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The effect of bovine embryo culture without proteins supplements until day 4 on transcription level of hyaluronan synthases, receptors and mtDNA content

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Summary

The effect of bovine embryo culture on a flat surface, (without a surface-active compound) on the level of mRNA expression of hyaluronan (HA) synthases (Has1, Has2 and Has3), Ha receptors RHAMM and C44 receptors was evaluated by mitochondrial DNA concentration and *in vitro* development. Cultures were evaluated up to 96 h post-insemination (hpi) using SOFaa medium. Of the three Has isoforms, Has2 expression only increased in the bovine serum albumin (BSA)-only supplemented groups regardless of time of BSA addition. Expression of RHAMM receptors was highly dependent on the addition of HA, irrespective of the presence of BSA in the medium. In contrast, expression of the CD44 receptor gene was not affected by any treatment. The cleavage rates and number of embryos that developed to ≤8-cell stage by day 4 were not affected by lack of BSA in the medium, but increased numbers of blastocysts developed in medium supplemented with BSA from days 1 or 4 with or without HA than in medium that had HA only. Addition of both HA and BSA at day 4 increased mtDNA copy numbers at the blastocyst stage. Data suggest that the addition of BSA and/or HA at 96 hpi increased expression of *RHAMM* and *Has2* genes, but not *CD44*, *Has1* or *Has3* genes. Higher expression levels of Has2 than Has1 and the three isoforms indicate that high- rather than low-molecular-weight HA should be used for preimplantation bovine embryo culture.

Keywords: Culture, Embryo, Gene expression, Hyaluronan, Mitochondria, Synthases

Introduction

Hyaluronan (HA) is a major component of mammalian follicular, oviductal and uterine fluids, and plays an important role in optimal embryo development. Hyaluronan is a simple glycosaminoglycan with very complex biological functions. It is composed solely of 2000–25 000 carbohydrates, glucuronic acid and *N*-acetylglucosamine, and can reach a molecular mass of approximately 4×10^6 Da (Laurent, 1998). Previously known as hyaluronic acid, it is now more often referred as hyaluronan, reflecting the fact that *in vivo* it exists as a polyanion and not in the acid form. There is ample evidence indicating that, during embryogenesis, HA participates in several biological processes that utilize its unique biochemical and physical properties. Extracellular matrices rich in HA expand the extracellular space, facilitating cell migration and differentiation (Toole, 1997). The viscoelastic properties of hyaluronan are essential for proper function of cartilage and joints (Gotoh *et al.*, 1993). Hyaluronan also acts as an antioxidant, protecting body tissue from radical oxygen species caused by enzymatically produced hydroxyl (OH) radicals (Presti & Scott 1994). Physiological importance of HA may be illustrated by the effects of hyaluronidase on certain biological processes, such as fertilization (Salustri *et al.* 1992), or from the correlation between HA accumulation and the presence of its receptors (CD44 and/or RHAMM), in mouse (Fenderson 1993), bovine (Furnus *et al.*, 2003; Stojkovic *et al.*, 2003) and human (Campbell *et al.*, 1995) embryo development. Hyaluronan is

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especially abundant during embryogenesis (Fenderson *et al.*, 1993) and is considered to contribute to the optimal development of the gametes and the embryo (Gardner *et al.*, 1999; Ulbrich *et al.*, 2004; Palasz *et al.*, 2007). In contrast with other glycosaminoglycans that are synthesized in the Golgi apparatus and are attached to proteins, HA is synthesized at the inner face of the plasma membrane and is not associated with any protein (Weigel *et al.*, 1997). Hyaluronan biosynthesis is determined by three related, yet distinct, hyaluronan synthase (Has) enzymes Has1, Has2 and Has3 (Itano *et al.*, 1999). The existence of three different Has isoforms with different characteristics implies that the wide range of biological and physiological functions are performed by HA (Spicer & McDonald, 1998). Each of them functions independently. Has3 is intrinsically more catalytically active than Has2, which is in turn intrinsically more active than Has1 (Spicer & Nguyen, 1999). Furthermore, HA synthesized by each Has protein *in vitro* indicated that HA chain length/molecular weight (MW) is determined by the specific Has protein. Has1 and Has2 proteins polymerize hyaluronan chains of similar lengths (MW up to 2×10^6 Da), whereas Has3 polymerized much shorter hyaluronan chains, in the range of $<2 \times 10^5$ Da to 3×10^5 Da (Tien & Spicer, 2005). During embryogenesis continuing changes of HA concentration and MW are prerequisite for embryo cell movement and proliferation. These functions require rapid polymerization and depolymerization of the HA chains, which in *in vitro* culture conditions is difficult to achieve or control. Presently, variable preparations of HA of different MW are used for embryo culture. Similarly, even concentrations as different as 6 mg/ml (Stojkovic *et al.*, 2003) and as low as 0.125 mg/ml of HA (Lane *et al.*, 2003) are used successfully for bovine embryo culture. Considering that almost all biological functions of HA depend on MW and on concentration, this finding may be the proof that full potential benefit of using HA in embryo culture has not yet been achieved. One important piece of information that may help to understand HA function, but was never tested in mammalian embryo culture, comes from the report by Kraemer & Barnhart (1978). They showed that cells cultured on solid surfaces, without surface active compound, produce larger amounts of HA than those grown in suspension. To verify this finding and to determine what specific MW of HA is synthesized during preimplantation bovine embryo development at different culture conditions, we quantified the gene expression patterns of HA synthases (Has 1, 2 and 3) and receptors CD44 and RHAMM in bovine day-7 blastocysts cultured up to day 4 (96 hpi) and without any protein or surface active compound. Quantification of mtDNA was used as an embryo quality assay. Considering that all biological functions of HA depend on MW and concentration we anticipate that results from this study will provide new scientific bases for the most effective use of HA for *in vitro* culture of bovine preimplantation embryos.

