

Effect of addition of hyaluronan to embryo culture medium on survival of bovine embryos in vitro following vitrification and establishment of pregnancy after transfer to recipients

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Abstract

Two experiments were conducted to determine whether addition of hyaluronan to culture medium could improve survival of bovine embryos after vitrification or following embryo transfer. In Experiment 1, embryos were produced in vitro and cultured for 7 days in modified synthetic oviductal fluid (SOF) containing one of four concentrations of hyaluronan (0, 0.1, 0.5, or 1 mg/mL), with or without 4 mg/mL of bovine serum albumin (BSA). On Day 7 after insemination, blastocysts and expanded blastocysts were vitrified using open-pulled straws. At a concentration of 1 mg/mL, hyaluronan increased ($P < 0.05$) the percentage of oocytes that were blastocysts and re-expansion rate at 24 h after warming. At 0.5 mg/mL, hyaluronan tended ($P < 0.10$) to increase re-expansion rate at 48 h after warming and increased ($P < 0.05$) embryo hatching rate at 24 and 72 h. Treatment with BSA caused a slight reduction in cleavage rate ($P < 0.05$), but only for cultures containing hyaluronan (BSA \times hyaluronan, $P = 0.10$), an increase in the percentage of oocytes that became blastocysts ($P < 0.001$), and a reduction in re-expansion rates ($P < 0.001$) and hatching rates ($P < 0.05$ or $P < 0.01$) at all times examined. In Experiment 2, embryos were produced in vitro and cultured in modified SOF containing 4 mg/mL BSA, with or without 1 mg/mL hyaluronan. At 159–162 h after insemination, grade 1 morula, blastocysts and expanded blastocysts were harvested for embryo transfer. Harvested embryos were transferred individually to lactating Holstein recipients with a palpable corpus luteum on Day 7 after presumptive ovulation. There was an interaction ($P < 0.05$) between hyaluronan and embryo stage on pregnancy rate. Recipients that received morula and blastocyst stage embryos treated with hyaluronan had a higher pregnancy rate than recipients that received control embryos of the same stage. There was no effect of hyaluronan on pregnancy rates of recipients that received expanded blastocysts. In conclusion, addition of hyaluronan to embryo culture enhanced blastocyst yield, improved survival following vitrification, and enhanced the post-transfer survival of fresh morula and blastocyst stage embryos.

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1. Introduction

In vitro embryo production has great potential for enhancing genetic selection, improving fertility, and optimizing crossbreeding schemes in beef and dairy cattle production systems [1,2]. Despite this potential, embryos produced in vitro represent only 30% of the total

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number of embryos transferred worldwide [3]. One reason for the limited commercial application of in vitro embryo transfer is that embryos produced in vitro are associated with altered ultrastructural and physiological features compared to embryos produced by superovulation [4–7]. Moreover, such alterations can affect post-culture viability. In particular, bovine embryos produced in vitro are more sensitive to cryopreservation [8–10], have reduced embryo and fetal survival following transfer [11,12], and result in an increased number of fetuses and calves with abnormalities [12–14].

Recent research using the sheep oviduct as a model for in vivo embryo development has highlighted the sub-optimal nature of embryo culture and the consequences of such an environment for post-culture viability [7–9,15]. Thus, a potential strategy for improving in vitro produced embryos is to modify culture conditions to more closely mimic the microenvironment found in vivo, through addition of growth factors, hormones and other regulatory molecules. Such molecules include the growth factor, insulin-like growth factor-1 (IGF-1), and the cytokine, granulocyte macrophage-colony stimulating factor, which have been reported to improve the post-transfer survival of embryos produced in vitro [16–18].

The glycoaminoglycan hyaluronan is another molecule that has the potential to improve post-culture viability of embryos produced in vitro. Hyaluronan plays an important role in several aspects of cell function, including cell proliferation, cell migration, cell adhesion and intracellular signaling [19,20]. Hyaluronan is present in the oviductal and uterine fluids of cattle [21] and receptors for hyaluronan are expressed on the preimplantation bovine embryo [22,23]. Addition of hyaluronan to bovine embryo culture has been reported to improve development to the blastocyst stage [24,25] and increase re-expansion and hatching rates following cryopreservation [26–28]. Hyaluronan can alter the abundance of several developmentally-important gene transcripts in bovine embryos [28,29] and promote the differentiation of extra-embryonic structures in mouse embryos [30].

