Critical role of hyaluronidase-2 during preimplantation embryo development

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ABSTRACT: Biological functions of hyaluronan (HA) depend on its molecular size. Small size HAs are known to regulate cell proliferation; however, the expression of HA synthases (HASs) and hyaluronidase-2 (HYAL2) and their role during early embryo development have not been previously identified. In this paper, we have shown by immunostaining that HA is produced by bovine in vitro-produced embryos at all stages of early development to blastocyst. Addition of HA-synthesis inhibitor (4-methylumbelliflorone; 4MU) to in vitro embryo culture inhibited blastocyst formation. HASs HAS2 and HAS3 mRNA were expressed at all stages of embryo development; however, relative mRNA expression of HAS2 was significantly reduced as the embryos develop to the blastocyst stage. HAS1 was detected during 2- and 4-cell stages but was barely detectable in subsequent stages. HYAL2 mRNA expression was detected in oviducts at the early luteal phase but was only detected in the embryos at morula and blastocyst stages (Day 6 and 7 post-fertilization). Addition of HYAL2 to embryo culture media at Day 2 post-fertilization increased phosphorylated mitogen-activated protein kinases (MAPK1 and 3) in the embryos and improved development to the blastocyst stage and increased embryo cell numbers. Addition of an anti-CD44 antibody or an MAPK inhibitor (U0126) abrogated the positive effects of HYAL2 on blastocyst rates. In conclusion, we demonstrate that the expression of different HAS genes and HYAL2 in bovine embryos varies according to the stage of development and that the supplementation of HYAL2 in vitro mimics oviductal conditions and is shown to improve the blastocyst rate and embryo quality, an effect which requires CD44 activity and MAPK signalling.

Key words: HYAL2 / blastocyst / CD44 / MAPK / IVF media

Introduction

IVF is a widely used treatment, but success rates remain low, and the number of IVF cycles performed and the number of patients being treated have continued to rise over the last few years (HFEA, 2010). Culture of IVF-produced embryos in the oviduct improves development to the blastocyst stage (Lazzari et al., 2010). This can be attributed to a number of supporting factors present in the oviduct, although it is fluid but still missing from routine IVF protocols. Among those factors is hyaluronic acid (HA), a ubiquitous component of the extracellular matrix of vertebrates. HA is present in the follicular, oviductal and uterine fluids and is known to play an important role in the regulation of many reproductive events like ovulation (Salustri et al., 1989), fertilization (Shimada et al., 2008) and embryogenesis (Camenisch et al., 2000).

HA is a linear polysaccharide which belongs to the family of glycosaminoglycans (GAGs). It is composed of repeating alternating units of D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) forming up to 25 000 disaccharides with a molecular mass of up to 10 million Da (Laurent, 1998). HA is synthesized by hyaluronic acid-synthase (HAS) enzymes (Itano and Kimata, 2002) located at the inner cytoplasmic face of the plasma membrane which adds units of GlcUA and GlcNAc while extruding the HA chain through the membrane during its synthesis to the outside of the cell (Laurent, 1998). There are three different mammalian HASs which synthesize HA chains with different molecular stability average lengths (molecular sizes) and different biological functions (Dougherty and van de Rijn, 1993; Crater and van de Rijn, 1995; Itano and Kimata, 2002; Stern, 2005). Polymer size, location and concentration govern the function of HA (Stern et al., 2006). The functions of high-molecular-weight (HMW) HA polymers (> 1000–5000 disaccharides) are mainly attributed to the hydrodynamical properties of the molecule. HMW HA is space-filling hydrating molecule that impedes cell differentiation and it is anti-angiogenic (Feinberg and Beebe, 1983) and immunosuppressive (McBride and Bard, 1979; Delmage et al., 1986). On the other hand, the functions of low-molecular-weight (LMW) HA is mainly mediated through their interaction with HA receptors, like CD44 and receptor for HA-mediated motility (RHAMM). LMW HA competes with the larger molecules for receptors and acts in an opposing manner; they are anti-apoptotic and function as a survival factor and stimulate cell proliferation (Xu et al., 2002). LMW HAs are also angiogenic (West et al., 1985) and immunostimulatory molecules (Alaniz et al., 2011).