Materials and methods

All chemicals for oocytes *in vitro* maturation, fertilization and embryo culture were purchased from Sigma Chemical Company unless otherwise stated.

In vitro maturation

Cumulus–oocyte complexes (COC) were aspirated from follicles of 2 to 7 mm in diameter from slaughterhouse-derived ovaries within 3 h of slaughter. After selection based on morphological evaluation (Shamsudin *et al.*, 1994), COC were washed once in TALP medium (Bavister *et al.*, 1983), buffered with 10 mM of MOPS (TALP–MOPS) and supplemented with 0.25 mM pyruvate and 0.5 mg/ml bovine serum albumin (BSA) fraction V, and then twice in the final maturation TCM-199 medium containing 10% fetal calf serum (FCS), 0.25 mM pyruvate and 100 ng/ml epidermal growth factor (EGF). Cumulus–oocyte complexes were matured in groups of 50 in 2 ml of medium (Avery *et al.* 1995) in Nunclon multi-well dishes (Nunc) without paraffin oil at 39 °C with 5% CO_2, 5% O_2 and 90% N_2 in humidified air.

In vitro fertilization

After 24 h of incubation in the maturation medium, oocytes were washed once in TALP–MOPS and then twice in the final Fert–Talp medium supplemented with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/ml BSA–FAF and 10 µg/ml heparin (Parrish *et al.* 1988). Oocytes were inseminated in Nunclon dishes in groups of 50 in 250 µl Fert–Talp with 1×10^6 ml spermatozoa selected after swim-up trough in Fert–Talp medium supplemented with 4 mg/ml BSA–FAF.

In vitro culture

Twenty hours post insemination cumulus cells were removed by vortexing (150 s) and washed twice in TALP–MOPS and then twice in the final culture medium. All presumptive zygotes were randomly allocated to treatment groups. Zygotes were cultured in Falcon dishes (Becton Dickinson & Co.) in modified (Holm *et al.*, 1999) Tervit's SOF culture medium (Tervit *et al.*, 1972) under paraffin oil. SOF culture medium contains: 0.1% (v/v) of non-essential amino acids (modified Eagle medium; MEM) and 0.2% (v/v) essential amino acids (basal medium Eagle; BME).

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Cleavage rates were recorded 48 hpi. Zygotes that developed to ≤8 cells on day 4 (96 h pi) and number of embryos that developed to blastocysts on days 7, 8 and 9. Embryos were cultured under paraffin oil at 39 °C with 5% CO_2, 5% O_2 and 90% N_2 in humidified air. All culture media used in this experiment were supplemented with 1 mM sodium pyruvate, 22 mM sodium lactate and 50 μg ml of gentamicin.

One to five day-7 blastocysts from each replicate and from each treatment were frozen for mRNA transcription and mtDNA copy analysis.

Assessment of embryo development

Cleavage rates were recorded at day 2 (48 hpi) as was the number of zygotes that developed to ≤8 cells on day 4 of culture (96 hpi). Developmental rates of embryos were based on the number of blastocysts that had developed at day 7, 8 and 9 of culture and were calculated based overall on the total number of cleaved oocytes, and separately on the number of zygotes that 96 hpi developed to ≤8 cells. Embryos with clearly visible blastocoel cavity up to two-thirds volume of the embryos were graded as blastocyst, as hatching blastocyst when the zona pellucida was open and part of embryo proper was out of the zona pellucida, and as hatched blastocyst when the entire embryo was out the zona pellucida.

Embryo's cell number counts

A total of 12 embryos from each treatment and from three replicates were taken randomly. Embryos were washed twice in 100 μl droplets of PBS containing 0.5 mg/ml ET–surfactant (Bioniche Inc.) and then fixed in a 100 μl droplet of 4% (w/v) paraformaldehyde solution in PBS, (pH 7.4) for 1 h at room temperature. Embryos were washed again three times in 100 μl droplets of PBS/ ET surfactant and transferred to a 50 μl microdrop of Hoechst 33342 dye. After 10 min of cell staining, embryos were transferred in a very small volume on previously coated glass with Sigmacoat (Sigma), embryos were allowed to settle and a coverslip was placed over the drop. Slides were stored in the dark at 4 °C until the nuclei count. The total number of nuclei was counted under a phase-contrast Nikon fluorescence microscope using a UV-2A/DH400 filter.

Experimental design

A total of 1966 presumptive zygotes in 11 replicates were cultured until 96 hpi in 30 μl (25 embryos per drop) of two culture media: Group 1, SOF supplemented with 5 mg/ml BSA; and Group 2, SOF without BSA supplement. On day 4 of culture, the number of zygotes that had developed to ≤8 cells was recorded and 20 μl of fresh culture medium were added as followed: Group 1a, SOF + BSA (no change); Group 1b, SOF + BSA + 1 mg/ml HA produced by bacteria fermentation (MAP-5; Bioniche Inc.); Group 2a SOF only (no change); Group 2b SOFaa + 1 mg/ml HA; and Group 3, SOF only (no change; negative control).

