013).

At the local cellular level, HA undergoes high rate of turnover which include de-polymerization by Hyaluronidases (Hyals). Hyal2 and Hyal1 are the major mammalian hyaluronidases in somatic tissues (Bastow, et al. 2008). They act sequentially to degrade high molecular weight hyaluronan to tetrasaccharides. Twenty-kDa hyaluronan fragments are generated at the cell surface by Hyal2, transported intracellularly by internalization (which is evident in bovine embryos (Furnus, et al. 2003)), and then further digested by Hyal1 to produce HA tetrasaccharide (Csoka, et al. 2001). The HA fragments generated by HA depolymerisation by Hyals are biologically active molecules that have diverse functions associated with embryo growth and survival, including stimulation of cytokine production, growth factor receptor expression, and protection against apoptosis (Stern, et al. 2006). Hyaluronan have been shown to regulate expression of factors such as insulin-like growth factors (IGFs) (Yevdokimova and Podpryatov 2005), heat shock proteins (Xu, et al. 2002), and interleukins, which are known to be important for early embryo development (Aviles, et al 2010). We have recently shown that the bovine oviductal epithelial cells (OECs) express *Hyal2* and the level of expression in the isthmus is much higher than in the ampulla (Marei, et al. 2013) suggesting their dependence on the oviductal secretions for endogenous HA breakdown. The presence of HA and its turnover in the vicinity of the oocyte during fertilization and the developing embryo suggest a biological role of HA in regulating these processes; however, this role is not well defined. We have demonstrated that in vitro supplementation of cleaved bovine embryos with Hyal2 significantly increased their development to blastocyst stage and increased embryonic cell numbers (Marei, et al. 2013). However, the differential effects of HA and its fragments during early embryo development have not been previously studied.

Building on the fundamental knowledge generated in our lab about the role of HA and Hyal2 during early embryo development *in vitro*, our objective in the present study was to compare size-specific effects of HA and its fragments on preimplantation embryo development. We compared the effects

of large-size HA (at the molecular weight produced by HAS3, 500–750 kDa), or fragmented HA (HA treated with Hyal2) versus vehicle control. Hyaluronidase 2 alone was also tested as an extra control to examine the effect of depolymerization of endogenous HA produced by oviduct and/or embryos. The effects on early embryo development were observed. In addition, the expression patterns of selected candidate genes expressed in the oviduct and known to affect embryo development were investigated in the OECs in each treatment group. Simultaneously, direct effects of the previously mentioned HA and Hyal2 treatments on embryo development were confirmed using in vitro–produced ovine embryos.

Materials and Methods

Experimental animals

The *in vivo* study was conducted at the Royal Veterinary College, after obtaining approval from the local ethical committee and authorization from the UK Home Office in compliance with Animal Scientific Procedures Act (1986). A total of 20 proven fertile, non-pregnant, 2-year old Welsh-mountain ewes, were used for this study during their breeding season. After a period of acclimatization, ewes were scanned to confirm the absence of pregnancy. Ewes were synchronized to a common estrus in five groups of four ewes every other day using vaginal sponges (Chronogest, Intervet, UK) for 12 days. Ewes were superovulated using a single injection of 700 IU pregnant mare serum gonadotropin (PMSG, intervet) and 230U Follitropin (Bioniche Animal Health, Belleville ON) 2 days before sponge removal to maximize the number of embryos recovered and increase the statistical power of the in vivo study. Receptal (8 µg Buserelin, GnRH agonist) was injected 24 hours after sponge removal to synchronize ovulations. Ewes in each synchronization group were then hand-mated by one of two proven fertile rams. Two days after mating, ewes were anesthetised, and reproductive tract was exposed by laparotomy. The number of corpora hemorrhagica (indicating number of ovulations) on each ovary was recorded, and oviductal treatments were infused.

Oviductal infusions and treatments

Oviducts were ligated with suture material (Silk) at the utero-tubal junction. A blunt needle attached to a syringe containing the treatment was introduced into the ampulla through the infundibulum. A loose ligation was placed at the base of the infundibulum around the needle and was tightened immediately after injection of 200 µL of one of the following four treatments into the oviduct. To minimize the effects of individual variation in nutritional, health, or hormonal environment on embryo development results, one oviduct in each ewe received either 0.5 mg/mL Hyalovet (0.5–0.75 × 106 Da of HA; Bioniche Animal Health, Ireland) or Hyalovet + Hyal2 (300 IU/mL), whereas the other oviduct received Dulbecco's Phosphate Buffered Saline (PBS, EGGTech, Wiltshire, UK) as a vehicle Control or Hyal2 (300 IU/mL) in a 2 × 2 factorial design within each group of four ewes. Hence, each treatment was infused into a total of 10 oviducts. All treatments were dissolved in PBS under complete sterile conditions and incubated for at least 2 hours at 37 °C before infusion. This period was enough to completely depolymerize HA by Hyal2 in the Hyalovet + Hyal2 treatment before infusion as tested by agarose gel electrophoresis and HA-binding protein staining described in our previous studies (Raheem, et al. 2013). Treatment concentrations were decided based on our previous results in the bovine (Marei, et al. 2013). After infusion, reproductive tract was gently placed back to its position in the abdomen, abdominal wall was sutured, and ewes were allowed to recover. Non-steroidal anti-inflammatory products were not used for postoperative analgesia as they may interfere with prostaglandin E2 (PGE2) signaling during embryo development. Instead, fentanyl transdermal patches were fitted on the forelimbs and changed after 48 hours. All ewes were killed 5 days after oviductal infusion, and reproductive tracts were recovered and immediately transferred to the laboratory at 37°C.

Embryo recovery

Oviducts were dissected from the surrounding tissue, washed, and placed in a sterile 90 mm dish in sterile PBS containing polyvinylpyrrolidone (1 mg/mL). The two ligated ends were cut, and the

oviductal contents were flushed out by injecting 1 mL PBS-PVP into the uterine end of the oviduct. The number and stage of development of the recovered embryos were recorded. Blastocysts were immediately assessed for quality by differential staining combined with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Fouladi-Nashta, et al. 2005). The OECs were harvested as previously described (Way, et al. 2006), pelleted and snap frozen for gene expression analysis. The number of ovulations was recorded for each ovary. Embryo recovery rate was calculated; the number of embryos recovered from each oviduct as a percentage of the number of ovulations (corpora hemorrhagica) in the ipsilateral ovary. Blastocyst rate was calculated in each oviduct as the proportion of blastocysts from the total number of recovered embryos.

Ewes were considered not fertilized at mating if only non-fertilized oocytes were recovered at the time of embryo recovery. These ewes were excluded from the data analysis. One ewe had to be euthanized because of illness before embryo recovery. The final data presented for this experiment are derived from n = 8 oviducts infused with Hyalovet, n = 8 with Hyal2, n = 7 with Hyalovet + Hyal2, and n = 7 oviducts infused with PBS.