Culture of mouse embryos in the presence of hyaluronan increased implantation rates and fetal development following embryo transfer [31]. In addition, treatment of ovine embryos with 6 mg/mL hyaluronan during culture increased pregnancy and lambing rates following the transfer of vitrified/warmed in vitro produced blastocysts [32]. The aim of the present study was to determine whether addition of hyaluronan to bovine embryo culture could improve survival following vitrification and increase pregnancy rates following embryo transfer.

2. Materials and methods

2.1. Materials

All materials were purchased from Sigma (St. Louis, MO, USA) or Fisher Scientific (Fairlawn, NJ, USA) unless specified otherwise. Hepes-Tyrode's Lactate and IVF-Tyrode's Lactate were purchased from Caisson Laboratories Inc. (Logan, UT, USA). These media were used to prepare Hepes-Tyrode's albumin lactate pyruvate (TALP) and IVF-TALP, as described previously [33]. Oocyte collection medium (OCM) consisted of Tissue Culture Medium-199 (TCM-199) with Hank's salts without phenol red (Atlanta Biologicals, Norcross, GA, USA) and supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR, USA), 2 U/mL heparin, 100 U/mL penicillin-G, 0.1 mg/mL streptomycin, and 1 mM glutamine. Oocyte maturation medium (OMM) was prepared as TCM-199 (Invitrogen, Carlsbad, CA, USA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 µg/mL estradiol 17-β, 20 µg/mL bovine FSH (Folltropin-V; Bioniche, Belleville, ON, Canada), 22 µg/mL sodium pyruvate, 50 µg/mL gentamicin sulfate, and 1 mM glutamine. Percoll[®] was from Amersham Pharmacia Biotech (Uppsala, Sweden). Recombinant human insulin-like growth factor-1 was purchased from Upstate Biotech (Lake Placid, NY, USA). A modified synthetic oviductal fluid (mSOF) was purchased from Millipore (Billerica, MA, USA). The formula was as described by Takahashi and First [34], except that phenol red was omitted and bovine serum albumin (BSA) was modified for the dictates of each specific experiment. The mSOF was further modified prior to use to contain 1.0 mM alanyl-L-glutamine, 5.3 mM sodium lactate, 0.5 mM tri-sodium citrate, 2.77 mM myo-inositol, 0.5 mM fructose, essential amino acids (basal medium Eagle), non-essential amino acids (minimum essential medium) and culture treatments as described below. Hyaluronan (1100 kDa from *Streptococcus pyrogenes*) was purchased from R&D Systems (Minneapolis, MN, USA), Syngro[®] holding medium was from Bioniche, and lidocaine was from Pro Labs (St. Joseph, MO, USA).

2.2. Experiment 1: Effect of bovine serum albumin and hyaluronan during culture on embryo development and survival following vitrification

2.2.1. Embryo production

All procedures related to in vitro embryo production were as described previously [35] unless noted otherwise. Immature cumulus oocyte complexes (COC) were

collected from ovaries (predominantly beef) obtained from Central Packing Co. (Center Hill, FL, USA). Harvested COCs were then allowed to mature for 21–24 h in a humidified atmosphere of 5% CO₂ in air. Following maturation, COC were washed once in Hepes-TALP and then co-incubated with a pool of sperm from three different bulls. To eliminate bull effects, a different combination of three bulls was used for each replicate. Spermatozoa and COC were allowed to co-incubate for 8 h. Following fertilization, presumptive zygotes were randomly assigned to one of eight different culture treatments. The experimental design was a 2 × 4 factorial design, with mSOF containing one of four concentrations of hyaluronan (0, 0.1, 0.5, and 1.0 mg/mL) with or without 4 mg/mL essentially fatty acid free bovine serum albumin (EFAF-BSA; Sigma, St. Louis, MO, USA). Presumptive zygotes were cultured in their respective treatment in either 25 µL microdrops in groups of 10–15, or 50 µL microdrops in groups of 25–30, depending on the total number of oocytes collected for each replicate. Within each replicate, the same drop size was used for all treatment groups. Culture drops were overlaid with mineral oil and placed at 38.5 °C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂ for 7 days. The proportion of oocytes that cleaved was recorded on Day 3 after insemination. The proportion of oocytes that developed to either the blastocyst, expanded blastocyst, hatching blastocyst or hatched blastocyst stages was recorded on Day 7 after insemination and defined as total blastocyst development. In addition, the proportion of oocytes that developed to either the expanded blastocyst, hatching blastocyst or hatched blastocyst stages was recorded on Day 7 after insemination and defined as advanced blastocyst development. The experiment was replicated 16 times, and embryos were vitrified for 13 of 16 replicates.

