Effects of high concentrations of hyaluronan in culture medium on development and survival rates of fresh and frozen–thawed bovine embryos produced *in vitro*

M. Stojkovic¹, S. Kölle², S. Peinl¹, P. Stojkovic¹,
V. Zakhartchenko¹, J. G. Thompson³, H. Wenigerkind⁴,
H-D. Reichenbach⁵, F. Sinowatz² and E. Wolf¹

¹Department of Molecular Animal Breeding and Biotechnology, Hackerstr. 27, D-85764 Oberschleissheim, Germany; ²Department of Veterinary Anatomy II, Veterinärstr. 13, D-80539 Munich, Germany; ³Obstetrics and Gynecology, University of Adelaide, TQEH, Woodville, SA 5011, Australia; ⁴Bavarian Research Center for Reproductive Biology (BFZF), D-85764 Oberschleissheim, Germany; and ⁵Bavarian Research Station for Animal Breeding, D-85586 Poing, Germany

Hyaluronic acid (HA) is the main glycosaminoglycan present in follicular, oviductal and uterine fluids. The main functions of HA include dynamic processes that are mediated through interaction with extracellular matrix components, regulation of gene expression, cell proliferation and cell differentiation. HA increases the viscosity of solutions and also has several physiological functions, including regulation of water distribution and water-binding capacity. The addition of 6 mg HA ml⁻¹ to synthetic oviduct fluid (SOF; SOF–HA) culture medium on day 5 (IVF = day 0) significantly ($P < 0.001$) increased the viscosity of the medium in comparison with SOF culture medium containing BSA (SOF–BSA). On day 8, rate of blastocyst development in SOF–HA culture medium was significantly ($P < 0.05$) higher than in SOF–BSA culture medium (38.2 versus 29.3%). The number of trophectoderm cells and the total number of cells of expanded blastocysts cultured in

the presence of HA were significantly ($P < 0.01$) higher in comparison with expanded blastocysts cultured in the presence of BSA (88.9 ± 7.3 versus 67.6 ± 3.0 and 130.1 ± 10.9 versus 104.8 ± 2.5 , respectively). After freezing and thawing, the percentage of day 8 embryos that re-expanded and hatched when cultured with SOF–HA was greater than that of embryos cultured with SOF–BSA (11.3 and 10.5% versus 75.5 and 36.8%, respectively). After thawing, the ATP contents of *in vivo*-derived, SOF–HA and SOF–BSA expanded blastocysts were similar. The embryos cultured with HA showed less ultrastructural deviation and de-differentiation after freezing and thawing than the embryos cultured with BSA. This study demonstrates that HA improves the developmental capacity of bovine embryos under *in vitro* conditions and is warranted as a supplement for *in vitro* production of bovine embryos, particularly if they are to be cryopreserved.

Introduction

The most critical step in the production of bovine embryos from oocytes matured and fertilized *in vitro* is perhaps the culture of the presumptive zygotes to the blastocyst stage. One of the most widely used supplements for *in vitro* culture of bovine embryos is serum, which reduces early developmental potential of embryos (Bavister, 1995), causes abnormal metabolic and ultrastructural configuration of embryos (Abe *et al.*, 1999; Crosier *et al.*, 2001; Stojkovic *et al.*, 2001a) and at the same time increases the sensitivity of embryos to cryopreservation and compromises viability of these embryos, as indicated by the rates of embryo survival after thawing (Vajta *et al.*, 1997; Ohboshi *et al.*, 1998) and pregnancy rates (Massip *et al.*, 1995).

Most studies have focused on creating suitable chemically defined serum-free media to avoid these problems and to increase the developmental potential of bovine embryos produced *in vitro*. Unfortunately, the physical, particularly rheological properties of culture media are often overlooked. Among those, viscosity of culture medium, defined as a property of a fluid to resist flowing when subjected to an applied force, may play an important role. Highly viscous fluids are often composed of large, complicated molecules that influence the resorption ability, kinetics and release of drugs and nutritional elements from these fluids (Jackson *et al.*, 1991; Nishida *et al.*, 2000).

Stojkovic *et al.* (1999a) demonstrated that a high concentration of hyaluronic acid, a natural high viscosity mucopolysaccharide composed of repeating disaccharides (Hoae *et al.*, 1993), is a prospective candidate for increasing the viscosity of culture medium because of its chemical and