In vitro embryo production

Ovine embryos were produced *in vitro* as previously described by Marei et al. 2012 with some modifications. Briefly, ovaries were collected from a local abattoir and transported to the laboratory where COCs were aspirated from 3 to 5-mm antral follicles. Grade 1 and 2 COCs characterized by dark homogenous ooplasm and more than four layers of compact cumulus cells were washed twice in TCM199 supplemented with 20-mM HEPES and 0.4% (w:v) BSA and once in maturation medium. Groups of 50 COCs were then cultured in four-well dishes containing 500-µL/well maturation medium; TCM199 medium supplemented with 10% (v:v) fetal bovine serum, 5-µg/mL follicle stimulating hormone (Follitropin; Bioniche Animal Health), 5-µg/mL luteinizing hormone (Lutropin; Bioniche Animal Health), 1-µg/mL estradiol, 10-ng/mL epidermal growth Factor, 100-µM cysteamine, 2-mM L-glutamine, 200-µM sodium pyruvate, and 50-µg/mL gentamycin. Cumulus oocyte complexes were

incubated for 24 hours at 38.5 °C under 5% CO2 in humidified air. Matured COCs were then fertilized (Day 0) in 4-well dishes containing 400-µL Fertilization-Tyrode's albumin lactate pyruvate (F-TALP) medium with 1 × 106 motile sperm/mL selected by swim-up in Sperm-TALP. Frozen semen of the same ejaculate from a proven fertile ram was used. Cumulus oocyte complexes were cultured for 18 hours at 38.5 °C in a humidified incubator of 5% CO2 in air. Presumptive zygotes were then denuded from cumulus cells by gentle pipetting and cultured in 500 µL of serum-free synthetic oviductal fluid (SOF) medium with amino acids, sodium citrate and myo-inositol (SOFaaci) that was supplemented with 0.4% (w:v) fatty acid-free BSA at 38.5 °C in a humidified incubator with 5% O2, 5% CO2, and 90% N2. In three independent repeats, good-quality cleaved embryos at 4-cell stage or more exhibiting symmetrical divisions and no fragmentation were pooled on Day 2 and randomly allocated in equal groups of 20 to 30 embryos into one of the four treatment groups as those used for the in vivo experiment (Control, Hyal2, Hyalovet, and Hyalovet + Hyal2) prepared in SOFaaci. A total of 298 embryos were used in three independent repeats. The culture was continued up to Day 8, and blastocysts (n = 88) were counted and stained for quality assessment.

Assessment of Blastocyst Quality by Differential Staining of Blastocysts Associated with TUNEL

Blastocysts recovered from the in vivo and in vitro experiments were differentially stained for counting cells in the inner cell mass (ICM) and trophectoderm (TE) as previously described (Fouladi-Nashta, et al. 2005). Briefly, fresh blastocysts were permeablized using Triton X-100 0.2% (v:v) in SOF containing 0.4% (w:v) BSA for 20 seconds then washed and incubated in 30-µg/mL propidium iodide for 10 minutes followed by washing and fixation in 4% paraformaldehyde containing 30 µg/mL bisbenzimide (Hoechst 33342). For detection of apoptotic cells, TUNEL labeling with a fluorescein isothiocyanate (FITC)-conjugated in situ cell death detection kit was applied (Roche, Penzberg, Germany). Briefly, embryos were permeabilized again in 0.1% Triton for 5 minutes and incubated in the kit reagent (1:10 dilution of the enzyme solution [terminal deoxynucleotidyl transferase from calf thymus] in label solution [nucleotide mixture]) in a humid chamber for 45 minutes at 37 °C. In vitro–produced

blastocysts treated with DNase were also used as positive controls. Embryos were then washed and mounted in small droplets of anti-fading Vectashield mounting medium (Vector laboratories, Inc. Burlingame, A94010) and examined under a Leica epiflourescent microscope (Leica, Wetzlar, Germany).

RNA isolation and reverse transcription

Total RNA was extracted from oviductal cells using an RNeasy Mini Kit (QIAGEN Ltd, West Sussex, UK) following the guidelines supplied by the manufacturer. The concentration and purity of the isolated RNA samples were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). All samples had an A260:280 ratio of absorbance between 1.9 and 2.1. RNA integrity was assessed using 1.5% denaturing formaldehyde agar gel electrophoresis by assessing intact 18S (1874 bp) and 28S (4781 bp) ribosomal RNA. All samples (500 ng total RNA per reaction) were then DNase treated for potential genomic DNA carryover and reverse transcribed using random hexamer primers in a single reaction (Reverse Transcription System Kit; Promega Corporation, Madison, WI, USA). A mastermix of reagents was prepared for the previously mentioned reaction to minimize potential variation, and all samples were reverse transcribed at the same time. Selected reverse transcription (RT)-negative control samples were also prepared by including all reagents as mentioned previously except the reverse transcriptase.

Primer design and optimization of RT-PCR

RT-PCR assays were designed for the genes listed in table 1 using Ovis aries nucleotide sequences derived from the coding domain sequence (CDS) from the GenBank published in the National Centre for Biotechnology Information database (NCBI, Bethesda, MD). All primer pairs were deigned to be intron spanning or flanking to avoid co-amplification of genomic DNA using the Primer3 web software (Rozen and Skaletsky 2000). Primer alignment specificity was checked using the BLAST search tool (NCBI). Sequence information, accession numbers and product lengths are provided in Table 1. All

oligonucleotides were commercially synthesized as highly purified salt-free products (MWG-Biotech AG, London, UK).

Table 1. Details for the primers used for real time PCR analysis

Gene	Forward and reverse primer sequences	Product size	NCBI Reference Sequence
IL1A	FW: accgaagaagAAATCATCAAGC	166	NM_001009808.1
	REV: TTAATGTAGCAGCCGTCATGTA		
IGFBP2	FW: TGAACTTGATGGGAGGTGGA	277	NM_001009436.1
	REV: gggatgtgtagggaatagaggt		
IGF2	FW: CATCGTGGAAGAGTGTTGCT	163	HQ711956.1
	REV: ccaggtgtcagattggaagaa		
IGF1	FW: TGCTCTCAACATCTCCCATCT	170	NM_001009774.2
	REV: ACTGGCATCTTCACCTGctt		
HSP70	FW: CGTGCTCATCTTTGACCTG	174	JQ807666.1
	REV: TCCTTCTTGTGCTTCCTCTTG		
B2M	FW: cgccagaagatggaaagc	167	NM_001009284.1
	REV: gaactcagcgtgggacaga		
GAPDH	FW: cactgtccacgccatcact	267	NM_001190390.1
	REV: gcctgcttcaccaccttct		
18S ribosomal RNA	FW: GCTCGCTCCTCTCCTACTTG	326	AY753190.1
	REV: CGTTTCTCAGGCTCCCTCT		
HPRT1	FW: agcgtggtgATTAGCGATG	219	NM_001034035.1
	REV: caacaggtcggcaaagaac		