HA has the high rate of turnover at cellular and tissue levels mainly by enzymatic hydrolysis by hyaluronidases (HYALs) which include HYAL1, HYAL2, HYAL3, HYAL4, HYALP1 and PH-20. Predominantly, HYAL1...
Materials and Methods

All chemicals were obtained from Sigma Chemical Company (Poole, Dorset, UK) unless stated otherwise.

Collection of ovaries and retrieval of COCs

Bovine ovaries were collected from a local abattoir and transported to the laboratory in phosphate-buffered saline (PBS) in a thermos container at 37°C within 2 h after slaughter, and they were washed in fresh PBS immediately after arrival. Follicular fluid was aspirated from antral follicles 3–8 mm in diameter with a 19-gage needle mounted on a 10-ml syringe and left to settle in a 50-ml conical centrifuge tube for 5 min. The cellular precipitate was transferred to a 90-mm sterile petri dish and mixed with washing medium; M-199 supplemented with 20 mM HEPES and 0.4% (w/v) bovine serum albumin (BSA). Groups of COCs, characterized by dark homogenous ooplasm and more than four layers of compact cumulus cells, were selected under a stereomicroscope as previously described by Fouladi-Nashta et al. (2007).

In vitro maturation

Selected COCs were washed twice in washing medium and twice in maturation medium; M-199 supplemented with 0.6% (w/v) fatty acid-free BSA, 5 μg/ml follicle-stimulating hormone (folltropin; Bioniche Animal Health, Belleville, ON, Canada), 5 μg/ml luteinizing hormone (leutropin; Bioniche Animal Health), 1 μg/ml estradiol and 50 μg/ml gentamycin. Groups of COCs were then cultured in 4-well dishes (NUNC, Thermo Fisher Scientific, Loughborough, Leicestershire, UK) using 20 μl maturation medium per COC. COCs were incubated for 24 h at 38.5°C under 5% CO2 in humidified air. About 30 COCs were used per treatment per repeat.

IVF and embryo culture

After in vitro maturation, oocytes were fertilized using frozen semen from a single bull as previously described by Fouladi-Nashta and Campbell (2006). Briefly, motel sperm were selected by swim-up for 45 min in calcium-free medium followed by centrifugation at 300 g at room temperature and re-suspension of the pellet in fertilization medium [TALP supplemented with 0.6% (w/v) fatty acid-free BSA, 1 μg/ml heparin, 50 ng/ml epinephrine and 50 ng/ml hypotaurine]. Groups of ~30 COCs were washed twice in fertilization medium and transferred into 400 μl of fertilization medium containing 1 × 10⁶ sperm/ml and cultured for 18 h at 38.5°C in a humidified incubator of 5% CO2 in air. At 24 h after fertilization, presumptive zygotes were denuded from cumulus cells by gentle pipetting and cultured in 500 μl of SOFaaci (Holm et al., 1999). Groups of ~30 COCs were washed twice in fertilization medium containing 1 × 10⁶ sperm/ml and cultured for 18 h at 38.5°C in a humidified incubator of 5% CO2 in air. At 24 h after fertilization, presumptive zygotes were denuded from cumulus cells by gentle pipetting and cultured in 500 μl of SOFaaci (Holm et al., 1999) supplemented with 0.4% (w/v) fatty acid-free BSA and cultured at 38.5°C in a humidified incubator with 5% O2, 5% CO2 and 90% N2. On Day 2 following fertilization, all embryos at the 4-cell stage or further were divided equally between treatment groups. The culture was continued up to Day 8. The rate of blastocyst per cleaved embryos was recorded on Day 8 and all blastocysts were differentially stained for quality assessment.

Differential staining of blastocysts associated with terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling

Day 8 blastocysts were differentially stained for counting cells in the inner cell mass (ICM) and trophoderm cell and apoptotic cells as previously described (Fouladi-Nashta et al., 2005) with some modifications. Briefly, embryos were permeabilized using 0.2% solution of Triton X-100 in SOFaaci containing 30 μg/ml propidium iodide (PI, stains the TE cells red) for 20 s. Embryos were then washed and fixed in 4% paraformaldehyde containing 30 μg/ml bisbenzimide (Hoechst 33342, stains all cells blue) for 20 min. Apoptotic cells were then detected by terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) using an FITC-conjugated in situ cell death detection kit, fluorescein (Roche, Penzberg, Germany) according to the manufacturer’s instructions. Briefly, embryos and HYAL2 are considered the major HA-degrading enzymes in somatic tissue, whereas PH-20 is abundant in spermatozoa (Bastow et al., 2008).