RNA Extraction and reverse transcription

Poly(A) RNA was prepared from groups of 10 day-7 *in vitro* blastocysts; 10 *in vivo* blastocysts exposed to PBS ~1 h were used as control. Blastocysts were washed in PBS supplemented with 0.1% (w/v) polyvinyl pyrrolidone and were stored at –80 °C until processed for real-time RT-PCR following the manufacturer's instructions using the Dynabeads mRNA Direct KIT (Dynal). Briefly, samples were lysed in 50 μl lysis/binding buffer (Dynal). After vortexing and a brief centrifugation, the samples were incubated at room temperature for 10 min. Ten microliters of pre-washed Dynabeads oligo (dT) 25 were added to each sample. After 5 min of hybridization, the beads were separated from the binding buffer using the Dynal magnetic separator. Then the beads were washed in buffer A and B (Dynal) and the poly(A) RNA was eluted from the beads by adding 11 μl of DEPC-treated water. The resultant poly(A) mRNA was used in the RT-PCR in a total volume of 20 μl using 2.5 μM random hexamer primer, 1× RT buffer, 20 IU RNase inhibitor, 50 IU MMLV reverse transcriptase enzyme (Promega, Madrid, Spain), 5 mM $MgCl_2$ and 1 mM of each dNTP. Tubes were heated to 70 °C for 5 min to denature the secondary RNA structure before the addition of RT enzyme (Palasz *et al.* 2008). The RT reaction were then incubated at room temperature for 10 min and then at 42 °C for 60 min to allow the reverse transcription of RNA, followed by 93 °C for 1 min to denature the RT enzyme.

Quantitative RT-PCR

Quantitation of all gene transcripts was carried out by real-time quantitative RT-PCR in three replicates (Rizos *et al.* 2007). PCR was performed using a Rotorgene 2000 Real Time CyclerTM (Corbett Research) and SYBR Green (Molecular Probes) as a double-stranded DNA-specific fluorescent dye. The PCR reaction mixture (25 μl) contained 2.5 μl 10× buffer, 3 mM $MgCl_2$, 2 U *Taq* Express (MWGAG Biotech), 100 μM of each dNTP, and 0.2 μM of each primer (Table 1). In addition, the double-stranded DNA dye, SYBR Green I, (1:3000 of 10 000× stock solution) was included in each reaction. The PCR protocol included an initial step of 94 °C (2 min), followed by 40 cycles of 94 °C (15 s), 56–59 °C (30 s) and 72 °C (30 s). Fluorescence data were acquired at 80–85 °C. The melting protocol consisted of holding at 40 °C for 60 s and then heating from 50 to 94 °C, holding at each temperature for

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Table 1 Details of primers used for RT-PCR.

	Gene				
Mice	Primer	Sequence (5′–3′)	Fragment size (bp)	Annealing temperature (°C)	GenBank accession no.
Histone H2a	5′	AGGACGACTAGCCATGGACGTGTG	208	59	NM_174809
	3′	CCACCACCAGCAATTGTAGCCTTG			
mtDNA	5′	AAATAATATAAGCTTCTGACTCC	190	59	EF568705.1
	3′	TCCTAAAATTGAGGAAACTCC			
CD44	5′	TATAACCTGCCGATATGCAGG	221	56	X62881
	3′	CAGCACAGATGGAATTGGG			
RHAMM	5′	TGTTGAATGAACATGGTGCAGCTC	249	59	AJ439694
	3′	CCTTAGAAGGGTCAAAGTGTTTGAT			
HAS1	5′	GGTACAACCAGAAGTTCCTGGG	184	59	AB017803.1
	3′	CGGAAGTACGACTTGGACCAG			
HAS2	5′	GGATGTGTCCAGTGCATTAGCGGAC	143	59	NM_174079.2
	3′	CAGCACTCGGTTCGTTAGATGCCTG			
HAS3	5′	ACAGGTTTCTTCCCCTTCTTCC	165	56	AJ293889
	3′	GCGACATGAAGATCATCTCTGC			

5 s while monitoring fluorescence. Product identity was confirmed by ethidium bromide staining and 2% agarose gel electrophoresis. As negative controls, tubes were always prepared in which RNA or reverse transcriptase was omitted during the RT reaction. The comparative CT method was used for quantitation of expression levels. The quantitation was normalized to the endogenous control histone H2a. Fluorescence data were acquired in each cycle in order to determine the threshold cycle or the cycle during the log–linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, each difference of one cycle is equivalent to a doubling of the amplified product of the PCR. According to the comparative CT method, the ΔCT value was determined by subtracting the H2a CT value for each sample from each gene CT value of the sample. Calculation of ΔΔCT involved using the highest sample ΔCT values (i.e. the sample with the lower target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of target were determined by using the formula, 2–ΔΔCT. The details of primers used for RT PCR are shown in Table 1.