Absolute quantification of candidate genes by Real time qPCR

Gene transcripts were quantified by real-time PCR as previously described (Wathes, et al. 2011). Primers were tested by conventional PCR amplification using Multiplex PCR kits (Qiagen), 25 ng complementary DNA and 20 µM primers. Polymerase chain reaction products were purified using QIAquick PCR purification columns (QIAGEN, Crawley, West Sussex, UK) for the preparation of standards for absolute quantification. The precise concentration of purified PCR product was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.), and the presence of a single product was confirmed by electrophoresis on a 2% (w:v) agarose gel. The identity of the PCR products was confirmed by DNA sequence analysis (Geneservice Ltd, Cambridge, UK). Standards (10−1 to 10−7 ng/mL) were diluted in nuclease-free water. The optimal annealing temperature which resulted in lowest cycle threshold (Ct) was defined using the temperature gradient function of the real-time PCR machine (qPCR, CFX 96 Real-Time PCR Detection System, Bio-Rad Laboratories, Inc.) using a set of eight identical reactions for each gene. A melting curve analysis was performed for each amplicon between 50 °C and 95 °C, and accordingly, the reading temperature for qPCR analysis of samples was set so that any smaller nonspecific products such as dimers were melted (if present) before fluorescence acquisition. For each qPCR assay, a mastermix was prepared that contained a final concentration of 2 × KAPA SYBR Green (Anachem Ltd, Bedfordshire, UK), 500-nM forward and reverse primers, and nuclease-free water. For all unknown samples measured, 20 µL reactions containing the previously mentioned mastermix plus 50 ng of reverse transcribed RNA were used. External standards were run on the same plate in duplicate. No-template controls were included in every plate for each gene. Absolute concentrations of the PCR product were calculated by comparing the Ct values of the unknown samples to the standard curve using the CFX Manager Software version 1.0.1035.131 (Bio-Rad Laboratories Inc, Hemel Hempstead, Hertfordshire). Efficiency of the assays (E) was greater than or equal to 95%, and the standard curve R2 values were greater than or equal to 0.999. Data are presented as fg/µg reverse transcribed RNA. Results were normalized

using correction factor generated by GenNorm software on the basis of expression of four house-keeping genes (B2M, GAPDH, 18S ribosomal RNA, and HPRT1) in the samples.

Statistical Analysis

All data were analyzed using SPSS (version 20). Binominal data from blastocyst development were analyzed using binary logistic regression in generalized linear model. The interaction between the ram used for mating and treatment was tested and removed from the model as it was found to be insignificant ($P > 0.05$). Numerical data of embryo cell numbers and gene expression analyses were analyzed using a Linear mixed model. If the main treatment effect was significant, pairwise comparisons with Bonferroni correction of P values were performed. Differences of values of $P < 0.05$ were considered as significant.

Results

Superovulation and recovery efficiency

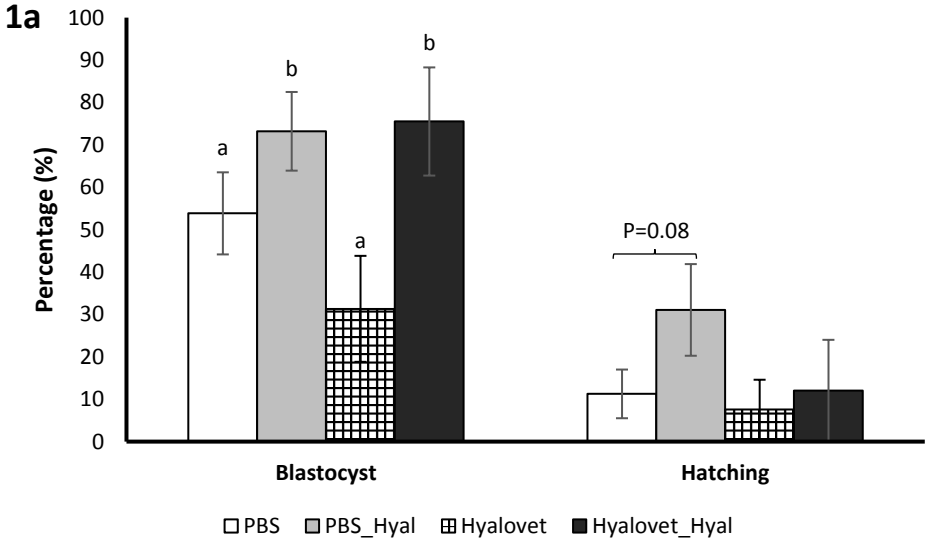
All ewes responded to the superovulation on both ovaries. The overall average number of ovulations was 7.53/ovary, and the average embryo recovery rate was 78.6%. The mean (± standard deviation) number of ovulations in ovaries ipsilateral to the oviducts infused with PBS (7.6 ± 3.47), Hyal2 (7.3 ± 2.50), Hyalovet (8.0 ± 2.38), and Hyalovet + Hyal2 (7.2 ± 2.49) did not differ between the groups ($P > 0.05$). Embryo recovery rate (the number of embryos recovered/the number of ovulations) was also similar among different treatment groups; 79.8% ± 31.10, 82.1% ± 38.84, 71.4% ± 29.36, and 77.8% ± 38.69, respectively, ($P > 0.05$).

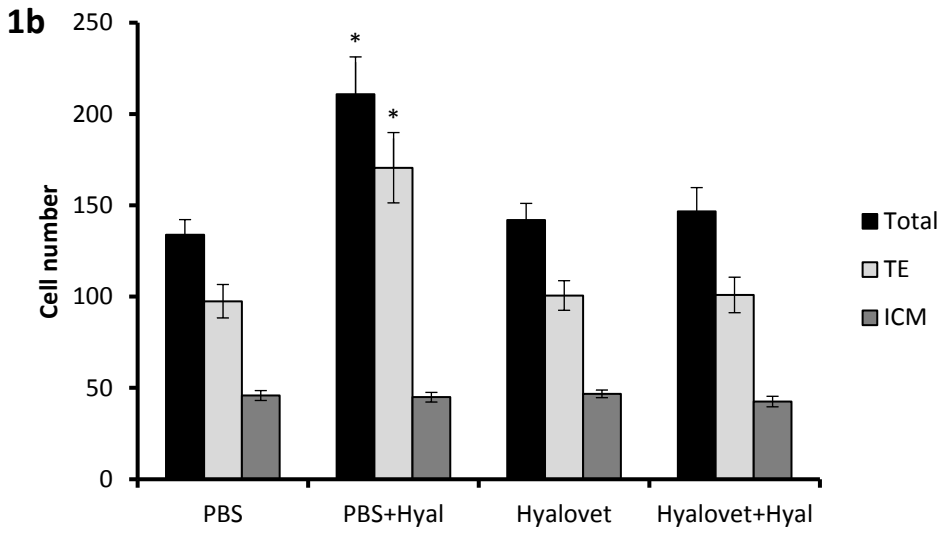
Effect of oviductal infusions on in vivo embryo development