As formulated by Stern (2003, 2004), a sequence of enzymatic reactions cleave the HMW HA progressively generating HA fragments of decreasing sizes. HYAL2 is a glycosylphosphatidylinositol-anchored enzyme attached to the external surface of the plasma membrane expressed in many tissues (Lepperdinger et al., 2001). HYAL2 initially cleaves HMW HA into fragments ~20,000 Da (50–60 saccharides in size. CD44–HYAL2 interaction facilitates the endocytosis of HA which undergoes further degradation by lysosomal HYAL1 into decreasing sizes. HYAL2 is a glycosylphosphatidylinositol-anchored enzyme expressed in many tissues (Lepperdinger et al., 2001), involving multiple signalling pathways like Rac1 mitogen-activated protein kinase (MAPK), PI3-AKT and NFκB (Toole, 2001), and in some cases, influences cell proliferation (Bourguignon et al., 1997).

Genes and proteins involved in HA synthesis and its receptor CD44 are expressed in cumulus–oocyte complexes (COCs) in different animal species (Tirone et al., 1997; Kimura et al., 2002; Schoenfelder and Einspanier, 2003) as well as human (Campbell et al., 1995) and were considered as valuable and indirect markers of oocyte competence in bovine and human (McKenzie et al., 2004; Assidi et al., 2008; Gebhardt et al., 2011). Moreover, HA receptors CD44 and RHAMM were shown to be expressed in all stages of embryo development (Furnus et al., 2003; Palasz et al., 2006; Choudhary et al., 2007). In some studies, the addition of HA to culture media has been shown to stimulate the in vitro embryonic development of cows (Furnus et al., 1998; Lane et al., 2003) and mice (Gardner et al., 1999). HA has also been shown to improve the cryotolerance of blastocysts, which then leads to increased birth rates in cows (Lane et al., 2003), mice (Palasz et al., 1993) and lambs (Dattena et al., 2007). HA is also an important component of oviductal fluids in which fertilization and early embryo development take place (Stojkovic et al., 2002). These data suggest that HA production may play an important role in cell proliferation and survival during early embryo development. However, the expression and the role of HYAL2 during this stage of embryo development have not been previously investigated. As mentioned above, HA fragments produced by the action of HYAL2 have been shown to stimulate intracellular signalling and promote mitosis and cell proliferation and have anti-apoptotic effect, all of which are critical for embryo development.

In this paper, we have investigated the expression of different HAS enzymes and HYAL2 in embryos at different stages of development and in the oviduct. We also studied the effect of HA fragments generated by HYAL2 supplementation on the development and quality of bovine embryos in vitro and whether this effect is mediated through HA-receptor CD44. In addition, we examined the effect of HA synthesis inhibitor 4-methylumbelliferone (4MU) on embryo development and quality. Bovine embryos were used for these experiments as they are readily available in adequate numbers, enabling a reliable assessment of effects.
were permeabilized by incubating in 0.1% Triton for 5 min and incubated in the kit reagent in a humid chamber for 45 min at 37°C. Embryos were then washed and mounted in small droplets of anti-fading Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, USA, A94010) and examined under a Leica epifluorescent microscope (Leica). Images were acquired for each fluorescent stain then merged as shown in Fig. 1. Because not all the cells are in focus at one plane image, cells in each compartment were counted directly under the epifluorescent microscope while finely changing the focus.

**RNA extraction and reverse transcription–polymerase chain reaction**

Bovine oviducts from healthy cows in the early luteal phase were collected ipsilateral to ovaries containing corpus haemorrhagica. Surrounding tissue was carefully removed, then segments from the ampulla and isthmus were frozen in dry ice within 15 min after slaughter. Bovine embryos were produced in vitro as mentioned above. Groups of 30 embryos were collected at each stage of development as previously described by Stojkovic et al. (2003). Briefly, 2-, 4-, 8-, ~16- and ~32-cell stages as well as compact morulas and blastocysts were collected at 24, 36, 48, 70, 120, 144 and 168 h post-insemination (hpi). Embryos were washed in SOFaaci and immediately snap frozen in liquid nitrogen. All samples were kept at −80°C until RNA extraction.

Total RNA was extracted from frozen samples using RNeasy kit (Qiagen) following the manufacturer’s instructions. The concentration and integrity of the RNA was determined using nanodrop. The extracted RNA was treated with RNase-free DNase (Promega) to exclude genomic DNA contamination before reverse transcription (RT).