2.2.2. Vitrification and post-warming survival

At Day 7 after insemination, all blastocysts and expanded blastocysts were harvested from culture and washed three times in Syngro[®] holding medium which was modified to contain an additional 0.1% (w/v) polyvinyl alcohol (mSHM). Embryos were then vitrified and warmed as described previously [36] with minor modifications. For vitrification, embryos were first placed into a solution consisting of 5 M ethylene glycol in mSHM for 3 min. Then, embryos were placed into a 15 µL drop that consisted of 7 M ethylene glycol, 18% (w/v) Ficoll[®] 70 and 0.5 M galactose in mSHM for 45 s. Immediately after placing the embryos into the second vitrification solution, embryos were loaded into 0.25 mL straws surrounded on both sides by columns of

dilution solution (0.5 M galactose in mSHM). Once 45 s had elapsed, straws were placed vertically into liquid nitrogen vapor for 1 min and then plunged into liquid nitrogen. For warming, straws were held in air for 8 s, and then placed in a water bath at 35–37 °C for 15 s. Straws were then removed from the water bath and shaken four times to mix the columns. The straws were then held at room temperature for 4 min; thereafter, embryos were expelled and washed three times in mSHM prior to being placed into culture. Embryos were cultured in 25 µL microdrops of mSOF + 10% fetal bovine serum and 50 µM dithiothreitol overlaid with mineral oil for 72 h in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 38.5 °C. Embryos were vitrified and warmed in groups containing similar numbers of embryos per treatment for each replicate. Re-expansion and hatching rates (including both hatching and hatched embryos) were recorded at 24, 48, and 72 h post-warming.

2.3. Experiment 2: Effect of hyaluronan during culture on embryo survival following transfer

2.3.1. Animals

The experiment was conducted between July and September 2007 at three dairy farms located near Mayo, FL (30.052N, 83.175W). Primiparous and multiparous, lactating Holstein cows between 68 and 944 days in milk (mean = 230.3) were used at each farm. At one farm, cows were housed in free-stall barns equipped with fans and sprinklers. At the other two farms, cows were maintained on pasture but had access to a cooling facility. At all three locations, cows were fed a total mixed ration and milked two times per day. None of the recipients were administered bovine somatotropin.

2.3.2. Embryo production

Holstein cumulus oocyte complexes were purchased from Evergen Biotechnologies (Storrs, CT, USA). Following collection, COCs were placed into 2 mL cryovials (approximately 100 COCs/cryovial) containing maturation medium and shipped overnight in a portable incubator at 39 °C to the laboratory in Gainesville, FL, USA. In vitro fertilization and embryo culture were conducted as described previously [35]. Following 21–24 h of maturation, COCs were washed once in Hepes-TALP and then co-incubated with semen. Three Holstein bulls (Accelerated Genetics, Baraboo, WI, USA) were used to produce embryos; a single bull was used for an individual fertilization drop. Semen from two bulls was treated to skew the sex ratio towards females using the Bovatel[™] technology. Spermatozoa

and COC's were co-incubated for 8 h. Following fertilization, presumptive zygotes were randomly assigned to one of two culture media: mSOF + 4 mg/mL EFAF-BSA and 100 ng/mL IGF-1; or mSOF + 4 mg/mL EFAF-BSA, 100 ng/mL IGF-1, and 1 mg/mL hyaluronan. Presumptive zygotes were cultured in 50 μ L microdrops overlaid with mineral oil in groups of 25–30 in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 38.5 °C. Cleavage rate was recorded on Day 3 after insemination. The proportion of oocytes that developed to the morula stage was recorded at 159–162 h after insemination. In addition, the proportion of oocytes that developed to the blastocyst, expanded blastocyst, hatching blastocyst or hatched blastocyst stages was recorded at 159–162 h after insemination and defined as total blastocyst development.