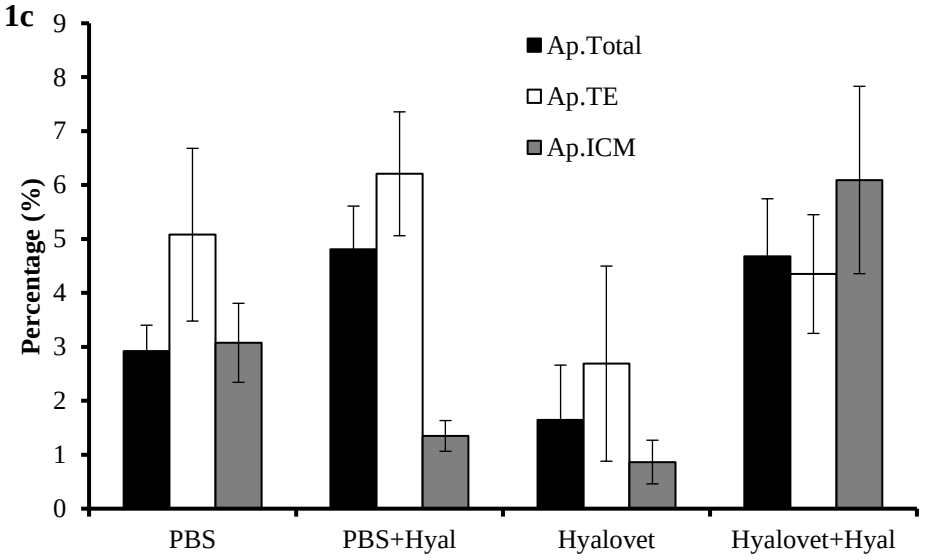
Recovered embryos from the in vivo experiment revealed that exposure of embryos starting at day 2 to Hyalovet resulted in a numerical reduction of blastocyst rate by day 7 (31%±12.5 vs. 54%±9.7 in PBS control; $P>0.05$) (fig. 1a). The proportion of embryos reaching the blastocyst stage was significantly

higher (P < 0.05) when Hyal2 was infused alone (Hyal2; 73% ± 9.3) or in combination with Hyalovet (Hyalovet + Hyal2; 76% ± 12.6; Fig. 1A). Interestingly, Hyal2 resulted in highest hatching rate (31% ± 10.8 vs. 11% ± 5.7 in PBS; P = 0.08), whereas Hyalovet tended to inhibit blastocyst hatching (7.5% ± 7) with mild nonsignificant improvement in the presence of Hyalovet + Hyal2 (14% ± 12; P > 0.05; Fig. 1A). All blastocysts were stained using differential staining and TUNEL labeling to determine their quality. Hyal2 infusion (PBS-Hyal2) significantly increased the number of trophectoderm cells and total cell number compared to PBS (Fig. 1B). Variation in the percentage of apoptotic cells was high within different groups in vivo, and no significant difference could be detected among different treatments (Fig. 1C).

Figure 1. The effects of oviductal infusion with different combinations of Hyal2 and Hyalovet on preimplantation embryo development **(1a)**, blastocyst cell counts **(1b)** and percentage of apoptotic cells **(1c)**. Oviducts of synchronised and superovulated ewes were infused 2 days after mating with 200 µl of either Hyal2 (300U/ml) or Hyalovet (0.5 mg/ml) while the other oviduct within each ewe received control vehicle (PBS) or PBS+Hyal2. Data are presented as means±SEM. Bars with different alphabets indicate significant differences at P<0.05. Asterisks denote significant difference compare to the control group at P<0.05. **ICM;** inner cell mass, **TE;** trophectoderm cells, **Ap;** apoptotic.




1b
250
200
150
100
50
0
Cell number
*
*
Total
TE
ICM
PBS
PBS+Hyal
Hyalovet
Hyalovet+Hyal


1c
9
8
7
6
5
4
3
2
1
0
Percentage (%)
Ap.Total
Ap.TE
Ap.ICM
PBS
PBS+Hyal
Hyalovet
Hyalovet+Hyal

Effect of HA and Hyal2 on in vitro embryo development

In vitro supplementation of the embryo culture media with Hyal2 resulted in a significant increase in blastocyst rate (64% ± 4.6 vs. 38% ± 7.9 in control; $P < 0.05$) with no effect on hatching rate. In contrast, Hyalovet had no effect on blastocyst rate ($P > 0.05$) and significantly inhibited hatching (0% compared to 31% in control; $P < 0.05$). Supplementation with Hyalovet + Hyal2 resulted in more blastocysts compared to Hyalovet (41% ± 0.8 vs. 32% ± 1.7; $P > 0.05$) both of which were not significantly different from the controls. Hyalovet + Hyal2 had significantly higher hatching rate (19% ± 13.2) compared to Hyalovet only ($P < 0.05$) and did not differ compared to controls ($P > 0.05$; Fig. 2). Blastocyst cell numbers were similar among different treatment groups with a numerical increase noticed in Hyal2 group ($P > 0.05$). Hyaluronidase-2 also decreased the percentage of apoptotic inner cell mass cells in Hyal2 and Hyalovet + Hyal2 groups (Table 2).

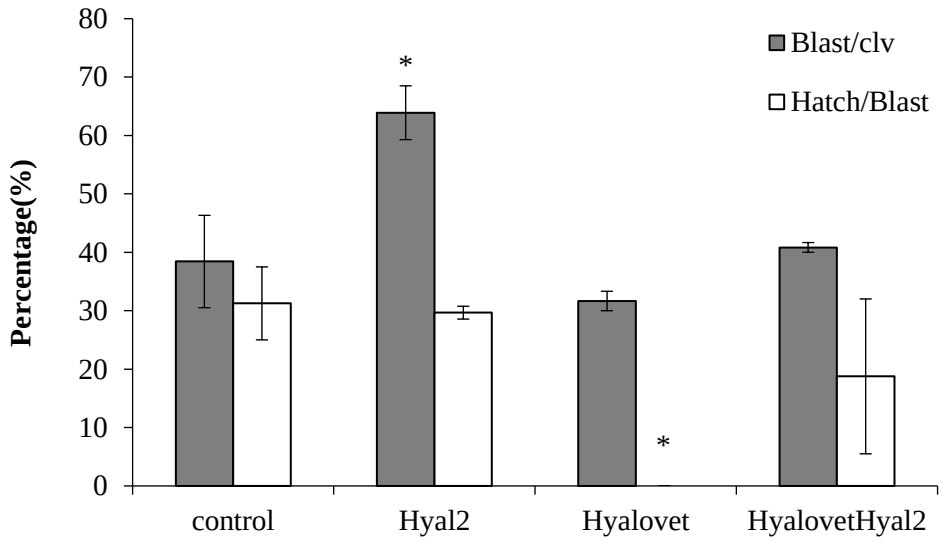


Figure 2. The effects of Hyal2 and Hyalovet supplementation during *in vitro* culture of ovine embryos on blastocyst and hatching rates. Day 2 cleaved embryos were cultured in SOF media containing Hyalovet (0.5 mg/ml) or Hyaluronidase-2 (300U/ml). Blastocyst rate and hatching were assessed on day 8. Data are presented as mean% ± SEM. Mean values with asterisks denote significant difference at $P<0.05$ compared to control within the same category.