Email: M.Stojkovic@gen.vetmed.uni-muenchen.de

physical properties, and for successfully replacing serum for *in vitro* culture of bovine embryos. Hyaluronic acid or hyaluronan (HA, which includes the different forms that the molecule can take, for example, the acid form, hyaluronic acid, and the salts, such as sodium hyaluronate, which is formed at physiological pH) is the major glycosaminoglycan present in follicular, oviductal and uterine fluids (Lee and Ax, 1979; Suchanek *et al.*, 1994; Rodriguez-Martinez *et al.*, 1998). Physiological concentrations of HA in follicular, oviductal and uterine fluids of pigs range from 0.04 to 1.83 mg HA ml⁻¹ (16–39% of all glycosaminoglycans; Kano *et al.*, 1998). In addition, HA is secreted by granulosa and expanding cumulus cells (Eppig, 1979; Nagyova *et al.*, 2000). Receptors for HA (CD44) are present in human (Campbell *et al.*, 1995) and bovine (Valcarcel *et al.*, 1999; M. Stojkovic, P. Stranieri, V. Zakhartchenko, E. Wolf, unpublished) oocytes, cumulus cells, early embryos and blastocysts. The main functions of HA include cell adhesion and migration, dynamic processes that are mediated through interaction with extracellular matrix components (including laminin, elastin, chondroitin sulphate, heparan sulphate), regulation of protein secretion, gene expression, and cell proliferation and differentiation (Scott, 1992; Fraser *et al.*, 1997). As a result of the physicochemical properties of HA solutions, various physiological functions have been assigned, including regulation of protein and water distribution, water-binding capacity, filtering effects and lubrication.

For these reasons, the aim of the present study was to investigate the effects of high concentrations of HA on the viscosity of culture media, as well as on the rates of cleavage and development to the blastocyst stage, and on survival after freezing of bovine embryos produced *in vitro*. In addition, the effects of HA on the ultrastructural configuration of fresh and frozen-thawed embryos cultured with or without HA were analysed. As glycosaminoglycans are actively involved in metabolic pathways (Laurent, 1992) and strongly influence adenylate cyclase activity and ATP concentration (Dittmann *et al.*, 1998), total ATP contents of fresh and frozen-thawed embryos of different origin were measured and compared.

Parts of this study were presented at the IETS Meetings 1999 (Stojkovic *et al.*, 1999a) and 2001 (Stojkovic *et al.*, 2001c).

Materials and Methods

Measurement of viscosity of culture media and follicular fluid

Viscosities of all culture media before and after filtration and of follicular fluid were measured at 39°C using a capillary viscometer (AVS-350; Schott, Hofheim). All samples were measured at least three times and the final viscosity was estimated using Hagenbach's correction coefficient.

Determination of HA concentrations in follicular fluid and oestrous cow serum (ECS)

Follicular fluid from ovaries of slaughtered German Simmental cows at unknown stages of the oestrous cycle was collected from follicles of 2–8 mm in diameter, and

frozen with other culture media at –20°C until further use. The concentrations of HA in follicular fluid and culture media were evaluated using a sensitive (detection limit: < 10 µg l⁻¹) radiometric assay (HA-Test; Pharmacia Upjohn Diagnostics AB, Uppsala), based on the reaction between HA in the sample and ¹²⁵I-(hyaluronic acid binding proteins)-labelled HA in the assay solution. The unbound ¹²⁵I-labelled-HABP was quantified by incubation with HA covalently coupled to Sepharose® particles. The radioactivity bound to the particles was measured in a gamma counter (CB 2111; Berthold, Wildbad). All measurements were repeated at least three times.

In vitro production of bovine embryos

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO).

In vitro maturation of oocytes. Bovine oocytes were obtained from cows slaughtered at an abattoir as described by Stojkovic *et al.* (1995a), with some minor modifications. In brief, follicles 2–8 mm in diameter were aspirated from ovaries using a 20 gauge needle and a vacuum pressure of approximately 100 mm Hg. The cumulus–oocyte complexes (COCs) were collected in a 50 ml centrifuge tube and washed twice with pre-incubated (39°C; 5% CO₂) tissue culture medium 199 (TCM-199) supplemented with 10% (v/v) ECS. Only COCs with a complete dense cumulus and a dark, evenly granulated cytoplasm (Stojkovic *et al.*, 2001b) were selected for *in vitro* maturation. The COCs were washed in TCM-199 supplemented with 10% (v/v) ECS and 0.01 iu b-FSH and b-LH (Sioux Biochem., Sioux Center, IA) and cultured for maturation in the same medium for 22–24 h at 39°C in 5% CO₂ in air and maximum humidity.

In vitro fertilization. After maturation, COCs were transferred to fertilization medium (Tyrode's albumin lactate pyruvate; Minitüb, Tiefenbach) containing 6 mg BSA (Fraction V) ml⁻¹ and 10 µg heparin ml⁻¹, and incubated with frozen-thawed semen containing 10⁶ spermatozoa ml⁻¹ (German Simmental bull) that had been subjected to a swim-up procedure (Parrish *et al.*, 1986). Gametes were cultured in this medium for 18 h under the same conditions as those used for *in vitro* maturation.