2.3.3. Estrous synchronization and embryo transfer

Cows were synchronized for timed embryo transfer using the standard OvSynch protocol [37]. Specifically, on Day –10, cows received 100 μ g gonadotropin releasing hormone (GnRH), im, followed 7 days later by 25 mg prostaglandin F_{2 α} im on Day –3. A second injection of GnRH was given on Day –1 (48 h after prostaglandin F_{2 α} treatment). Day 0 was defined as the presumptive day of ovulation. On Day 7 after presumptive ovulation, the presence or absence of a corpus luteum was diagnosed either by transrectal palpation or by ultrasonography with an Aloka 500 ultrasound scanner equipped with a 5 MHz linear-array transducer (Aloka, Tokyo, Japan). All cows that were

diagnosed as having a corpus luteum ($n = 310$) received an epidural block (5 mL of 2% lidocaine, w/v) and a single embryo was transferred to the uterine horn ipsilateral to the ovary with the corpus luteum. Pregnancy was diagnosed by transrectal palpation at 35–90 days of gestation.

Embryos used for embryo transfer were grade 1 morula, blastocyst, and expanded blastocyst stage embryos [38], harvested at 159–162 h post-insemination. Embryos were loaded into 0.25 mL French straws in holding medium (Hepes-TALP containing 10% fetal bovine serum and 100 μ M β -mercaptoethanol) and straws containing selected embryos were then placed horizontally into a portable incubator (Minitube, Verona, WI, USA) at 39 °C and transported to the respective farm. Straws containing embryos were loaded into a 53 cm transfer pipette (IMV Technologies, L'Aigle, France) and transferred to recipients. Each cow was assigned an embryo randomly. Of the harvested embryos that were transferred, 80 were morula, 71 were blastocysts, and 159 were expanded blastocysts.

2.4. Statistical analysis

Percentage data were subjected to arcsin transformation prior to analysis. For Experiment 1, the proportion of oocytes that cleaved on Day 3 after insemination, the proportion of oocytes that became a blastocyst of any category (percent total blastocysts), and the proportion of oocytes that became an advanced blastocyst (expanded, hatching and hatched blastocysts) on Day

Table 1

Effect of hyaluronan and bovine serum albumin (BSA) on development of bovine embryos (Experiment 1)^a.

Hyaluronan (mg/mL)	BSA	No. oocytes	Percent cleaved (Day 3)	Percent total blastocysts (Day 7)	Percent advanced blastocysts (Day 7) ^b
0	–	467	76.8 \pm 2.0	14.2 \pm 2.2	10.1 \pm 1.9
0.1	–	538	76.6 \pm 2.0	12.6 \pm 2.2	7.5 \pm 1.9
0.5	–	559	79.6 \pm 1.8	17.4 \pm 2.0	12.3 \pm 1.7
1.0	–	594	80.3 \pm 1.8	16.9 \pm 2.0	13.0 \pm 1.7
0	+	559	77.3 \pm 1.9	17.9 \pm 2.1	13.2 \pm 1.8
0.1	+	573	73.1 \pm 2.0	21.6 \pm 2.2	14.8 \pm 1.9
0.5	+	599	73.4 \pm 1.9	19.2 \pm 2.1	14.7 \pm 1.8
1.0	+	583	75.9 \pm 2.1	25.5 \pm 2.3	17.9 \pm 2.0
Statistical analysis					
Hyaluronan, main effect			NS	NS	NS
0 vs 0.1			NS	NS	NS
0 vs 0.5			NS	NS	NS
0 vs 1.0			NS	<0.05	<0.05
BSA			<0.01	<0.001	<0.01
Hyaluronan \times BSA			=0.10	NS	NS

^a Data represent least-squares means \pm SEM of results from 16 replicates.

^b Advanced blastocysts were expanded, hatching or hatched.

Table 2
Effect of hyaluronan and bovine serum albumin (BSA) on survival of bovine embryos after vitrification (Experiment 1)^a.