Table 2. The effects of Hyal2 and Hyalovet supplementation during *in vitro* culture of ovine embryos on blastocyst quality.

	Total cells	TE	ICM	Total Apoptotic cells (%)	Apoptotic TE (%)	Apoptotic ICM (%)
Control	101±14.2	79±11.9	22±2.6	1.5±0.44	1.3±0.46	2.0±0.89
Hyal2	114±9.4	89±8.1	25±2.1	0.7±0.19	0.8±0.23	0.2±0.22*
Hyalovet	101±9.4	78±7.5	22±2.4	0.8±0.62	0.8±0.53	1.1±1.10
Hyalovet+Hyal2	94±6.0	75±5.6	19±1.4	1.0±0.53	1.3±0.67	0.0±0.0*

Data are presented as mean% ± SEM. Mean values with asterisks indicate significant difference at P<0.05 compared to control within the same category.

Effect of HA and Hyal2 on the gene expression in the oviductal epithelial cells.

The expression of selected candidate genes was examined in the epithelial oviductal cells collected from the in vivo experiment at the time of embryo recovery. Hyalovet significantly decreased IGF2 and IGFBP2 mRNA expression (P < 0.05). Hyaluronidase-2 resulted in significantly higher HSP70 mRNA expression (P < 0.05) in both Hyal2 and Hyalovet + Hyal2 groups compared to PBS and Hyalovet group, which did not differ from each other. IGF1 and ILα mRNA expression in oviductal cells were not affected by Hyalovet or Hyal2 oviductal infusions (Table 3).

Table 3. The effects of oviductal infusion with different combinations of Hyal2 and Hyalovet on mRNA expression of selected candidate genes

	IGF1	***IGF2***	***IGFBP2***	***ILα***	***HSP70***
Control	871±229.8	997±295.9^{a}	3117±603.1^{a}	64±8.7	160±56.2^{a}
Hyal2	763±114.7	1003±414.9^{a}	2792±936.6^{a}	85±12.2	**441±157.0^{b}**
Hyalovet	737±186.1	**295±98.6^{b}**	**912±591.1^{b}**	70±24.1	130±52.9^{a}
Hyalovet+Hyal2	868±144.5	387±113.5^{b}	**1197±797.6^{b}**	58±3.7	**512±235.3^{b}**

Data are shown as mean±SEM (n=8 per treatment). Mean values with different superscripts within a column are significantly different at P<0.05.

Discussion

To understand the size-dependent effects of HA we designed an in vivo experiment in which we altered the oviductal environment 2 days after natural mating of superovulated ewes with HA of different molecular sizes. We used sheep as an experimental model because of its good response to superovulation regimes (Ryan, et al. 1991) and the feasibility in surgical handling of oviducts. We used Hyalovet^{TM}, a commercial HA with a defined molecular weight of 0.5-0.7 x 10^6 Da, and Hyalovet treated with Hyal2 which is known to depolymerise HA into 20 kDa fragments (Stern, et al. 2006). Verifying our results by conducting an in vitro experiment was useful to dissociate effects on embryos via HA/Hyal2-oviductal interactions.

The results presented here show that sheep oviduct were better able to support embryo development in the absence of Hyalovet (which caused about 20% reduction in blastocyst rate compared to PBS-infused oviducts). In addition, Hyalovet did not enhance development to the blastocyst stage when supplemented in vitro compared to the controls and did not influence apoptotic cell indices either in vitro or in vivo. Looking at previously reported effects for HA during embryo development, it is clear that results are variable among different studies. Few studies have actually reported positive influence of HA in terms of increased development to blastocyst stage (Furnus, et al. 1998), whereas others reported either no effects (Lane, et al. 2003) or a significant decrease (with 1.0 mg HA/ml) in the percentage of blastocyst rates (Torner, et al. 2014). Nevertheless , we have previously shown strong evidence that endogenous HA is critically essential for oocyte maturation and embryo development since addition of HA-synthesis inhibitor (4-methylumbelliferone; 4MU) to in vitro embryo maturation or culture medium completely inhibited blastocyst formation (Marei, et al. 2012, Marei, et al. 2013). So, the question remained to verify whether exogenous HA supplementation (in the oviduct or in vitro) would be beneficial for blastocyst development or quality. The controversy among different studies can be attributed to differences in culture medium or combination with other supplements as suggested by Block, et al. (2011), but also we suggest that the source of HA (its molecular size) is rather

an important variable. The source of the HA used is not mentioned in some of the previous studies. In this notion, here we show evidence that 0.5 to 0.7 × 106 kDa of HA is not supportive for embryo development. In contrast, in the Hyalovet + Hyal2 group where Hyalovet was treated with Hyal2 before infusion (to produce 20 kDa fragments), negative effects of Hyalovet were completely alleviated and resulting blastocyst rates were ∼20% higher than PBS-infused oviducts and ∼40% higher than Hyalovet-infused group. Because Hyalovet + Hyal2 treatment in vitro resulted only in a 10% increase in blastocyst rate ($P > 0.05$) compared to Hyalovet alone, this shows that part of the effect of Hyalovet + Hyal2 group in vivo might be mediated through interaction between HA-fragments and the oviductal cells.