In vitro culture. On day 1 (IVF = day 0), cumulus cells were removed from presumptive zygotes by vortexing (120 s) and gentle pipetting. Presumptive zygotes were washed three times in synthetic oviduct fluid medium (Tervit *et al.*, 1972) supplemented with 0.1% (v/v) minimum essential medium (MEM), 0.2% (v/v) basal medium eagle amino acids (SOF) and 1 mg BSA ml⁻¹ (Fraction V, fatty acid free and low endotoxin; ICN Biomedicals, Eschwege) (SOF–BSA). In nine replicates, 2012 presumptive zygotes were cultured in groups of 10–12 in 20 µl droplets of SOF–BSA until day 5. Subsequently, each group of 10–12 embryos was transferred randomly to either 20 µl droplets of fresh SOF–BSA (in total 992 embryos) or 20 µl droplets of fresh SOF–BSA supplemented with 6 mg HA–sodium salt

ml⁻¹ (SOF-HA) (in total 1020 embryos), recovered from *Streptococcus equi* sp. (537474; Fluka, Buchs, molecular weight: 1.69×10^6). The concentration and the *in vitro* culture schedule with HA used in the present study were chosen because it had been demonstrated previously that HA markedly increased the viscosity of the medium and successfully supported *in vitro* development of bovine embryos (Stojkovic *et al.*, 1999a). HA was solubilized for at least 45 min at 39°C. In addition, presumptive zygotes were allocated in our standard conditions, that is, groups of 30–35 presumptive zygotes in 400 µl SOF supplemented with 10% (v/v) ECS (SOF-ECS) for 8 days. Before use, all culture media were pre-equilibrated for 12–14 h at 39°C in a humidified atmosphere of 5% CO₂ in air. Osmolarity of all culture media was measured using a freeze point osmometer (Bachofar, Reutlingen): the readings were 271, 288 and 274 mOsm for SOF-BSA, SOF-HA and SOF-ECS, respectively. The pH values of culture media before use were measured using pH-meter (WTW Microprocessor, Weilheim) and were 7.46, 7.44 and 7.41 for SOF-BSA, SOF-HA and SOF-ECS, respectively. For all treatments, presumptive zygotes were allocated to all culture media from each *in vitro* fertilization experiment and cultured in four-well dishes (Nunc, Roskilde) and covered with pre-equilibrated paraffin oil at 39°C in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂. The numbers of embryos at the five- to eight-cell, morula and blastocyst stages on days 3, 7 and 8, respectively, were recorded. Day 7 and day 8 expanded blastocysts developed in SOF-ECS and day 8 blastocysts developed in SOF-BSA or SOF-HA were frozen immediately or allocated for determination of ATP content and number of cells.

Collection of *in vivo*-derived embryos

In vivo-derived embryos were recovered as described by Stojkovic *et al.* (1999b). In brief, German Simmental donor cows were synchronized by one i.m. injection of 500 µg PGF_{2α} (Estrumate; Mallinckrodt Vet GmbH, Burgwedel) at mid-oestrous cycle. Only cows with a functional corpus luteum on day 8 of the oestrous cycle were superovulated between day 9 and day 14 using 20 iu (NIH) FSH-p (Schering Pharmaceuticals, Kenilworth, NJ). FSH was administered twice each day at a decreasing dosage in a series of eight injections over 4 days. Animals received a double dose of PGF_{2α} i.m. at the time of the seventh and the eighth FSH injection and were observed twice each day for signs of oestrus. During oestrus, cows were artificially inseminated (day 0) two to three times with frozen-thawed semen from a selected bull of the same breed. After collection, day 7 expanded blastocysts were frozen immediately or allocated for determination of ATP content and number of cells.

Determination of the numbers of inner cell mass (ICM) and trophectoderm (TE) cells of blastocysts

Equal numbers of embryos (15 expanded blastocysts per treatment group) recovered on day 7 or day 8 after insemination

were subjected to differential cell staining as described by Stojkovic *et al.* (1998a). In brief, blastocysts were washed several times in Dulbecco's PBS to remove excess protein. Blastocysts were treated with 0.5% (w/v) pronase (Protease, Type XXV) in PBS for 3–5 min to dissolve the zona pellucida. Subsequently, blastocysts were incubated in a 1:2 dilution in PBS of rabbit antiserum raised against recombinant bovine interferon tau and other trophoblastic secretions (Stojkovic *et al.*, 1997) for 45 min at 39°C in a humidified atmosphere of 5% CO₂ in air. Embryos were then washed five times in PBS warmed at 39°C, and incubated in PBS supplemented with 5% (v/v) guinea-pig complement (ICN Immunobiologicals, Costa Mesa, CA) and 50 µg propidium iodide ml⁻¹ for 45 min at 39°C in a humidified atmosphere of 5% CO₂. Blastocysts were then washed in PBS and placed in cold absolute ethanol (Merck, Darmstadt) containing 25 µg fluorochrome bisbenzimidazole 33258 ml⁻¹ (Riedel-de Haen AG, Seelze, Hannover) for 30 min at 4°C. Finally, embryos were washed in absolute ethanol, mounted in undiluted glycerol (Merck) and squashed on a glass slide. The stained embryos were observed using a fluorescent microscope (Axiovert 135; Zeiss, Jena) with a mercury lamp under transmittance illumination and an UV excitation filter of 365 nm and a barrier filter of 420 nm.