**RT–polymerase chain reaction**

RT was carried out at 37°C for 1 h using the Omniscript RT kit (Qiagen) for oviduct samples (500 ng/reaction) or Sensiscript RT kit (Qiagen) for embryo samples (50 ng/reaction), in combination with oligo-dT and random hexamer primers (Ambion). Conventional polymerase chain reaction (PCR) was performed using Multiplex plus Q-solution master mix (Qiagen) in a thermal cycler (G-Storm GSX machine, GRI, Braintree, UK) for 40 cycles. About 25 ng of cDNA per reaction and 0.2 μM PCR primers specific for HAS1, HAS2, HAS3, HYAL2 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Table I) were used. PCR products were visualized on 2% agarose gel (w/v) using ethidium bromide staining. Images were obtained using Syngene Gel doc facility (SYNGENE, Cambridge, UK). PCR bands were quantified using Quantity-one software (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) to calculate relative expression compared with GAPDH.

**Immunofluorescence staining of HA**

Ten to 15 embryos from each stage were removed from SOFaaci medium, washed in PBS and placed on polylysine-coated slides, dried, fixed with 4% paraformaldehyde in PBS for 10 min and dehydrated in increasing concentrations of ethanol (30–100%). Slides were kept at −20°C until all embryo stages were collected and ready for staining. For HA staining, slides were treated with RNase-free DNase (Promega) to exclude genomic DNA contamination before reverse transcription (RT).

**Figure 1** Representative images for differential staining of embryos associated with TUNEL. (A) Trophoectoderm stained with PI (red), (B) all cells stained with Hoechst (blue), (C) apoptotic cells labelled with TUNEL (green) and (D) image merge of the PI and Hoechst staining.
thawed, rehydrated in decreasing concentrations of ethanol (100–30%) and washed in PBS. Slides were blocked in normal goat serum (1:20 in PBS) for 2 h using avidin–biotin blocking kit for 15 min at room temperature. Slides were then incubated with biotinylated-HA-binding protein (b-HABP) at 1:3000 in PBS at 4°C overnight, followed by washing and incubation with FITC-conjugated streptavidin (ebioscience, San Diego, CA, USA) for 2 h at room temperature in the dark. Slides were then counterstained with Hoechst (30 μg/ml in PBS), washed and mounted in small droplets of anti-fading Vectashield mounting medium (Vector Laboratories, Inc.) and examined under a Leica epifluorescent microscope (Leica).

### Western blotting for detection of phosphorylated and total MAPK1 and 3

Cleaved bovine embryos (in groups of 30–50 embryos) were randomly allocated into SOFaaci media supplemented with or without Hyal2 (300 unit/ml). After 24 h in culture, embryos were washed in protein-free SOFaaci containing 0.4% polyvinylpyrrolidone, then transferred into 1 ml microcentrifuge tubes and snap-frozen in liquid nitrogen awaiting analysis. Embryos were lysed by repeated freezing and thawing in liquid nitrogen in 10 μl of lysis buffer containing Tris (63.5 mM, pH 6.8), 10% glycin, 4% SDS, sodium orthovanadate 2 mM, sodium fluoride 200 μM and protease inhibitor cocktail 1× (Calbiochem, Darmstadt, Germany). After lysis, β-mercapto ethanol was added and samples were boiled at 100°C for 10 min. Lysates were loaded on a SDS–polyacrylamide gel electrophoresis of 4% stacking gel over-laid with a 15% resolving gel. Pre-stained protein ladder (10 μl; Fermentas, Loughborough, UK) was loaded in the first lane. Electrophoresis was carried out at 170 V ~ 24 mA for 1 h35 m. Afterwards, the proteins were transferred from the gel to polyvinylidene difluoride membrane (GE Healthcare, Hatfield, UK) at 100 V ~ 51 mA for 1 h40 m. The membranes were then blocked in 10% Skimmed milk overnight. On the next day, membranes were washed for 5 min in PBS-Tween-20 (0.05% v/v) and blotted with primary antibodies against phosphorylated MAPK (1:500 dilution; Cell Signaling, Boston, MA, USA) followed by thorough washing (6 × 15 min) and probing with donkey anti-rabbit IgG-horse-radish peroxidase-conjugated (1:125 000) secondary antibody (SantaCruz, CA, USA). The membranes were then developed using ECL Select (GE Healthcare) and a gel documentation facility (SYNGENE). After saving the image from phosphorylated bands, the membranes were stripped by washing in the stripping buffer (Tris–HCl, pH 6.8, 2% SDS, 1% Tween-20, 0.1% 2-mercaptoethanol) for 15 min with maximum agitation. Then, the membranes were blocked and re-probed as above using total rabbit anti-MAPK1 and three primary antibodies (1:500 dilution; Cell Signaling).