Regarding the effects of Hyal2 in the absence of exogenous HA, the dramatic increase in blastocyst rate *in vitro* (doubled blastocyst rate compared to control) is in accordance with our previously reported Hyal2 effects on bovine embryos (Marei, et al. 2013). It was also interesting to see that Hyal2 retains the same effects when infused in the oviduct. We and others have previously shown that oviductal epithelial cells and embryos at all stages of development are equipped with the machinery to synthesise HA as they express different HA synthases and are positively stained when immunolabelled with biotinylated HA-binding protein (Marei, et al. 2013, Ulbrich, et al. 2004). However, we have previously shown that Hyal2 mRNA was also expressed in oviducts at the early luteal phase and was only detected in the embryos at morula and blastocyst stages (Day 6 and 7 post-fertilization) but not at earlier stages of development (Marei, et al. 2013). The degradation of HA by HYAL2 into smaller fragments is known to initiate CD44 cell signaling (Ohno-Nakahara, et al. 2004), which involves multiple signaling pathways like Rac1-MAPK, PI3-AKT, and NFkB (Toole 2001) that may support embryo development and increase cell proliferation (Bourguignon, et al. 1997). Therefore, we suggest that the increase in blastocyst rates in the present study associated with Hyal2 supplementation in vivo or in vitro is mediated by fragmentation of endogenous HA produced by the oviduct and/or embryos resulting in cell signaling. In fact, we have previously reported that the increase in blastocyst rate and cell numbers observed with Hyal2 supplementation to bovine embryos

in vitro were associated with and dependent on increased phosphorylated mitogen-activated protein kinases (MAPK1 and 3) in the embryos through CD44 signaling (Marei, et al. 2013).

Through binding to its specific receptors like CD44 and HARE (Pandey and Weigel 2014), hyaluronan and its fragments are involved in signalling pathways that can influence cellular functions though regulation of gene expression. For example, HA is associated to increased growth factors expression during particular healing processes including epidermal growth factor (EGF), insulin-like growth factor (IGF) (Saliba, et al. 2014). Therefore, in an attempt to further understand the role of HA in the oviduct-embryo interaction, we collected the OECs 5 days after infusion at the time of embryo recovery and investigated gene expression of some candidate genes that are known to be important for embryo development at this stage; IGF1, IGF2, IGFBP2, IL1α, and HSP70.

Therefore, in an attempt to further understand the role of HA in the oviduct-embryo interaction, we collected the oviductal epithelial cells 5 days after infusion at the time of embryo recovery, and investigated gene expression of some candidate genes that are known to be important for embryo development at this stage; *IGF1, IGF2, IGFBP2, IL1α,* and *HSP70*.

The use of a combination of recombinant growth factors and cytokine, as IGF1, IGF2, bFGF, TGF-beta1, LIF, and GM-CSF, produces similar results to 10% fetal calf serum for the development of *in vitro*-produced bovine embryos (Neira, et al. 2010). Transcripts for *IGF1* and *IGF2* have been detected in the oviduct of bovine (Schmidt, et al. 1994) in addition to their regulatory binding proteins IGFBP1-6 (Fenwick, et al. 2008). The expression of *IGF1* and *IGF2* receptors were also detected in bovine oocytes and embryos at different stages of development (Wang, et al. 2009). IGF system in the oviduct was suggested to act as an important modulator of oviductal function (Fenwick, et al. 2008).

Messenger RNA expression of the IGF system (IGF1, IGF2) and their binding proteins (IGFBP2, 3, and 4) have been reported in ovine (Stevenson and Wathes 1996) and bovine (Schmidt, et al. 1994; Fenwick, et al. 2008) oviducts. In ovine, IGF1 and IGF2 expression was found to be higher in the

mucosal layer during late-follicular to early-luteal phase of the estrous cycle (Stevenson and Wathes 1996). IGFBPs (2–5) are also expressed by bovine and ovine preimplantation embryos (Watson, et al. 1999). The IGF system in the oviduct was suggested to act as an important modulator of oviductal function (Fenwick, et al. 2008). The maintenance of embryos in an IGF-rich environment while free living in the oviduct and its influence on enhancing development to the blastocysts stage was previously reviewed (Watson, et al. 1999).

Looking at specific effects of IGF2, it was shown that its supplementation to SOF media increased the total blastocyst rate when added to bovine IVM or IVC medium (Wang, et al. 2009) and also resulted in early blastocyst formation on day 6, and hastened blastocyst hatching (higher hatching rate on day 7 and lower hatching rate on day 8) compared to control embryos with no growth factor supplementation (Neira, et al. 2010). In the present study, Hyalovet was found to significantly decrease *IGF2* expression in oviductal cells compared to oviducts infused with saline. In contrast, *IGF2* expression was not affected in Hyal2-infused group. This may explain, at least in part, its effect on the observed embryo development and hatching. In addition, IGFBP2, the second most abundant IGFBP, plays an important role in the regulation of several cellular processes such as proliferation, cell migration, and adhesion as in case of tumor progression (Tombolan, et al. 2011). Here, Hyalovet but not Hyal2 was observed to decrease expression of IGFBP2. This might be secondary to the reduced IGF2 expression because IGFBP2 has higher affinity to IGF2 (Oesterreicher, et al. 2005).

On the other hand, in the present study, *IGF1* expression was the same among different experimental groups. IGF1 is linked to embryo development. IGF1 is locally produced by the oviduct (Pushpakumara, et al. 2002) and the uterus (Robinson, et al. 2000) and can affect embryos though its receptors on the embryo. The bovine embryo can respond to IGF1 treatment in vitro and improved development to the blastocyst stage was demonstrated in many studies (see Block, et al. 2011 for review). In human, follicular fluid IGF1 was considered to be a plausible biochemical marker of embryo quality and implantation rate as it was associated with higher rates of fertilization, cleavage, blastocyst formation

and top grade embryos as well as high top grade embryos and clinical pregnancy rates (Mehta, et al. 2013). Although *IGF1* was not altered by Hyalovet, it is possible that hyalovet may block its delivery to the embryo. A previous study demonstrated that the addition of high levels (5.00 mg/mL) of HA can entrap IGF1 and interfere with the delivery of IGF1 to chondrocytes (Yoon, et al. 2009).

We also noticed that both Hyal2 and Hyalovet-Hyal2 infusions increased mRNA expression of *HSP70*. Heat shock proteins are known to play an important role to protect cells from environmental stresses and they are among the first genes to be expressed by the embryo (Hahnel, et al. 1986). *HSP70* is also expressed in the oviduct and has been shown to be responsive to hormonal changes during the estrous cycle in the rat oviduct (Mariani, et al. 2000). It is also an active component of the ewe oviduct that is required for maintenance of sperm viability (Lloyd, et al. 2009). The presence of anti-HSP70 in culture medium from day 3 to day 9 of development increased apoptosis in bovine embryos and significantly reduced the number of embryos reaching the blastocyst stage (Matwee, et al. 2001) illustrating its importance during this period. Therefore, the increased HSP70 expression in ovine oviducts as reported in the present study may mediate its positive effects on blastocyst rate and increased cell numbers.

In conclusion, we show evidence that 500-750kDa HA fragments do not enhance development of embryos to blastocyst stage and inhibit hatching possibly via a disruption in the IGF system. In contrast, we provide further evidence that Hyal2 improves blastocyst rate and quality, and increases hatching by maintaining IGF system and an increased expression of HSP70 mRNA.