Hyaluronan (mg/mL)	BSA	No. embryos	Percent re-expansion			Percent hatching ^b		
			24 h	48 h	72 h	24 h	48 h	72 h
0	–	50	22.2 ± 7.4	33.7 ± 7.7	37.5 ± 7.8	0.8 ± 2.9	7.2 ± 4.3	10.0 ± 6.2
0.1	–	57	43.8 ± 7.8	46.7 ± 8.1	49.1 ± 8.2	7.7 ± 3.0	11.3 ± 4.5	20.4 ± 6.5
0.5	–	70	38.9 ± 7.0	50.0 ± 7.3	45.9 ± 7.5	8.4 ± 2.7	25.5 ± 4.1	36.1 ± 5.9
1.0	–	84	51.8 ± 6.7	51.9 ± 7.0	48.2 ± 7.1	7.6 ± 2.6	12.4 ± 3.9	21.6 ± 5.6
0	+	79	14.9 ± 7.0	14.6 ± 7.3	13.9 ± 7.4	1.8 ± 2.7	6.2 ± 4.1	9.1 ± 5.9
0.1	+	101	16.5 ± 7.4	18.8 ± 7.7	28.9 ± 7.8	3.6 ± 2.9	5.6 ± 4.3	12.3 ± 6.1
0.5	+	98	21.6 ± 7.0	23.1 ± 7.3	21.7 ± 7.4	1.7 ± 2.7	8.4 ± 4.1	10.8 ± 5.9
1.0	+	122	14.9 ± 7.4	18.1 ± 7.7	13.3 ± 7.8	0.2 ± 2.9	2.2 ± 4.3	7.0 ± 6.2
Statistical analysis								
Hyaluronan, main effect			<0.05	NS	NS	NS	NS	<0.09
0 vs 0.1			NS	NS	NS	NS	NS	NS
0 vs 0.5			NS	<0.10	NS	NS	<0.05	<0.05
0 vs 1.0			<0.05	NS	NS	NS	NS	NS
BSA			<0.001	<0.001	<0.001	<0.05	<0.01	<0.01
Hyaluronan × BSA			NS	NS	NS	NS	NS	NS

^a Least-squares means ± SEM of results from 13 replicates.

^b Includes embryos that were hatching or hatched.

7 after insemination were recorded for each replicate ($n = 16$). Treatment effects on these variables (Table 1) were analyzed by least-squares analysis of variance, using the General Linear Models procedure of SAS (SAS for Windows, Version 9.0, SAS Institute Inc., Cary, NC, USA). The model included the main effects of replicate, BSA, hyaluronan and their interactions. Contrasts were used to analyze differences between each of the three concentrations of hyaluronan and the control. For vitrification data (Table 2), the proportion of vitrified embryos that re-expanded and underwent hatching at 24, 48, and 72 h post-warming was calculated for each replicate and analyzed by analysis of variance using the General Linear Models procedure of SAS. The model included the main effects of replicate, BSA, hyaluronan, and their interactions.

For Experiment 2, the proportion of oocytes that cleaved on Day 3 after insemination and the proportion of morula and total blastocysts at 159–162 h post-insemination were calculated for each replicate ($n = 13$). The model included the main effects of replicate and treatment. All values reported are least-squares mean ± SEM of untransformed data. Pregnancy rate data in Experiment 2 were analyzed by logistic regression using the LOGISTIC procedure of SAS to analyze the proportion of recipients that were pregnant at Days 35–90 of gestation. The model included the main effects of replicate, treatment and embryo stage as well as their interactions.

For all analyses, all treatment effects that were associated with a probability of 0.10 or less are reported.

3. Results

3.1. Experiment 1: Effect of bovine serum albumin and hyaluronan during culture on embryo development and survival following vitrification

Overall, there was no main effect of hyaluronan treatment on the proportion of oocytes that cleaved or on the proportion of total blastocysts or advanced blastocysts on Day 7 after insemination (Table 1). However, addition of 1 mg/mL hyaluronan increased ($P < 0.05$) the proportion of oocytes that became blastocysts on Day 7 and the proportion of oocytes that became advanced blastocysts on Day 7 when compared to controls without hyaluronan (Table 1). There was no effect of hyaluronan at 0.1 or 0.5 mg/mL on cleavage rate or embryo development when compared to controls cultured without hyaluronan. Addition of BSA to embryo culture reduced slightly the proportion of oocytes that cleaved ($P < 0.01$). There was a tendency for an interaction with hyaluronan ($P = 0.10$) because the negative effect of BSA occurred only for cultures containing hyaluronan. Despite the slight negative effect on cleavage, BSA increased the yield of total blastocysts ($P < 0.001$) and advanced blastocysts ($P < 0.01$; Table 1). There was no interaction between hyaluronan and BSA on embryo development.