Statistical analysis

Embryo development was analysed by chi-squared test with the Fisher's Exact test correction. Differences in embryo development between replicates were analysed by analysis of variance (ANOVA). Data on mRNA expression were analysed using the SigmaStat (Jandel Scientific) software package. One-way repeated-measures ANOVA (followed by multiple pair-wise comparisons using the Student–Newman–Kleus method) were used for the analysis of differences in mRNA expression assayed by quantitative RT-PCR.

Results

Embryo development

There was no effect of BSA on cleavage and embryo development until day 4 of culture. Zygotes that had been cultured in SOF supplemented with both non-essential (modified Eagle medium; MEM) and essential amino acids (basal medium Eagle; BME), sodium pyruvate, sodium lactate and L-glutamine, but without BSA or serum, all cleaved and developed to ≤8 cells 96 hpi at similar rates as in medium supplemented with BSA (Table 2). Although present results of embryo culture without any protein source until day 4 were not expected, the same patterns of cleavage and development were very consistent and did not differ among the 11 replicates. The majority of embryos developed to the blastocyst stage at day 7: Group 1, 23.1%; Group 1a, 19.2%; Group 2, 21.8%; Group 2a, 12.0% and Group 3, 3.5%. The total number of the blastocysts developed at day 7 and 8 is showed in Table 2. The relatively modest blastocyst production in our study may be related to the quality of the oocytes that we observed previously during summer time.

Total cell numbers

The total cell number of day-7 blastocysts from Group 1, cultured in SOF + BSA or SOF + BSA + HA and Group 2, SOF+BSA (added on day 4 of culture) or

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Table 2 Embryo development in SOF culture medium with or without BSA until day 4 and then supplemented with/without 0.5% BSA or 1 mg/ml HA.

Group	No. of oocytes	Cleavage day 2 no. (%)	Embryo development no. (%) day 4 No.	Media	Blastocysts days days 7 and 8	Hatching/hatched** day 9
Control (+) SOF + BSA	419	333 (79.5)	158	No change	89 (26.7)$_a$	34/15 (44.1)^a
	444	339 (76.3)	156	+ HA	77 (22.7)^a	30/21 (70.0)^b
SOF only	332*	256 (77.1)	113	+ BSA	71 (27.7)^a	31/ 12 (38.7)^a
	437	340 (77.8)	152	+ HA	59 (17.3)^b	18/5 (27.7)^{a,b}
Control (–) SOF only	334*	256 (76.6)	129	No change	23 (8.9)^c	5/0 (0.0)^c

^{a,b,c}Percentages within a columns with values not in common differ ($p < 0.05$).
*Eight replicates.
**Based on the number of blastocysts remained in culture at day 8 (less blastocysts taken at day 7 for the PCR).

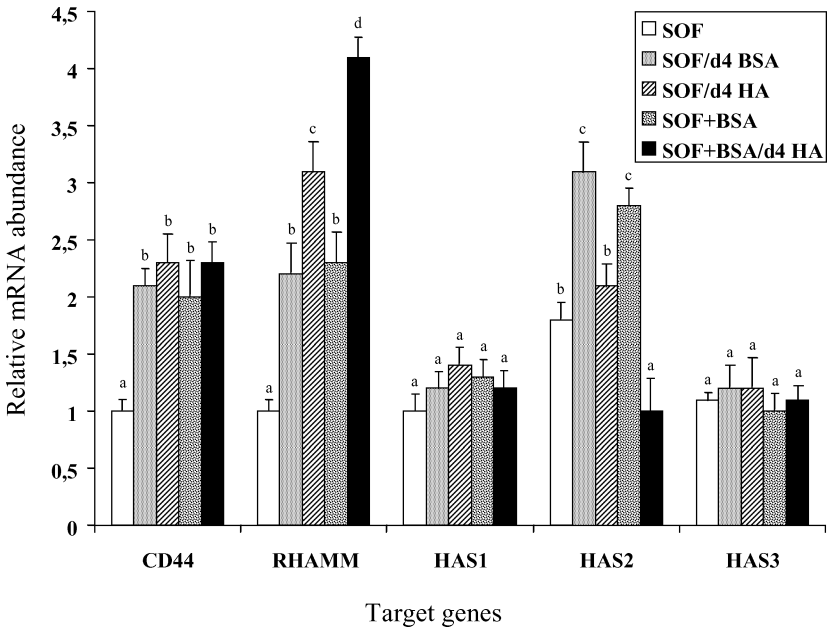

Figure 1 Relative abundance of hyaluronan receptors RHAMM and CD44 and hyaluronan synthases Has1, Has2 and Has3 in bovine blastocysts developed in SOF culture medium without BSA supplement until day 4 of culture.

SOF + HA (added on day 4 of culture) were 99 ± 15.8, 108 ± 11.5, 77 ± 21.1 and 73 ± 26.2 respectively and were not different within the group but differed between the groups ($p < 0.05$). The total cell numbers of blastocysts from Group 3, cultured until day 7 in SOF only were 69 ± 26.1 and significantly ($p < 0.05$) lower than blastocysts from Group 1, but not different from blastocysts of Goup 2.