Acknowledgement

The studies presented here were funded by BBSRC New Investigator Award to A.A. Fouladi-Nashta (BB/G008620/1)

References

Aruffo, A, I Stamenkovic, M Melnick, CB Underhill, and B Seed 1990 CD44 is the principal cell surface receptor for hyaluronate. *Cell* **61** 1303-1313.

Aviles, M, A Gutierrez-Adan, and P Coy 2010 Oviductal secretions: will they be key factors for the future ARTs? *Mol Hum Reprod* **16** 896-906.

Bastow, ER, S Byers, SB Golub, CE Clarkin, AA Pitsillides, and AJ Fosang 2008 Hyaluronan synthesis and degradation in cartilage and bone. *Cell Mol Life Sci* **65** 395-413.

Bergqvist, AS, M Yokoo, P Heldin, J Frendin, E Sato, and H Rodriguez-Martinez 2005 Hyaluronan and its binding proteins in the epithelium and intraluminal fluid of the bovine oviduct. *Zygote* **13** 207-218.

Block, J, PJ Hansen, B Loureiro, and L Bonilla 2011 Improving post-transfer survival of bovine embryos produced in vitro: actions of insulin-like growth factor-1, colony stimulating factor-2 and hyaluronan. *Theriogenology* **76** 1602-1609.

Bourguignon, LY, H. Zhu, A. Chu, N. Iida, L. Zhang, M.C. Hung 1997 Interaction between the adhesion receptor, CD44, and the oncogene product, p185HER2, promotes human ovarian tumor cell activation. *J Biol Chem* **272** 27913-27918.

Choudhary, M, X Zhang, P Stojkovic, L Hyslop, G Anyfantis, M Herbert, AP Murdoch, M Stojkovic, and M Lako 2007 Putative role of hyaluronan and its related genes, HAS2 and RHAMM, in human early preimplantation embryogenesis and embryonic stem cell characterization. *Stem Cells* **25** 3045-3057.

Csoka, AB, GI Frost, and R Stern 2001 The six hyaluronidase-like genes in the human and mouse genomes. *Matrix Biology* **20** 499-508.

Dicker, KT, LA Gurski, S Pradhan-Bhatt, RL Witt, MC Farach-Carson, and X Jia 2014 Hyaluronan: a simple polysaccharide with diverse biological functions. *Acta Biomater* **10** 1558-1570.

Fenwick, MA, S Llewellyn, R Fitzpatrick, DA Kenny, JJ Murphy, J Patton, and DC Wathes 2008 Negative energy balance in dairy cows is associated with specific changes in IGF-binding protein expression in the oviduct. *Reproduction* **135** 63-75.

Fouladi-Nashta, AA, R Alberio, M Kafi, B Nicholas, KH Campbell, and R Webb 2005 Differential staining combined with TUNEL labelling to detect apoptosis in preimplantation bovine embryos. *Reprod Biomed Online* **10** 497-502.

Furnus, CC, DG de Matos, and AG Martinez 1998 Effect of hyaluronic acid on development of in vitro produced bovine embryos. *Theriogenology* **49** 1489-1499.

Furnus, CC, A Valcarcel, FN Dulout, and ALa Errecalde 2003 The hyaluronic acid receptor (CD44) is expressed in bovine oocytes and early stage embryos. *Theriogenology* **60** 1633-1644.

Georgiou, AS, E Sostaric, CH Wong, AP Snijders, PC Wright, HD Moore, and A Fazeli 2005 Gametes alter the oviductal secretory proteome. *Mol Cell Proteomics* **4** 1785-1796.

Hahnel, AC, DJ Gifford, JJ Heikkila, and GA Schultz 1986 Expression of the major heat shock protein (hsp 70) family during early mouse embryo development. *Teratog Carcinog Mutagen* **6** 493-510.

Hess, AP, S Talbi, AE Hamilton, DM Baston-Buest, M Nyegaard, JC Irwin, F Barragan, JS Kruessel, A Germeyer, and LC Giudice 2013 The human oviduct transcriptome reveals an anti-inflammatory, anti-angiogenic, secretory and matrix-stable environment during embryo transit. *Reprod Biomed Online* **27** 423-435.

Lane, M, JM Maybach, K Hooper, JF Hasler, and DK Gardner 2003 Cryo-survival and development of bovine blastocysts are enhanced by culture with recombinant albumin and hyaluronan. *Mol Reprod Dev* **64** 70-78.

Laurent, TC 1998 The chemistry, biology and medical applications of Hyaluronan and its derivatives, Wenner-Gren INternational series. London: Portland Press Ltd.

Lesley, J, R Hyman, and PW Kincade 1993 CD44 and its interaction with extracellular matrix. *Adv Immunol* **54** 271-335.

Lloyd, RE, RM Elliott, A Fazeli, PF Watson, WV Holt 2009 Effects of oviductal proteins, including heat shock 70 kDa protein 8, on survival of ram spermatozoa over 48 h in vitro. *Reprod Fertil Dev* **21** 408-418.

Marei, WF, F Ghafari, and AA Fouladi-Nashta 2012 Role of hyaluronic acid in maturation and further early embryo development of bovine oocytes. *Theriogenology* **78** 670-677.

Marei, WF, M Salavati, and AA Fouladi-Nashta 2013 Critical role of hyaluronidase-2 during preimplantation embryo development. *Mol Hum Reprod* **19** 590-599.

Mariani, ML, M Souto, MA Fanelli, and DR Ciocca 2000 Constitutive expression of heat shock proteins hsp25 and hsp70 in the rat oviduct during neonatal development, the oestrous cycle and early pregnancy. *J Reprod Fertil* **120** 217-223.

Matwee, C, M Kamaruddin, DH Betts, PK Basrur, and WA King 2001 The effects of antibodies to heat shock protein 70 in fertilization and embryo development. *Mol Hum Reprod* **7** 829-837.

Mehta, BN, NM Chimote, MN Chimote, NN Chimote, and NM Nath 2013 Follicular fluid insulin like growth factor-1 (FF IGF-1) is a biochemical marker of embryo quality and implantation rates in in vitro fertilization cycles. *J Hum Reprod Sci* **6** 140-146.

Mohey-Elsaeed, O, WF Marei, AA Fouladi-Nashta, and AA El-Saba 2015 Histochemical structure and immunolocalisation of the hyaluronan system in the dromedary oviduct. *Reprod Fertil Dev*.

Morris, SA, and M Zernicka-Goetz 2012 Formation of distinct cell types in the mouse blastocyst. *Results Probl Cell Differ* **55** 203-217.

Neira, JA, D Tainturier, MA Pena, and J Martal 2010 Effect of the association of IGF-I, IGF-II, bFGF, TGF-beta1, GM-CSF, and LIF on the development of bovine embryos produced in vitro. *Theriogenology* **73** 595-604.

Ohno-Nakahara, M, K Honda, K Tanimoto, N Tanaka, T Doi, A Suzuki, et al. Induction of CD44 and MMP expression by hyaluronidase treatment of articular chondrocytes. *J Biochem* 135 567-575.