Addition of hyaluronan during embryo culture affected re-expansion and hatching rate after thawing, but in a dose-dependent manner (Table 2). At a concentration of 1 mg/mL, hyaluronan increased ($P < 0.05$)

the re-expansion rate at 24 h after warming but had no significant effect on other measures of survival to vitrification. At 0.5 mg/mL, hyaluronan tended ($P < 0.10$) to increase re-expansion rate at 48 h after warming and increased ($P < 0.05$) embryo hatching rate at 48 and 72 h. Culture of embryos in the presence of BSA reduced ($P < 0.001$) post-warming re-expansion rate at 24, 48, and 72 h. Furthermore, addition of BSA to embryo culture reduced embryo hatching rate at 24 h ($P < 0.05$), 48 h ($P < 0.01$), and 72 h post-warming ($P < 0.01$; Table 2). There was no interaction between hyaluronan and BSA on the post-warming re-expansion or hatching rate.

3.2. Experiment 2: Effect of hyaluronan during culture on embryo survival following transfer

Addition of 1 mg/mL hyaluronan to embryo culture had no effect on cleavage rate at Day 3 after insemination ($64.2 \pm 1.1\%$ vs $62.0 \pm 1.1\%$; control vs hyaluronan). There was also no effect of hyaluronan on the proportion of oocytes that became morula and total blastocysts ($15.6 \pm 1.1\%$ vs $14.5 \pm 1.1\%$) or total blastocysts ($10.0 \pm 1.2\%$ vs $9.2 \pm 1.2\%$) alone at 159–162 h post-insemination.

Pregnancy rates among recipients that received expanded blastocyst stage embryos were higher ($P < 0.05$) than for recipients that received morula and blastocyst stage embryos (Table 3). Overall, pregnancy rates among recipients that received embryos cultured in hyaluronan were not different from those recipients that received control embryos. There was, however, a hyaluronan \times stage interaction ($P < 0.05$). Treatment of embryos with hyaluronan increased pregnancy rates among recipients that received morula and blastocyst stage embryos compared to control embryo recipients that received similar-stage embryos. There was, however, no effect of hyaluronan on pregnancy rates for recipients that received expanded blastocysts (Table 3).

Table 3
Effect of hyaluronan and embryo stage on pregnancy rates following transfer to lactating recipients (Experiment 2)^a.

Treatment	Embryo stage	Pregnancy rate (Days 35–90)
Control	Morula + blastocysts	15/81 = 18.5%
Hyaluronan	Morula + blastocysts	20/70 = 28.6%
Control	Expanded blastocysts	31/81 = 38.3%
Hyaluronan	Expanded blastocysts	23/78 = 29.5%

^a Treatment effect was $P > 0.10$, stage effect was $P < 0.05$ and treatment \times stage effect was $P < 0.05$.

4. Discussion

The commercialization of in vitro embryo transfer into cattle production systems is limited by problems associated with bovine embryos produced in vitro. Specifically, bovine embryos produced in vitro are more sensitive to cryopreservation [8–10] and have reduced embryo and fetal survival following transfer [11,12]. The aim of the present study was to determine whether addition of hyaluronan to bovine embryo culture could improve survival following vitrification and increase pregnancy rates following embryo transfer. Addition of 0.5 mg/mL hyaluronan to bovine embryo culture increased re-expansion and hatching rates following vitrification. Moreover, treatment with 1 mg/mL hyaluronan treatment during embryo culture increased blastocyst yield, re-expansion rate of vitrified embryos at 24 h after warming, and the post-transfer survival of morula and blastocyst stage embryos, while not affecting post-transfer survival of more advanced embryos.