Freezing-thawing of *in vivo*-derived and *in vitro*-produced expanded blastocysts

Day 7 blastocysts cultured with SOF-ECS and *in vivo*-derived expanded blastocysts, and day 8 expanded blastocysts cultured with SOF-BSA, SOF-HA or SOF-ECS were frozen as described by Stojkovic *et al.* (1995b). In brief, blastocysts were equilibrated in 1.5 mol ethylene glycol (EG) l⁻¹ in EmcareTM (ICP, Auckland) medium for 10 min at room temperature (21°C), loaded (2–4 embryos per straw) into plastic straws (Minitüb) and placed for 1 min at –7°C in the freezing machine (Haake, Karlsruhe). After seeding was induced, embryos were cooled at 0.5°C min⁻¹ to –35°C, after which they were immersed in liquid N₂ and stored until use. Embryos were thawed by warming for 10 s in air at room temperature and 20 s in a 30°C waterbath, washed six times in SOF-ECS medium and then cultured in 400 µl SOF-ECS. Re-expansion and hatching rates were evaluated at 24, 48 and 72 h after thawing. After thawing, expanded blastocysts were used immediately for ultrastructural analysis or determination of ATP content as described below.

Measurement of the ATP content of fresh *in vivo*-derived and *in vitro*-produced expanded blastocysts

The ATP content of both fresh and frozen-thawed *in vivo*-derived expanded blastocysts or expanded blastocysts developed in SOF medium in the presence of ECS, BSA or HA was measured using a commercial assay based on the luciferin-luciferase reaction (Bioluminescent Somatic Cell

Table 1. Mean viscosity and hyaluronic acid (HA) concentration of follicular fluid and different culture media before and after filtration

Sample	Viscosity (mm ² s ⁻¹)		HA (µg l ⁻¹)	
	Before filtration	After filtration	Before filtration	After filtration
SOF-ECS	1.25 ^a	1.13 ^{ab}	18.10 ^d	17.40 ^d
SOF-BSA	0.99 ^a	0.93 ^a	2.22 ^e	1.59 ^e
SOF-HA	82.90 ^b	52.03 ^c	5908.33 × 10 ^{3f}	4915.33 × 10 ^{3g}
Follicular fluid	4.03 ^c	1.74 ^b	37.19 × 10 ^{3g}	15.87 × 10 ^{3h}

SOF-ECS: synthetic oviduct fluid–oestrous cow serum; SOF-BSA: synthetic oviduct fluid–bovine serum albumin; SOF-HA: synthetic oviduct fluid–hyaluronic acid.

Values with different superscripts (^{a–c} for viscosity; ^{d–h} for HA concentration) within columns and rows are significantly different ($P < 0.05$; ANOVA followed by Bonferroni *post hoc* test).

Assay Kit, FL-ASC) according to the technique described by Stojkovic *et al.* (1999c). Fresh blastocysts (15–21 per treatment) were washed four times in sample buffer (99.0 mmol NaCl l⁻¹, 3.1 mmol KCl l⁻¹, 0.35 mmol NaH₂PO₄ l⁻¹, 21.6 mmol sodium lactate l⁻¹, 10.0 mmol Hepes l⁻¹, 2.0 mmol CaCl₂ l⁻¹, 1.1 mmol MgCl₂ l⁻¹, 25.0 mmol NaHCO₃ l⁻¹, 1.0 mmol sodium pyruvate l⁻¹, 0.1 mg gentamicin ml⁻¹ and 6.3 mg BSA ml⁻¹). Embryos were transferred individually in 50 µl of sample buffer and placed into separate plastic tubes, which were transferred into iced water. Subsequently, 50 µl ice-cold somatic cell reagent (FL-SAR) was added to each tube and tubes were held in iced water for 5 min. After this procedure, 100 µl ice-cold assay mix (FL-AAM) (diluted 1:25 with ATP Assay Mix Dilution Buffer, FL-AAB) was added and the tubes were held for 5 min at room temperature in the dark. After thawing, groups of 10–12 (in total 52–63 embryos) *in vivo*-derived and *in vitro*-produced expanded blastocysts were cultured in 400 µl SOF-ECS. The ATP content of frozen–thawed expanded blastocysts was measured at 0.0, 0.5, 1.0, 3.0 and 6.0 h after thawing. The ATP content of the samples was measured individually using a luminometer (Bioluminat Junior; Berthold, Wildbad). A seven point standard curve (0–6 pmol tube⁻¹) was routinely included in each assay. The ATP content was determined from the formula for the standard curve.

Semithin sections and transmission electron microscopy

The effects of culture condition on ultrastructure of embryos was investigated by removing 20 day 8 expanded blastocysts cultured with SOF-BSA and 21 day 8 expanded blastocysts cultured with SOF-HA from the culture medium. The blastocysts were washed in cacodylate buffer (0.2 mmol sodium cacodylate l⁻¹, pH 7.2) and fixed in Karnovsky's fluid (2.5% (v/v) glutaraldehyde and 2% (v/v) formaldehyde in 0.1 mol cacodylate buffer l⁻¹). Embryos were then fixed in 1% (w/v) OsO₄ and 1.5% (w/v) KFe(CN)₆ and transferred to a drop of 20% (w/v) BSA in cacodylate buffer. The addition of 25% (v/v) glutaraldehyde resulted in the polymerization of BSA to a pellet containing the

embryo. The pellet was dehydrated in a graded series of ethanol and embedded in Epon (Polysciences, Eppelheim). Semithin sections (1 µm) were cut and stained with methylene blue to assess the cellular compartments of the embryos. Ultrathin sections (50 nm) were mounted on grids, stained with OsO₄ and examined under a Zeiss electron microscope.