Oesterreicher, S, WF Blum, B Schmidt, T Braulke, and B Kubler 2005 Interaction of insulin-like growth factor II (IGF-II) with multiple plasma proteins: high affinity binding of plasminogen to IGF-II and IGF-binding protein-3. *J Biol Chem* **280** 9994-10000.

Pandey, MS, and PH Weigel 2014 Hyaluronic acid receptor for endocytosis (HARE)-mediated endocytosis of hyaluronan, heparin, dermatan sulfate, and acetylated low density lipoprotein (AcLDL), but not chondroitin sulfate types A, C, D, or E, activates NF-kappaB-regulated gene expression. *J Biol Chem* **289** 1756-1767.

Pushpakumara, PG, RS Robinson, KJ Demmers, GE Mann, KD Sinclair, R Webb, and DC Wathes 2002 Expression of the insulin-like growth factor (IGF) system in the bovine oviduct at oestrus and during early pregnancy. *Reproduction* **123** 859-868.

Raheem, KA, WF Marei, K Mifsud, M Khalid, DC Wathes, AA Fouladi-Nashta 2013 Regulation of the hyaluronan system in ovine endometrium by ovarian steroids. *Reproduction* 145 491-504.

Robinson, RS, GE Mann, TS Gadd, GE Lamming, and DC Wathes 2000 The expression of the IGF system in the bovine uterus throughout the oestrous cycle and early pregnancy. *J Endocrinol* **165** 231-243.

Rossant, J, and PP Tam 2009 Blastocyst lineage formation, early embryonic asymmetries and axis patterning in the mouse. *Development* **136** 701-713.

Rozen, S, and H Skaletsky 2000 Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132** 365-386.

Russell, DL, and A Salustri 2006 Extracellular matrix of the cumulus-oocyte complex. *Semin Reprod Med* **24** 217-227.

Ryan, JP, JR Hunton, and WM Maxwell 1991 Increased production of sheep embryos following superovulation of Merino ewes with a combination of pregnant mare serum gonadotrophin and follicle stimulating hormone. *Reprod Fertil Dev* **3** 551-560.

Saliba, I, M Alzahrani, T Zhu, and S Chemtob 2014 Growth factors expression in hyaluronic acid fat graft myringoplasty. *Laryngoscope* **124** E224-230.

Schmidt, A, R Einspanier, W Amselgruber, F Sinowatz, and D Schams 1994 Expression of insulin-like growth factor 1 (IGF-1) in the bovine oviduct during the oestrous cycle. *Exp Clin Endocrinol* **102** 364-369.

Seytanoglu, A, AS Georgiou, E Sostaric, PF Watson, WV Holt, and A Fazeli 2008 Oviductal cell proteome alterations during the reproductive cycle in pigs. *J Proteome Res* **7** 2825-2833.

Stern, R, AA Asari, and KN Sugahara 2006 Hyaluronan fragments: an information-rich system. *Eur J Cell Biol* **85** 699-715.

Stevenson, KR and DC Wathes 1996 Insulin-like growth factors and their binding proteins in the ovine oviduct during the oestrous cycle. *J Reprod Fertil* **108** 31-40.

Taniyama, A, Y Watanabe, Y Nishino, T Inoue, Y Taura, M Takagi, C Kubota, and T Otoi 2011 Assisted hatching of poor-quality bovine embryos increases pregnancy success rate after embryo transfer. *J Reprod Dev* **57** 543-546.

Tienthai, P, N Kimura, P Heldin, E Sato, and H Rodriguez-Martinez 2003 Expression of hyaluronan synthase-3 in porcine oviducal epithelium during oestrus. *Reprod Fertil Dev* **15** 99-105.

Tombolan, L, F Orso, V Guzzardo, S Casara, A Zin, M Bonora, C Romualdi, C Giorgi, G Bisogno, R Alaggio, P Pinton, C De Pitta, D Taverna, A Rosolen, and G Lanfranchi 2011 High IGFBP2 expression correlates with tumor severity in pediatric rhabdomyosarcoma. *Am J Pathol* **179** 2611-2624.

Toole, BP 2001 Hyaluronan in morphogenesis. *Semin Cell Dev Biol* **12** 79-87.

Torner, E, E Bussalleu, MD Briz, M Yeste, and S Bonet 2014 Embryo development and sex ratio of in vitro-produced porcine embryos are affected by the energy substrate and hyaluronic acid added to the culture medium. *Reprod Fertil Dev* **26** 570-577.

Ulbrich, SE, M Schoenfelder, S Thoene, and R Einspanier 2004 Hyaluronan in the bovine oviduct--modulation of synthases and receptors during the estrous cycle. *Mol Cell Endocrinol* **214** 9-18.

Van Soom, A, B Mateusen, J Leroy, and A De Kruif 2003 Assessment of mammalian embryo quality: what can we learn from embryo morphology? *Reprod Biomed Online* **7** 664-670.

Way, AL 2006 Isolation and culture of bovine oviductal epithelial cells for use in the anatomy and physiology laboratory and undergraduate research. *Adv Physiol Educ* 30 237-241.

Wang, LM, HL Feng, Y Ma, M Cang, HJ Li, Z Yan, P Zhou, JX Wen, S Bou, and DJ Liu 2009 Expression of IGF receptors and its ligands in bovine oocytes and preimplantation embryos. *Anim Reprod Sci* **114** 99-108.

Wathes, DC, Z Cheng, MA Fenwick, R Fitzpatrick, and J Patton 2011 Influence of energy balance on the somatotrophic axis and matrix metalloproteinase expression in the endometrium of the postpartum dairy cow. *Reproduction* **141** 269-281.

Watson, AJ, ME Westhusin, QA Winger 1999 IGF paracrine and autocrine interactions between conceptus and oviduct. *J Reprod Fertil Suppl* **54** 303-315.

Weigel, PH, VC Hascall, and M Tammi 1997 Hyaluronan synthases. *J Biol Chem* **272** 13997-14000.

Xu, H, T. Ito, A. Tawada, H. Maeda, H. Yamanokuchi, K. Isahara, et al. 2002 Effect of hyaluronan oligosaccharides on the expression of heat shock protein 72. *J Biol Chem* **277** 17308-17314.

Yoon, DM, S Curtiss, AH Reddi, and JP Fisher 2009 Addition of hyaluronic acid to alginate embedded chondrocytes interferes with insulin-like growth factor-1 signaling in vitro and in vivo. *Tissue Eng Part A* **15** 3449-3459.

Yevdokimova, NY and SE Podpryatov 2005 Hyaluronic acid production and CD44 expression in cultured dermal fibroblasts of patients with non-insulin-dependent diabetes mellitus with and without chronic ulcers on the lower extremity. *Wound Rep Reg* **13** 181-188.