Images obtained from the developed membranes were analysed using Alpha Ease FC software v.3.1.2 (Alphalnnotech; Protein Simple, CA, USA) for band densitometry (integrated density value). The background value was deduced automatically from the analysis. The percentage of phosphorylated MAPK1 and 3 was calculated from the total MAPK1 and 3, respectively, as described by Marei et al. (2009).

### Statistical analysis

In all the experiments, the data were from at least three independent repeats. Binomial data from all experiments were converted to percentages. All data were tested for homogeneity by Levene’s test and were normally distributed. Data were then analysed in SPSS (version 19) using a linear mixed model with randomized block analysis. The effect of different treatments was considered the fixed effect, whereas the different batches of ovaries were taken into consideration as a random effect. If the main treatment effect was significant, Bonferroni post hoc tests were performed for pairwise comparisons. Differences in P-values of ≤0.05 were considered as significant.

### Results

#### mRNA expression of different HAS and HYAL2 in bovine embryos

HAS2 and HAS3 were expressed in all stages of embryo development, whereas HAS1 was expressed predominantly in 2- and 4-cell stages and was faintly detectable thereafter. In contrast, HYAL2 expression was only detectable at Days 6 and 7 in compact morula and blastocyst stages but not detected earlier (Fig. 2A). The relative expression of these genes to GAPDH showed that HAS1 and HAS2 expression dramatically decreased during development to the blastocyst stage, whereas HAS3 was more stable (Fig. 2B).

#### HYAL2 expression in bovine oviduct

HYAL2 was expressed in the isthmus and ampulla of bovine oviducts collected at the early luteal phase (Fig. 3A). The relative expression of HYAL2 to GAPDH in isthmus was significantly higher than in the ampulla (Fig. 3B).

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**Table 1 Details of primers used for RT–PCR.**

<table>
<thead>
<tr>
<th>PCR product (bp)</th>
<th>Annealing (°C)</th>
<th>Primer sequence</th>
<th>Accession number</th>
</tr>
</thead>
</table>
| HAS1*            | 184            | 59             | Forward: GGTACAACCAGAAGTTCCCGG
|                  |                |                | Reverse: CCGAAGTACGACTTGGACCCAG |
|                  | 150            |                | Forward: GGGGGGAGATGTCAGAGGTT |
|                  |                |                | Reverse: ATGCAGCTGAGCAACTCCGA |
|                  | 134            | 57.5           | Forward: TGATGTCGCTCTACTCCCTCTT |
| HAS2             | 125            |                | Reverse: GATGAGCCCAATAAGATTCACC |
| HAS3             | 134            | 57.5           | Forward: GGACGACCAACACTCCGTCG |
| HYAL2            | 280            | 59             | Reverse: ACCCTCAGTAGGATGGACCCT |
| GAPDH            | 218            | 59             | Forward: CCAACGTGTCTGTTGGATGGAG |
|                  |                |                | Reverse: GAGCTTGCGAAAGTGGTGAG |

*As in Palasz et al. (2006).
Immunofluorescence staining for HA in embryos

Immunostaining with the b-HABP followed by FITC-conjugated streptavidin (green) show the presence of HA in all stages of early embryo development. Staining at 2- and 4-cell stages was less intense compared with subsequent stages (Fig. 4).

The effect of HYAL2 treatment of cleaved embryos on the blastocyst rate and quality

As shown in Table II, the treatment of cleaved embryos with HYAL2 resulted in higher blastocyst rates and increased total cell number, TE and ICM of the embryos, which was significantly different at 300 unit/ml (P < 0.05) compared with the control group. HYAL2 did not affect the percentage of apoptotic cells in the treated embryos or the TE/ICM ratio.