Statistical analysis

The rates of early cleavage stage embryo, morula and blastocyst development and survival after cryopreservation were evaluated using the binomial model and Logit Link by general linear mixed models procedure (GLIMMIX, SAS, version 6.12; SAS Institute, Inc., Cary, NC). The general linear model (GLM) was used to estimate fixed effects of culture condition and age of embryos, and interactions between these factors on the re-expansion, hatching and total survival rates of frozen–thawed embryos. When a significant ($P < 0.05$) F value was found, the GLM procedure was used to calculate least squares means, which were compared with t tests. The numbers of ICM and TE cells and the total number of cells were compared using Mann–Whitney U test. When an F value was significant ($P < 0.05$), HA concentration of follicular fluid and the various culture media, as well as ATP contents of fresh and frozen–thawed blastocysts, were compared using ANOVA followed by Bonferroni *post hoc* test. Differences were considered significant at $P < 0.05$.

Results

Viscosity of used culture media

The mean viscosities of follicular fluid and SOF medium with different supplements (ECS, BSA, HA) are presented (Table 1). HA significantly ($P < 0.001$) increased the viscosity of culture medium when compared with other samples measured. Follicular fluid had a higher viscosity than SOF-BSA and SOF-ECS media, but only the difference between follicular fluid and SOF-BSA was significant ($P < 0.05$).

Concentration of HA in follicular fluid and culture media

Before filtration, concentrations of HA in SOF-ECS ($18.10 \pm 0.18 \mu\text{g HA l}^{-1}$) were significantly ($P < 0.05$) higher than those in SOF-BSA ($2.22 \pm 0.01 \mu\text{g HA l}^{-1}$) (Table 1). Within non-filtered groups, significantly ($P < 0.001$) more HA was present in SOF-HA medium than in follicular fluid, SOF-BSA and SOF-ECS media. The same finding was observed after filtration of samples. The concentration of HA in filtered samples was always lower compared with non-filtrated samples, but the differences were significant ($P < 0.05$) only for the SOF-HA and follicular fluid samples.

Cleavage and rates of blastocyst development

Early cleavage rate (embryos with five- to eight-cells on day 3 after insemination) was significantly ($P < 0.05$) higher in embryos cultured with SOF-BSA than with SOF-ECS (73.8 ± 2.4 versus 66.9 ± 3.6) (Table 2). On day 7, significantly ($P < 0.05$) more morulae developed in SOF-HA medium than in SOF-ECS or SOF-BSA medium. However, on day 7, significantly ($P < 0.01$) more blastocysts developed in SOF-ECS than in SOF-HA or SOF-BSA medium. On day 7, total embryo rate in SOF-HA tended to be higher than in SOF-BSA (35.6 ± 2.8 versus 31.0 ± 1.5). This difference was significant ($P < 0.05$) in day 8 embryos (38.2 ± 3.4 versus 29.3 ± 2.1). The highest total development rate on either day 7 or day 8 was obtained using SOF-ECS (Table 2).

Differential staining of ICM and TE cells

Day 7 *in vivo*-derived expanded blastocysts possessed more ICM cells (48.4 ± 0.6 ; Fig. 1a) than did day 7 expanded blastocysts cultured with SOF-ECS (45.6 ± 2.4) and day 8 expanded blastocysts cultured with SOF-ECS (40.3 ± 1.4), SOF-BSA (37.2 ± 1.4) or SOF-HA (41.2 ± 4.5). Expanded blastocysts cultured in the presence of HA possessed more ICM cells than did other embryos cultured *in vitro*, but the differences were not significant. However, HA significantly ($P < 0.01$) increased the number of TE cells (Fig. 1b) and the total numbers of cells (Fig. 1c) compared with expanded blastocysts cultured in the presence of BSA (88.9 ± 7.3 versus 67.6 ± 3.0 and 130.1 ± 10.9 versus 104.8 ± 2.5 , respectively). The addition of HA to the culture medium consistently increased the number of TE cells and the total number of cells, which were similar to those of *in vivo*-derived embryos.

ATP content of *in vivo*-derived and *in vitro*-produced bovine embryos

Fresh day 8 expanded blastocysts cultured in the presence of ECS contained significantly ($P < 0.01$) more ATP ($2.34 \pm 0.25 \text{ pmol}$) than did day 7 expanded blastocysts ($1.21 \pm 0.08 \text{ pmol}$) cultured under the same conditions, day 8 blastocysts cultured with SOF-BSA ($0.75 \pm 0.06 \text{ pmol}$) or SOF-HA ($0.61 \pm 0.04 \text{ pmol}$) and day 7 *in*

vivo-derived expanded blastocysts ($1.05 \pm 0.07 \text{ pmol}$). However, *in vivo*-derived expanded blastocysts contained significantly ($P < 0.05$) more ATP than expanded blastocysts developed in the presence of BSA or HA (Fig. 2).