Hyaluronan receptors CD44 and RHAMM and synthases Has1, Has2 and Has3 gene expression patterns

Addition of BSA or HA, or both, 96 hpi to SOFaa medium with or without BSA have various effects on the levels of gene expression of HA receptors CD44 and RHAMM and synthases Has1, 2 and 3. Expression of RHAMM receptors was highly dependent on HA addition to media that contained BSA and were the highest ($p < 0.05$) in groups supplemented with BSA from day 1 of culture (24 hpi) and when HA was added at day 4, and lowest ($p < 0.05$) in the SOF-only group, as compared with both BSA-only supplemented groups that were not different. However, there was no effect of any treatment on the expression of CD44 genes except in the SOF-only group culture from days 1 to 9, without either BSA or HA (Fig. 1).

Out of the three Has isoforms, only Has2 gene expression was increased ($p < 0.05$) in both BSA-only supplemented groups, but not in the

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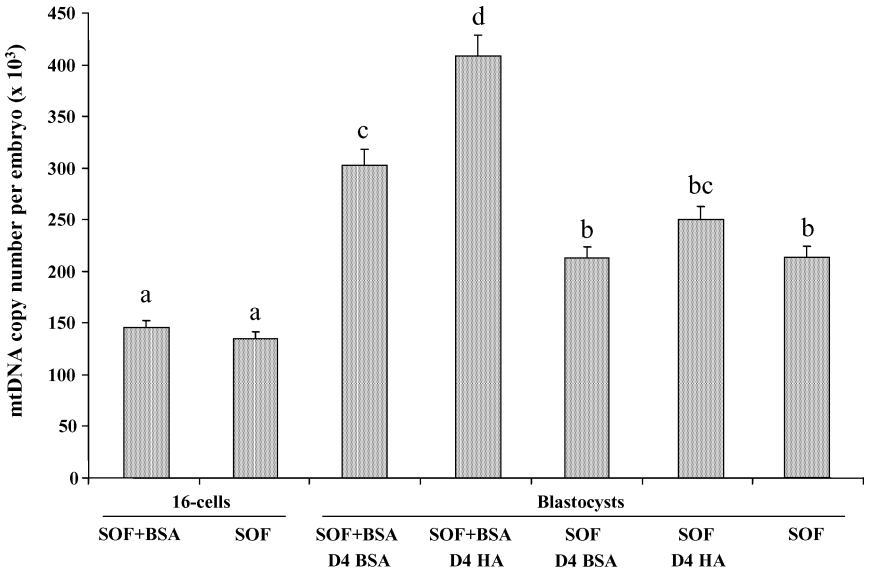

Figure 2 Mitochondrial copy number of bovine 16-cell and blastocysts embryo culture in SOF medium.

remaining three groups in which expression did not differ.

Concentration of mitochondrial DNA

Mitochondrial DNA concentration was correlated to BSA presence in the culture medium from day 1 of embryo culture; addition of HA at day 4 to medium containing BSA further increased ($p < 0.05$) mtDNA copy number. However, addition of HA or BSA at day 4 to embryos that were culture in SOF-only medium had reduced ($p < 0.05$) expression of mtDNA copy number as compared with embryos cultured in SOF supplemented with BSA from day 1 (Fig. 2).

Discussion

The results of the present study demonstrate for the first time that preimplantation bovine embryos predominantly synthesise and utilize high- not low-molecular weight hyaluronan and are dependent on the composition of the culture medium.

Expression levels of all hyaluronan synthases Has1, 2 and 3 and corresponding HA receptors CD44 and RHAMM in day-8 blastocysts appear to be positive indicators and important prerequisites for the developing embryo. Of the three Has isoforms, only Has2 was expressed at an elevated level as compared with Has1 and 3; Has2 increased in both BSA-only groups but not in HA groups regardless of the time of BSA and/or HA addition. In contrast, the remaining synthases Has1 and 3 were at lower levels and were not affected by any changes in culture conditions. The lower expression levels of Has1 and Has3 syntheses in this study is most probably related to different catabolic activities of each HA synthase that differ at different stages of cell differentiation and development. The existence of three Has isoforms with distinctive characteristics suggests the broad variability of biological and physiological functions of each isoform are controlled in a different fashion (Itano & Kimata, 2002). Analysis of hyaluronan MW synthesized separately by each Has protein *in vitro* indicated that hyaluronan MW is determined by the specific Has protein. Has2 is the most expressed HA synthase throughout embryonic development in the mouse (McDonald & Camenisch, 2003). It is significantly more active than Has1 and synthesizes high-molecular-weight HA (up to 2×10^6 Da; Camenisch *et al.*, 2000). Thus, the high expression of Has2 in our study indicates greater production of high-molecular-weight HA that could have significant effects on cell movement, differentiation (Scott & Heatley, 2002) and volume (development of blastocyst cavities). Mice deficient in Has2 activity have been found to have severe developmental defects, whereas mice deficient in Has1 or Has3 were viable (Camenisch *et al.*, 2000). These findings may suggest that either Has1 or Has3 is not required at early stages of embryonic development synthesis. However, Has3 is the most catabolically active of the three Has synthases, but is expressed late in embryonic development and synthesizes low-molecular-weight hyaluronan. Another possible cause that affects HA synthase activity is the alteration of molecular composition of the embryo membranes during *in vitro* culture, because HA is the only glycosaminoglycan that is synthesized

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at the inner layer of the cell membrane (Prehm, 1984). Any change in the phospholipid bilayer, which in *in vitro* conditions spontaneously loses phospholipids (Douard *et al.*, 2000), may disrupt the HA synthesis process and can be reversed and missing lipids taken up from the culture medium if they are available. This scenario has been demonstrated with sperm cells (Spector & Yorek, 1985; Graham & Foot, 1987; He *et al.*, 2001) but not with oocytes or embryos.