The effect of 4MU treatment of cleaved embryos on the blastocyst rate and quality

Cleaved embryos were treated with increasing concentration of the hyaluronan synthesis inhibitor (4MU) in embryo culture medium starting from Day 2. A concentration-dependent inhibition of blastocyst formation was observed in 4MU-treated embryos where no blastocyst was produced in the presence of 1 mM 4MU (P < 0.05 compared with the control). 4MU at 0.1 or 0.5 mM did not affect the blastocyst quality as shown by cell counts (Table III).

Embryo development in the presence of HYAL2 with or without anti-CD44 antibody

Bovine embryos were treated with either HYAL2 (300 unit/ml), anti-CD44 antibody (2 μg/ml) or both, starting at 48 hpi until Day 8. Blastocyst formation from embryos treated with anti-CD44 tended to be lower but was not significantly different from control (12 ± 3.5 versus 20 ± 0.7; P = 0.63). Supplementation of embryos with HYAL2...
resulted in a significant increase in the blastocyst rate (35 ± 2.9; \( P = 0.017 \)); however, this effect was abolished in the presence of anti-CD44 (17 ± 0.9; \( P = 1.0 \) when compared with control; Fig. 5A). Hyal2 supplementation resulted in an increase in the total cell count \( (P < 0.05) \). This increase was abrogated in the presence of an anti-CD44 antibody (Fig. 5B).

### Effect of HYAL2 on phosphorylated MAPK1 and 3

Supplementation of the cleaved embryo (on Day 2) with HYAL2 (300 unit/ml) in SOFaaci medium for 24 h significantly increased the percentage of phosphorylated/total MAPK1 and 3 in embryos when compared with the controls (Fig. 6).

### Embryo development in the presence of HYAL2 with or without MAPK kinase inhibitor (U0126)

Bovine embryos were treated with either HYAL2 (300 unit/ml), MAPK kinase inhibitor U0126 (10 \( \mu \)M) or both, starting at 48 hpi until Day 8. Supplementation of embryos with HYAL2 resulted in a significant increase in the blastocyst rate (48 ± 4.1 versus 28 ± 3.0; \( P < 0.01 \)). The blastocyst rate from embryos treated with U0126 was significantly

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**Figure 4** Localization of HA in bovine embryos at different stages of development. Immunofluorescence staining of HA was done using b-HABP followed by streptavidin-FITC. Green, HA staining; Blue, counter staining with Hoechst; hpi, hours post insemination; -ve control, blastocyst was used as a negative control with the omission of the HABP.

**Table II** The blastocyst rate and quality following treatment of Day 2 cleaved embryos with increasing concentration of HYAL2.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>50 U</th>
<th>100 U</th>
<th>300 U</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blastocyst (%)</td>
<td>21.8 ± 0.4</td>
<td>22.6 ± 5.9</td>
<td>29.6 ± 5.6</td>
<td>38.1 ± 4.8*</td>
<td>0.03</td>
</tr>
<tr>
<td>Number of total cells</td>
<td>78.0 ± 14.5</td>
<td>86.3 ± 5.8</td>
<td>96.7 ± 6.4</td>
<td>114.6 ± 13.3*</td>
<td>0.03</td>
</tr>
<tr>
<td>ICM</td>
<td>22.3 ± 3.9</td>
<td>26.0 ± 3.6</td>
<td>26.2 ± 1.4</td>
<td>34.4 ± 3.4*</td>
<td>0.00</td>
</tr>
<tr>
<td>TE</td>
<td>55.8 ± 14.2</td>
<td>60.3 ± 6.7</td>
<td>66.5 ± 5.7</td>
<td>75.2 ± 14.0</td>
<td>0.05</td>
</tr>
<tr>
<td>ICM:TE ratio</td>
<td>0.50 ± 0.19</td>
<td>0.48 ± 0.12</td>
<td>0.32 ± 0.19</td>
<td>0.61 ± 0.13</td>
<td>0.34</td>
</tr>
<tr>
<td>Apoptotic (%)</td>
<td>5.6 ± 3.52</td>
<td>2.9 ± 0.3</td>
<td>4.5 ± 0.7</td>
<td>6.3 ± 2.9</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SEM from five independent repeats. ICM, inner cell mass; TE, trophoectoderm cells.

* Significant difference at \( P < 0.05 \) compared with the control.
lower than control (6 ± 0.9; P < 0.05). Combined treatment with HYAL2 and U0126 was also significantly lower than control (15 ± 6.0; P < 0.05; Fig. 7).