It has been previously reported in mice that culture of embryos in the presence of 0.5 mg/mL hyaluronan increased implantation rates and fetal development following transfer [31]. In addition, treatment of ovine embryos with 6 mg/mL hyaluronan during culture increased pregnancy and lambing rates following the transfer of vitrified/warmed in vitro produced blastocysts [32]. To our knowledge, this is the first study to test whether the addition of hyaluronan to embryo culture would increase the post-transfer viability of bovine embryos produced in vitro. In Experiment 2, addition of 1 mg/mL hyaluronan to embryo culture increased pregnancy rates for recipients that received morula and blastocyst stage embryos. However, there was no effect of hyaluronan treatment for recipients that received expanded blastocysts. In recent work with bovine embryos, hyaluronan altered the abundance of some developmentally-important gene transcripts [28,29]. These or other genes that are differentially regulated by hyaluronan could enhance post-transfer survival. One gene down-regulated by hyaluronan is *Bax* [28,29], an anti-apoptotic gene; perhaps reduced apoptosis could facilitate embryonic survival. *Glut-1* is a gene found to be upregulated by hyaluronan [29] and enhanced capacity for glucose transport might also facilitate survival after transfer. Effects of hyaluronan on gene expression may have a more pronounced effect on the post-transfer viability of morula and blastocyst stage embryos, because such embryos are slower-developing when compared to expanded blastocysts.

In addition to affecting post-transfer viability, hyaluronan treatment also improved survival following

vitrification. In Experiment 1, embryos treated with 0.5 mg/mL hyaluronan were more likely to survive vitrification than embryos not treated with hyaluronan, as indicated by a tendency for improved rate of re-expansion at 48 h after warming and improved rates of hatching at 48 and 72 h after warming. Embryos cultured with 1 mg/mL hyaluronan before vitrification also had increased rates of re-expansion at 24 h after warming, when compared to untreated controls. These results were consistent with previous reports in which addition of hyaluronan during embryo culture at low concentrations (0.125–1.0 mg/mL) increased the re-expansion and hatching rates of bovine embryos following cryopreservation following conventional freezing in ethylene glycol [26], glycerol [27] or propylene glycol [28]. Given the positive effects of hyaluronan on survival after vitrification in vitro and on survival of fresh embryos following transfer to recipients, further research should be conducted to determine whether hyaluronan treatment during culture can increase pregnancy rates following the transfer of vitrified/warmed embryos produced in vitro.

Addition of BSA to culture medium increased the yield of blastocysts. However, BSA also dramatically reduced re-expansion and hatching rates after vitrification. Bovine serum albumin is purified from blood products and thus it is possible that the batch of BSA utilized in the present experiments was contaminated with a molecule that was detrimental to cryosurvival. In previous reports, there was considerable variation among preparations and batches of BSA in their ability to stimulate embryo development in vitro [39,40]. Moreover, Lane et al. [27] reported that bovine embryos cultured with recombinant human albumin had increased re-expansion and hatching rates compared to embryos cultured in the presence of BSA. Therefore, we inferred that there are molecules in commercially prepared BSA preparations that can reduce the survival of bovine embryos following vitrification.

In Experiment 1, addition of 1 mg/mL hyaluronan increased the proportion of oocytes that developed to the blastocyst stage at Day 7 after insemination compared to culture medium without hyaluronan. However, there was no effect of 0.1 or 0.5 mg/mL of hyaluronan on embryo development. In Experiment 2, there was no effect of 1 mg/mL hyaluronan during embryo culture on the proportion of oocytes that became morula and blastocysts or blastocysts at 159–162 h after insemination. Previous studies evaluating the effect of low concentrations of hyaluronan (0.1–1.0 mg/mL) during culture on bovine embryo development in vitro were also inconsistent. For instance,

Furnish et al. [24] reported that 1 mg/mL hyaluronan increased blastocyst yield, but there was no effect of 0.5 mg/mL hyaluronan. In contrast, Jang et al. [25] reported improved embryo development in the presence of 0.5 mg/mL hyaluronan but not 0.1 or 1.0 ng/mL. Perhaps differences between Experiments 1 and 2 in the composition of embryo culture media altered the effect of hyaluronan on embryo development. In Experiment 2, the embryo culture medium included IGF-1, but IGF-1 was not in the medium used for Experiment 1. The addition of IGF-1 improved development of bovine embryos in vitro [16,41,42]. Thus, inclusion of IGF-1 in the embryo culture medium used in Experiment 2 may have masked the positive effect of hyaluronan on embryo development in Experiment 1.

In conclusion, the addition of hyaluronan to bovine embryo culture increased pregnancy rates obtained when morula and non-expanded blastocyst stage embryos produced in culture were transferred to recipients. Moreover, addition of hyaluronan to embryo culture improved survival following vitrification of bovine embryos produced in vitro. Taken together, hyaluronan has the potential to improve the efficacy of embryo transfer systems utilizing in vitro produced bovine embryos.

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