Expression of the CD44 transmembrane receptor was not affected by any treatment but was significantly lower in blastocysts developed in SOF-only medium. Despite being widely distributed in the intracellular matrix, lack of expression of this receptor in CD44-null mice showed no obvious deleterious morphological defects (Schmits *et al.*, 1997). However, addition of HA at day 4 of culture dramatically increased expression of the RHAMM receptors and this effect was further magnified in presence of BSA. Such an outcome may partially explain the importance of exogenous HA and BSA in bovine embryo development that was previously demonstrated (Lane *et al.*, 2003). Specifically, addition of HA and BSA significantly improved the fine structure of embryo (Palasz *et al.*, 2006) and is most probably one of the reasons for enhanced embryo cryo-survival (Lane *et al.*, 2003; Palasz *et al.*, 2007). RHAMM intracellular receptors may play a part in embryo fine structure enhancement. It has been shown that RHAMM interacts with microtubules and actin filaments (Assmann *et al.*, 1999), which may have further consequences regarding cytoskeleton organization. Increased expression of RHAMM proteins by HA in our study may indicate a more effective use of many of the growth factors (e.g. EGF, TGF-2α; Tzanakakis *et al.*, 1995; Tirone *et al.*, 1997; Syrokou *et al.*, 1999) and cytokines that have been shown to stimulates oocytes and embryo development *in vitro* (Salustri, 1992) and have HA affinity (Ruoslahti & Yamaguchi, 1991). Increased synthesis of RHAMM receptors also means that, when needed, they bind to and immobilize large amounts of HA in specific locations.

We were not able to demonstrate that embryo culture in medium without surface active components, such as BSA, leads to more HA synthesis. Exclusion of exogenous protein from SOF containing essential and non essential amino acids, glutamine, sodium lactate and pyruvate did not affect cleavage and embryo development up to the $\leq$8-cell stage, but considerably reduced blastocysts development when compared with medium containing BSA. However, addition of BSA at day 4 of culture produced similar development and hatching rates to embryos cultured from day 1 with BSA supplement. Further improvement of embryo quality/hatching rates, but not blastocysts number, was observed when HA was added at day 4 of culture ($\leq$8 cells stage) to media containing BSA. The positive effect of HA and BSA confirmed previously published reports (Lane *et al.*, 2003; Stojkovic *et al.*, 2003; Palasz *et al.*, 2006). Normal embryo development in this study in medium without any proteins or surface active compound until day 4 of culture may come as a surprise, however we previously provided evidence that bovine follicular fluid and TCM-199 without protein supplementation showed similar values of surface tension (Palasz *et al.*, 2000). For this reason this may be less of a surprise as TCM-199, as well as SOF culture medium, used in this study contains amino acids that possess some surface-active properties. This inclusion may facilitate the use of other components present in the culture medium and support embryo development until day 4 of culture. Also, the presence of glutamine in our media along with non-essential amino acids seems to be sufficient to support embryo development to $\leq$8 cell stage, which was previously shown with bovine (Steeves & Gardner, 1999) and porcine (Suzuki & Yoshioka, 2006) embryos.

Visible changes in mitochondrial DNA expression were noticed in day-7 blastocysts cultured from day 1 in medium supplemented with BSA and these changes increased further after addition of HA at day 4 of culture. Whereas blastocysts that had been developed in media without BSA until day 4 showed lower and similar levels of mtDNA expression that were not affected by later addition of BSA or HA. This finding seems to be in agreement with the study by Hsien *et al.* (2004) that showed that level of mitochondrial expression is closely related to fertilization/cleavage and, in general, the developmental potential of the embryo. The active transcription of mitochondrial DNA starts in bovines at the 8–16-cell stage (Thompson *et al.*, 2000), but mechanisms of activation are not very well established.

In summary, our data suggest that embryos cultured without BSA and/or HA up to 96 hpi increased gene expression of Has2 synthase and RHAMM receptors, but not Has1 and Has3 and CD44 receptors. Higher expression of the Has2 isoform compared with the Has1 and 3 isoforms indicates that only high-molecular-weight, not low-molecular-weight hyaluronan should be used for preimplantation embryo culture. Addition of both HA and BSA at day 4 increased mtDNA copy number at the blastocyst stage, which may indicate a higher developmental potential of embryos cultured under these conditions.

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References

Bavister, B.D., Leibfried, M.L. & Lieberman, G. (1983). Development of preimplantation embryos of the golden hamster in a defined culture medium. *Biol. Reprod.* **28**, 235–47.

Bavister, B.D., Rose-Hellekant, T.A. & Pinyopummintr, T. (1992). Development of *in vitro* matured/*in vitro* fertilized bovine embryos into morulae and blastocysts in defined culture media. *Theriogenology* **37**, 111–26.

Biggers, J.D. (1987). Pioneering mammalian embryo culture. In *The Mammalian Preimplantation Embryo: Regulation of Growth and Differentiation* In Vitro. (ed. B.D. BAVISTER), pp. 1–22. New York: Plenum Press.