Table III The blastocyst rate and quality following after treatment of Day 2 cleaved embryos with increasing concentration of 4MU.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.1 mM</th>
<th>0.5 mM</th>
<th>1.0 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blastocyst (%)</td>
<td>22.7 ± 4.8</td>
<td>19.2 ± 5.9</td>
<td>9.3 ± 5.4*</td>
<td>0.0 ± 0.00*</td>
</tr>
<tr>
<td>Total cells</td>
<td>82.6 ± 6.9</td>
<td>86.4 ± 5.5</td>
<td>85.3 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>TE</td>
<td>54.2 ± 4.2</td>
<td>49.2 ± 6.4</td>
<td>60.3 ± 11.3</td>
<td></td>
</tr>
<tr>
<td>ICM</td>
<td>28.3 ± 4.1</td>
<td>37.2 ± 7.6</td>
<td>25.0 ± 8.2</td>
<td></td>
</tr>
<tr>
<td>ICM:TE ratio</td>
<td>0.5 ± 0.1</td>
<td>0.8 ± 0.3</td>
<td>0.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>% of apoptosis</td>
<td>5.2 ± 2.7</td>
<td>12.0 ± 4.3</td>
<td>5.3 ± 5.3</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SEM from five independent repeats. ICM, inner cell mass; TE, trophoectoderm cells. *Significant difference at P < 0.05 compared with the control.

Discussion

The aim of this study was to investigate the expression of different HASs and HYAL in embryos at different stages of development and in the oviduct, as well as to study the importance of HA synthesis and the effect of HYAL2 supplementation on the development and quality of embryos in vitro and whether this effect is mediated through the HA-receptor CD44/MAPK pathway.
In the present study, we have shown by immunofluorescence that HA is produced by bovine embryos at all stages of early development particularly after the 4-cell stage. This observation is in accordance with Stojkovic et al. (2003) who showed that embryos secrete HA into culture medium in vitro. HA is known to be produced by actively dividing cells during mitosis (Brecht et al., 1986). We also show that the inhibition of HA synthesis by 4MU supplementation resulted in a partial to complete inhibition of blastocyst formation in a concentration-dependent manner. These findings suggest that the presence of HA is vital for embryo development. In vivo oviduct also contributes to HA production, since HAS2 and HAS3 were found to be expressed in the bovine oviduct, and HA could be detected by immunostaining (Ulbright et al., 2004; Bergqvist et al., 2005).

The expression of different HAS genes is tissue and cell specific (Weigel et al., 1997). Here, we demonstrated that the expression of different HAS genes in bovine embryos varies according to the stage of development. HAS2 and HAS3 were expressed at all stages of embryo development; however, the relative expression of HAS2 was significantly reduced as the embryos develop to the blastocyst stage. HAS1 was detected during 2- and 4-cell stages but was barely detectable in subsequent stages. Considering the fact that HAS2 produces the largest molecular weight HA (>2 × 10^5 Da), whereas HAS3 produces the smaller size HA (0.1–1 × 10^5 Da; Stern et al., 2006), our observations suggest that the HA fragments produced by the embryos may get smaller in size as the embryos develop.

In addition, we detected HYAL2 expression in compact morulae and blastocysts at Days 6 and 7 post-fertilization. We also found that HYAL2 is expressed in the oviduct collected at the early luteal phase and was significantly higher in the isthmus region compared with the ampulla. To our knowledge, HYAL2 expression in embryos and oviduct has not been shown previously. HYAL2 is normally located on cell surface and is critical for degrading the HMW HA to fragments of ~20 kDa in size (Duterme et al., 2009). These findings suggest that the depolymerization of HA by HYAL2 into smaller fragments may be required during embryo development and that HYAL2 will not be available in vitro, since embryos do not express it before the morula stage. To test this hypothesis, we examined the effect of HYAL2 supplementation on early embryo development in vitro.