Effects of culture conditions and age of frozen-thawed blastocysts on the re-expansion, hatching and total survival rates

The percentages of day 8 embryos re-expanding and hatching, and the total number of embryos that continued to develop (that is, sum of embryos re-expanding and hatching) were affected by culture condition. The effects of embryo age and the interaction of culture condition $\times$ age were not significant and were omitted from the model. By 24 h of culture after thawing, the total percentage of embryos that continued to develop was significantly ($F = 4.75$, $P < 0.01$) affected by culture conditions, with a lower performance of frozen-thawed embryos developed in SOF-BSA than embryos in the other two culture groups (which behaved similarly). This effect continued after extended culture to 48 and 72 h. Comparison between SOF-ECS embryos frozen on day 7 or day 8 revealed that day 8 embryos appeared to tolerate freezing and thawing better than day 7 embryos (Table 3).

In vivo-derived embryos, and day 8 blastocysts cultured with SOF-ECS and SOF-HA all behaved in a similar way after thawing (Table 3).

ATP content of frozen-thawed *in vivo*-derived and *in vitro*-produced bovine embryos

After thawing, day 8 expanded blastocysts cultured with ECS contained significantly ($P < 0.01$) more ATP than did day 7 SOF-ECS, *in vivo*-derived or SOF-BSA and SOF-HA expanded blastocysts (Fig. 3). The same tendency was observed after 1, 3 and 6 h, except that after 6 h the ATP content of day 7 blastocysts cultured with SOF-ECS was also significantly ($P < 0.001$) higher than that of blastocysts cultured with SOF-BSA or SOF-HA or of *in vivo*-derived blastocysts. The ATP contents of *in vivo*-derived, and SOF-HA and SOF-BSA expanded blastocysts were similar.

Semithin sections and transmission electron microscopy of fresh and frozen-thawed 8-day-old embryos cultured with SOF-BSA or SOF-HA

The 8-day-old embryos cultured with SOF-BSA showed a clear differentiation of TE and ICM cells surrounding a blastocoel. Although the polyhedral ICM cells had a spherical nucleus with large nucleoli, the flattened trophoblast cells were characterized by an oval nucleus and occasional mitotic figures. The microvilli of the apical membrane of the trophoblast cells were well developed. The most conspicuous and abundant organelles were mitochondria of different sizes characterized by a round or elongated shape and transverse cristae (Fig. 4a). Embryos cultured with SOF-BSA had Golgi apparatus and numerous ballooned

Table 2. Developmental rates of *in vitro*-produced bovine embryos cultured in synthetic oviduct fluid (SOF) with different supplements

Culture	Oocytes	Day 3		Day 7 ⁺				Day 8 ⁺			
		Five- to eight-cell		Morulae (%)	Blastocysts (%)	Expanded blastocysts (%)	Total (%)	Blastocysts (%)	Expanded blastocysts (%)	Hatched blastocysts (%)	Total (%)
		(%)	(%)								
BSA	2012	1485 (73.8) ^a	992	187 (18.9) ^a	101 (10.2) ^a	19 (1.9)	307 (31.0) ^a	119 (12.0)	124 (12.5) ^a	47 (4.7)	290 (29.3) ^a
HA	—	—	1020	256 (25.1) ^b	91 (8.9) ^a	17 (1.7)	364 (35.6) ^a	158 (15.5)	174 (17.1) ^{ab}	57 (5.6)	389 (38.2) ^b
ECS	1615	1082 (66.9) ^b	1615	301 (18.6) ^a	281 (17.5) ^b	151 (9.3)	733 (45.4) ^c	203 (12.6)	319 (19.7) ^b	217 (13.4)	739 (45.7) ^c

*In nine replicates, day 5 fertilized oocytes cultured in synthetic oviduct–bovine serum albumin (SOF–BSA) were divided into two groups: SOF–BSA or synthetic oviduct fluid–hyaluronic acid (SOF–HA).
^aData are based on oocytes and embryos transferred in fresh medium on day 5.

ECS: oestrous cow serum.

Values with different superscripts differ significantly (^a versus ^b and ^b versus ^c, $P < 0.05$; ^a versus ^c, $P < 0.01$; least squares means compared by *t* tests).

Fig. 1. The effect of hyaluronic acid (HA) supplementation on the number of (a) inner cell mass (ICM) cells, (b) trophoblast (TE) cells and (c) the total number of cells of bovine embryos produced *in vitro*. Data are presented as mean $\pm$ SEM (error bars) from 15 expanded blastocysts analysed per experimental group. ^{a-c}Within each graph, bars with different superscripts are significantly different ($P < 0.05$); * $P = 0.06$ oestrous cow serum (ECS) 7 versus ECS 8 (Mann–Whitney U test). *In vivo* 7: day 7 *in vivo*-derived embryos; ECS 7: day 7 embryos cultured with ECS; ECS 8: day 8 embryos cultured with ECS; BSA 8: day 8 embryos cultured with BSA; HA 8: day 8 embryos cultured with HA.

rough endoplasmic reticulae, implying high rates of protein synthesis (Fig. 4a). In the embryonic cells, numerous lysosomes and lipid vacuoles were visible. Cell debris accumulated regularly in the ICM (Fig. 4a) and TE cells, and between the trophoblast cells and the zona pellucida.