Camenisch, T.D., Spicer, A.P., Brehm-Gibson, T., Biesterfeldt, J., Augustine, M.L., Calabro, A. Jr., Kubalak, S., Klewer, S.E. & McDonald, JA. (2000). Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J. Clin.* Invest. **106**, 349–60.

Campbell, S., Swann, H.R., Aplin, J.D., Seif, M.W., Kimber, S.J. & Elstein, M. (1995). CD44 is expressed throughout pre-implantation human embryo development. *Hum. Reprod.* **10**, 425–30.

Douard, V., Hermier, D. & Blesbois, E. (2000). Changes in turkey semen lipids during liquid *in vitro* storage. *Biol. Reprod*. **63**, 1450–6.

Fenderson, B.A., Stamenkovic, I. & Aruffo, A. (1993). Localization of hyaluronan in mouse embryo during implantation, gastrulation and organogenesis. *Differentiation* **54**, 85–98.

Furnus, C.C., Valcarcel, A., Dulout, F.N. & Errecalde, A.L. (2003). The hyaluronic acid receptor (CD44) is expressed in bovine oocytes and early stage embryos. *Theriogenology* **60**, 1633–44.

Gardner, D.K., Rodriguez-Martinez, H. & Lane, M. (1999). Fetal development after transfer is increased by replacing protein with the glycosaminoglycan hyaluronan for mouse embryo culture and transfer. *Hum. Reprod.* **14**, 2575–80.

Gotoh, S., Onaya, J., Abe, M., Miyazaki, K., Hamai, A., Horie, K. & Tokuyasu, K. (1993). Effects of the molecular weight of hyaluronic acid and its action mechanisms on experimental joint pain in rats. *Ann. Rheum. Dis.* **52**, 817–22.

Graham, J.K. & Foote, R.H. (1987). Effect of several lipids, fatty acyl chain length, and degree of unsaturation on the motility of bull spermatozoa after cold shock and freezing. *Cryobiology* **24**, 42–52.

He, L., Bailey, J.L. & Buhr, M.M. (2001). Incorporating lipids into boar sperm decreases chilling sensitivity but not capacitation potential. *Biol. Reprod.* **64**, 69–79.

Hsieh, R.H., Au, H.K., Yeh, T.S., Chang, S.J., Cheng, Y.F. & Tzeng, C.R. (2004). Decreased expression of mitochondrial genes in human unfertilized oocytes and arrested embryos. *Fertil. Steril.* **81**, 912–8.

Itano, N. & Kimata, K. (2002). Mammalian hyaluronan synthases. *Life* **54**, 195–9.

Itano, N., Sawai, T., Yoshida, M., Lenas, P., Yamada, Y., Imagawa, M., Shinomura, T., Hamaguchi, M., Yoshida, Y., Ohnuki, Y., Miyauchi, S., Spicer, A.P., McDonald, J.A. & Kimata, K. (1999). Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. *J. Biol. Chem.* **274**, 25085–92.

Itano, N., Sawai, T., Atsumi, F., Miyaishi, O., Taniguchi, S., Kannagi, R., Hamaguchi, M. & Kimata, K. (2004). Selective expression and functional characteristics of three.ammalian hyaluronan synthases in oncogenic malignant transformation. *J. Biol. Chem*. **279**, 18679–87.

Keskintepe, L., Burnely, C.A. & Brackett, B.G. (1995). Production of viable bovine blastocysts in defined *in vitro* conditions. *Biol. Reprod*. **52**, 1410–7.

Kimura, N., Konno, Y., Miyoshi, K., Matsumoto, H. & Sato, E. (2002). Expression of hyaluronan synthases and CD44 messenger RNAs in porcine cumulus–oocyte complexes during *in vitro* maturation. *Biol. Reprod*. **66**, 707–17.

Kraemer, P.M., Barnhart, B.J. (1978). Elevated cell-surface hyaluronate in substrate-attached cells. *Exp. Cell Res.* **114**, 153–7.

Lane, M., Maybach, J.M., Hooper, K., Hasler, J.F. & Gardner, D.K. (2003). Cryo-survival and development of bovine blastocysts are enhanced by culture with recombinant albumin and hyaluronan *Mol. Reprod. Dev.* **64**, 70–8.

Laurent, T.C. (ed.) (1998). *The Chemistry, Biology and Medical Applications of Hyaluronan and its Derivatives*. Wenner-Gren International Series vol. 72. London: Portland Press.

Marchal, R., Caillaud, M., Martoriati, A., Gerard, N., Mermillod, P. & Goudet, G. (2003). Effect of growth hormone (GH) on *in vitro* nuclear and cytoplasmic oocyte maturation, cumulus expansion, hyaluronan synthases, and connexins 32 and 43 expression, and GH receptor messenger RNA expression in equine and porcine species. *Biol. Reprod*. **69**, 1013–22.

McDonald, J.A. & Camenish, T.D. (2003). Hyaluronan: genetic insights into the complex biology of a simple polysaccharide. *Glycoconjugate J*. **19**, 331–9.

Palasz, A.T., Thundathil, J., Verrall, R.E. & Mapletoft, R.J. (2000). The effect of macromolecular supplementation on the surface tension of TCM-199 and the utilization of growth factors by bovine oocytes and embryos in culture. *Anim. Reprod. Sci.* **58**, 229–40.