In the present study, we show evidence that HYAL2 supplementation to embryos at 48 h post-fertilization are beneficial for preimplantation embryo development and embryo quality. We observed an increase in the blastocyst rate and embryonic cell numbers when embryos were treated with 300 unit/ml of HYAL2. In contrast, a previous study shows that the addition of HA (~700,000 Da) to bovine embryos culture 48 h post-fertilization suppressed embryo development to the blastocyst stage (Palasz et al., 2006). Based on our observations, small HA fragments may be required to support embryonic development following fertilization. In contrast to large size HA which functions mainly depend on its structure and physical characteristics, small HA fragment functions are receptor mediated (Laurent, 1998). The degradation of HA by HYAL2 into smaller fragments is known to initiate CD44 cell signalling (Ohno-Nakahara et al., 2004). Recently, it has been shown that fragmented HA produced by HAS2-HYAL2/CD44 system on the plasma membrane has autocrine effects in cancer cells (Saito et al., 2011). CD44 which is expressed on the cell surface during all stages of bovine embryo development (Furnus et al., 2003) can induce the signaling cascade that augment the phosphorylation of MAP kinases and protein kinase B (Sohara et al., 2001) both of which are important molecular regulators for cell division and apoptosis during embryo development (Riley et al., 2005). In the present study, we have shown that HYAL2 increases the phosphorylation of MAPK1 and 3 in developing embryos and that the improvement of the blastocyst rate and embryo quality by HYAL2 supplementation is abrogated in the presence of an anti-CD44 antibody or an MAPK-kinase inhibitor (U0126), suggesting that HYAL2 effect is mediated through CD44/MAPK signalling. HA-CD44 binding can also result in direct or indirect interaction with other signalling receptors like the epidermal growth factor (EGF) receptor and thus may enhance EGF activity which again depends on MAPK signalling (Wang and Bourguignon, 2006; Kim et al., 2008). EGF has been shown to regulate embryo development in bovine (Buyalos and Cai, 1994; Lonergan et al., 1996).

Based on our findings, it can be suggested that, under in vitro conditions, the addition of HYAL2 can improve early embryo development by cleaving HMW HA produced by the embryos generating biologically active fragments that compete with HMW HA in binding to CD44 on the cell surface of the embryo. The activation of CD44 will stimulate the activation of the MAPK pathway which is required for embryo development. In this context, it is also important to mention that the presence of HMW HA is also required mainly for later stages during the hatching of the blastocyst and implantation and may improve embryo survival after cryopreservation. Palasz et al. (2006) stated that the addition of HA to bovine embryos at 96 hpi did not affect embryo development to blastocyst but increased hatching rates. Also, Lane et al. (2003) reported that the addition of HA to bovine embryos did not affect the rate of blastocyst development, degree of expansion, hatching, total cell number, ICM or TE. However, HA-treated embryos had significantly higher re-expansion and hatching following cryopreservation. In the present study, HYAL2 had no effect on hatching. In vivo HA produced by cumulus cells during expansion, as well as HA produced by the embryo and the oviduct together with the presence of HYAL2 expressed by the oviduct and embryo, create a balance of different fragment sizes of HA that provide a wide range of biological functions supporting early embryo development.
The continued rise in the need for IVF treatments has been associated with the fast growth of companies specialized in the assisted reproductive technology and the development of new media formulations trying to mimic the conditions naturally found in the mother’s tissue which protects the embryo and make it stronger and more robust in the early implantation period. It is noticed, however, that the success rate of IVF treatments (~24% live birth per IVF cycle in UK) is increasing at a slow rate (~0.4% annual increase; HFEA, 2010). Our results suggest that HYAL2 is a naturally occurring oviductal factor that can be directly added to embryo culture media to achieve higher rates of transferable embryos of better quality; however, further studies are needed to verify these effects in human. The likelihood of adverse effects following transfer is considered minimal, as these products are naturally occurring in the female genital tract and in male and female gametes and embryos of animals and human.

In conclusion, our results show that HYAL2 is only expressed by embryos at compact morula and blastocyst stages but not earlier which suggest that in vitro embryo production is devoid from HYAL2 until these stages. Since HYAL2 is expressed by the oviduct just after ovulation, supplementation of HYAL2 may mimic in vivo conditions and is shown to improve embryo development and quality via CD44-MAPK signalling. These findings present new aspects of the role of HA during embryo development and give a scientific basis for new IVF media formulation.

**Authors’ roles**

W.F.A.M. contributed to research ideas and experimental design, collected all samples and did all the experiments and data analysis except Western blotting and wrote and edited the manuscript; M.S. optimized and analysed samples by Western blotting and contributed to writing and data analyses; A.A.F.-N. is the project supervisor and contributed with the main research ideas and experimental design and critically revised the data and edited the manuscript.

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**Conflict of interest**

None declared.

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