In a similar manner to the embryos cultured with BSA, embryos treated with HA revealed a differentiation in trophoblast cells and ICM. However, in the embryos

Fig. 2. ATP content of fresh *in vivo*-derived and *in vitro*-produced bovine embryos cultured with different supplements. Data are presented as the mean $\pm$ SEM from 15 expanded blastocysts analysed per experimental group. ^{a-c}Bars with different superscripts are significantly different ($P < 0.05$; ANOVA followed by Bonferroni *post hoc* test). *In vivo* 7: day 7 *in vivo*-derived embryos; ECS 7: day 7 embryos cultured with oestrous cow serum (ECS); ECS 8: day 8 embryos cultured with ECS; BSA 8: day 8 embryos cultured with BSA; HA 8: day 8 embryos cultured with hyaluronic acid.

cultured with HA, the blastocoel was distinctly smaller than in the embryos cultured with BSA, implying a slower formation of the blastocoel in these embryos. The number of organelles in the cytoplasm of the embryonic cells seemed to be increased in embryos cultured with HA compared with those cultured in BSA. Marked differences were seen in the morphology of the mitochondria. Although mitochondria of embryos cultured with BSA were of the typical hooded appearance (Fig. 4a), embryos cultured with HA showed exclusively mitochondria with very few cristae or mitochondria with ballooned cristae (Fig. 4b). Further differences between the two groups of embryos were seen in the formation of lysosome-like structures and in the accumulation of cell debris, which was distinctly greater in embryos cultured with HA (Fig. 4b). Microvilli seemed to be more abundant in embryos cultured with HA compared with embryos cultured with BSA (Fig. 4c).

Cryopreservation resulted in distinct morphological alterations in both groups of embryo. The most conspicuous alteration was extended vacuolization of the cytoplasm of the embryonic cells and the formation of large intercellular cracks (Figs 5 and 6a). The number of cell organelles was distinctly reduced in both groups (Fig. 6a). The accumulation of cell debris was markedly increased (Fig. 6a). The nuclei of the embryonic cells were often irregular in shape and only rarely was heterochromatin detected (Fig. 5a,b). Generally, embryonic cells revealed irregular forms and showed signs of deviation; the differentiation in trophoblast and ICM cells was no longer visible (Fig. 5a,b).

In general, the embryos cultured with HA showed less deviation after freezing than did embryos cultured with BSA. In HA embryos, morphologically intact cells could be detected (Fig. 6b), which was not possible in BSA embryos.

Table 3. Re-expansion and hatching of frozen–thawed bovine blastocysts produced *in vitro*

Culture (day)	n*	24 h			48 h			72 h		
		Re-expansion (%)	Hatching (%)	Total (%)	Re-expansion (%)	Hatching (%)	Total (%)	Re-expansion (%)	Hatching (%)	Total (%)
<i>In vivo</i> (7)	37	23 (62.2) ^a	6 (16.2) ^{ab}	29 (78.4) ^a	9 (24.3)	24 (64.9) ^a	33 (89.2) ^a	4 (10.8)	30 (81.1) ^a	34 (91.9) ^a
SOF–ECS (7)	49	11 (22.4) ^b	15 (30.6) ^a	26 (53.1) ^{bc}	13 (26.5)	15 (30.6) ^{b†}	28 (57.1) ^{bc}	0 (0)	27 (55.1) ^{bc}	27 (55.1) ^b
SOF–ECS (8)	45	27 (60.0) ^a	6 (13.3) ^{ab}	33 (73.3) ^{ac}	9 (20.0)	25 (55.6) ^{ab}	34 (75.6) ^{ab}	1 (2.2)	31 (68.9) ^{ab}	32 (71.1) ^{ab‡}
SOF–BSA (8)	57	19 (33.3) ^b	2 (3.5) ^b	21 (36.8) ^b	12 (21.0)	17 (29.8) ^b	29 (50.8) ^c	6 (10.5)	21 (36.8) ^c	27 (47.3) ^b
SOF–HA (8)	53	32 (60.4) ^a	5 (9.5) ^b	37 (69.8) ^{ac}	13 (24.5)	33 (62.2) ^a	46 (86.7) ^a	6 (11.3)	40 (75.5) ^a	46 (86.8) ^a

*All embryos that were frozen in 1.5 mol ethylene glycol l⁻¹ were recovered after thawing.

In four replicates, two to four expanded blastocysts from the *in vivo* group, the synthetic oviduct fluid–oestrous cow serum (SOF–ECS) day 7 group, SOF–ECS day 8 group, the synthetic oviduct fluid–bovine serum albumin (SOF–BSA) group, and synthetic oviduct fluid–hyaluronic acid (SOF–HA) group were loaded per straw into 10, 13, 12, 15 or 14 straws, respectively.