Palasz, A.T., Rodriguez-Martinez, H., Beltran-Breña, P., Perez-Garnelo, S., Martinez, M.F., Gutierrez-Adan, A. & De la Fuente, J. (2007). The effect of hyaluronan, BSA and serum on bovine embryo *in vitro* development, ultrastructure and gene expression patterns. *Mol. Reprod. Dev*. **73**, 1503–11.

Palasz, A.T., Beltrán-Breña, P., Martinez, M.F., Perez-Garnelo, S.S., Ramirez, M.A., Gutiérrez-Adán, A. & De la Fuente, J. (2008). Development, molecular composition and freeze tolerance of bovine embryos cultured in TCM-199 supplemented with hyaluronan. *Zygote* **16**, 39–47.

Prehm, P. (1984). Hyaluronate is synthesized at plasma membranes. *Bioch. J*. **220**, 597–600.

Presti, D. & Scott, J.E. (1994). Hyaluronan-mediated protective effect against cell damage caused by enzymatically produced hydroxyl (OH.) radicals is dependent on hyaluronan molecular mass. *Cell Biochem. Funct*. **4**, 281–8.

Ruoslahti, E. & Yamaguchi, Y. (1991). Proteoglycans as modulators of growth factor activities. *Cell* **64**, 867–9.

Salustri, A., Yanagishita, M., Underhill, C.B., Laurent, T.C. & Hascall, V.C. (1992). Localization and synthesis of

hyaluronic acid in the cumulus cells and mural granulosa cells of the preovulatory follicle. *Dev. Biol.* **151**, 541–51.

Schmits, R., Filmus, J., Gerwin, N., Senaldi, G., Kiefer, F., Kundig, T., Wakeham, A., Shahinian, A., Catzavelos, C., Rak, J., Furlonger, C., Zakarian, A., Simard, J.J., Ohashi, P.S., Paige, C.J., Gutierrez-Ramos, J.C. & Mak, T.W. (1997). CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. *Blood* **90**, 2217–33.

Schoenfelder, M. & Einspanier, R. (2003). Expression of hyaluronan synthases and corresponding hyaluronan receptors is differentially regulated during oocyte maturation in cattle. *Biol. Reprod.* **69**, 269–77.

Scott, J.E. & Heatley, F. (2002). Biological properties of hyaluronan in aqueous solution are controlled and sequestered by reversible tertiary structures, defined by NMR spectroscopy. *Biomacromolecules* **3**, 547–53.

Spector, A.A. & York, M.A. (1985). Membrane lipid composition and cellular function. *J. Lipids Res.* **26**, 1015–35.

Spicer, A.P. & McDonald, J.A. (1998). Characterization and molecular evolution of a vertebrate hyaluronan synthase gene family. *J. Biol. Chem.* **273**, 1923–32.

Spicer, A.P. & Nguyen, T.K. (1999). Mammalian hyaluronan synthases: investigation of functional relationships *in vivo*. *Biochem. Soc. Trans*. **27**, 109–15.

Steeves, T.E. & Gardner, D.K. (1999). Temporal and differential effects of amino acids on bovine embryo development in culture. *Biol. Reprod.* **61**, 731–40.

Stojkovic, M., Krebs, O., Kolle, S., Prelle, K., Assmann, V., Zakhartchenko, V., Sinowatz, F. & Wolf, E. (2003). Developmental regulation of hyaluronan-binding protein (RHAMM/IHABP) expression in early embryos. *Biol. Reprod.* **68**, 60–6.

Suzuki, C. & Yoshioka, K. (2006). Effects of amino acid supplements and replacement of polyvinyl alcohol with bovine serum albumin in porcine zygote medium. *Reprod. Fertil. Dev.* **18**, 789–95.

Syrokou, A., Tzanakakis, G.N., Hjerpe, A. & Karamanos, N.K. (1999). Proteoglycans in human malignant mesothelioma. Stimulation of their synthesis induced by epidermal, insulin and platelet-derived growth factors involves receptors with tyrosine kinase activity. *Biochimie* **81**, 733–44.

Thompson, J.G., McNaughton, C., Gasparrini, B., McGowan, L.T. & Tervit, H.R. (2000). Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured *in vitro*. *J. Reprod. Fertil*. **118**, 47–55.

Tien, J.Y. & Spicer, A.P. (2005). Three vertebrate hyaluronan synthases are expressed during mouse development in distinct spatial and temporal patterns. *Dev. Dyn.* **233**, 130–41.

Tirone, E., D'Alessandris, C., Hascall, V.C., Siracus, A.G. & Salustri, A. (1997). Hyaluronan synthesis by mouse cumulus cells is regulated by interactions between follicle-stimulating hormone (or epidermal growth factor) and a soluble oocyte factor (or transforming growth factor beta1). *J. Biol. Chem.* **272**, 4787–94.

Toole, B.P. (1997). Hyaluronan in morphogenesis. *J. Intern. Med.* **242**, 35–40.

Tzanakakis, G.N., Karamanos, N.K. & Hjerpe, A. (1993). Effects on glycosaminoglycan synthesis in cultured human mesothelioma cells of transforming, epidermal, and fibroblast growth factors and their combinations with platelet-derived growth factor. *Exp. Cell Res.* **220**, 130–7.

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

Weigel, P.H., Hascall, V.C. & Tammi, M. (1997). Hyaluronan synthases. *J. Biol. Chem.* **272**, 13997–4000.