†SOF–ECS day 7 versus SOF–ECS day 8, $P = 0.07$; ‡SOF–ECS day 8 versus SOF–BSA, $P = 0.06$.

Values with different superscripts within columns differ significantly (^a versus ^b, $P < 0.05$ and ^a versus ^c, $P < 0.01$; least squares means compared by *t* tests).

Fig. 3. Comparison of the ATP contents of frozen–thawed *in vivo*-derived and *in vitro*-produced bovine embryos measured at different time points after cryopreservation. Mean values from 10–12 expanded blastocysts were analysed between different culture treatments using ANOVA followed by Bonferroni *post hoc* test ($P < 0.05$). (●): Day 7 *in vivo*-derived embryos; (■): day 7 embryos cultured with oestrous cow serum (ECS); (△): day 8 embryos cultured with ECS; (▲): day 8 embryos cultured with BSA; (○): day 8 embryos cultured with hyaluronic acid. ^a versus ^b, $P < 0.01$; ^a versus ^c and ^b versus ^c, $P < 0.001$.

Embryos cultured with BSA showed not only irregular, deviated cells, but also large areas of cytoplasm without any organelles (Fig. 6a). The cells of the embryos cultured with BSA always lost contact with the zona pellucida after freezing (Fig. 5a), a phenomenon which was not seen in embryos cultured with HA (Fig. 5b). Lysosome-like structures in the cells and accumulation of cell debris were seen more frequently in embryos cultured with BSA than in

embryos cultured with HA (Fig. 6a). Similar to the fresh embryos, after freezing, the microvilli in embryos cultured with HA seemed more prominent and dense than in embryos cultured with BSA.

Discussion

In the present study, the effects of the high concentrations of HA on early embryonic development and sensitivity to cryopreservation of *in vitro*-derived bovine blastocysts were evaluated.

The viscosity of the basic medium used (synthetic oviduct fluid) was markedly increased after the addition of HA in both non-filtered and filtered samples. To our knowledge there are no studies that describe the viscosity of bovine oviduct and uterine fluid. Bovine embryos are able to develop to the blastocyst stage in highly viscous medium supplemented with Guar Gum (Stojkovic *et al.*, 1998b) or hydroxypropylmethylcellulose (M. Stojkovic, unpublished) and with HA (Stojkovic *et al.*, 1999a; this study). The viscosity of HA solutions varies with molecular weight and concentration. The commercially available material has molecular weights ranging from $< 1 \times 10^6$ to as much as 8×10^6 , and this certainly influences the biological and physicochemical properties of HA. In addition, our preliminary results demonstrate that the origin of HA (human umbilical cord, rooster comb or *Streptococcus equi* sp.) influences bovine embryo development *in vitro* (data not presented). In the present study, a high concentration of HA (molecular weight: 1.69×10^6) was used to increase the viscosity of culture medium.

Marquant-Le Guenne *et al.* (1998) demonstrated that HA can successfully substitute for sera for *in vitro* maturation of bovine oocytes. In support of the results from the present study, Stojkovic *et al.* (1999a) and several other studies have demonstrated that supplementation of culture medium with HA improves *in vitro* development of bovine (Furnus *et al.*, 1998) and pig (Miyoshi *et al.*, 1999) embryos

Fig. 4. Ultrastructure of 8-day-old expanded bovine blastocysts cultured with (a) synthetic oviduct fluid–bovine serum albumin (SOF–BSA) or (b,c) synthetic oviduct fluid–hyaluronic acid (SOF–HA). The inner cell mass cells of embryos cultured with BSA possess ballooned endoplasmic reticulum implying high protein synthesis (arrowhead). In addition, (a) embryos cultured with BSA have mitochondria with the typical hooded appearance (arrows), whereas (b) embryos cultured with HA show exclusively mitochondria with very few cristae or mitochondria with ballooned cristae (arrows). The accumulation of cell debris (*) is distinctly increased in (b) embryos cultured with HA compared with (a) embryos cultured with BSA. Microvilli seem to be more abundant in embryos cultured with (c) HA as compared with embryos cultured with BSA. Scale bars represent 2 µm.

up to the blastocyst stage. In mice, increased rates of implantation and fetal development after blastocyst transfers were observed when HA was added to the culture medium (Gardner *et al.*, 1999).

In the present study, blastocysts cultured in the presence of HA contained a similar total number of cells to *in vivo*-derived embryos. There was an increase in the number of TE cells of embryos cultured with SOF–HA in comparison to embryos cultured in the presence of BSA, demonstrating that HA plays an important role in cell proliferation. Induction of proliferation by HA has been widely demonstrated in regenerating or healing tissues (Laurent, 1992). HA is also involved in cell migration and proliferation, critical events in tissue remodelling during development, morphogenesis and normal tissue homeostasis (Toole, 1997). In mice, HA plays a crucial role in the attachment of the blastocyst and outgrowth of the trophectoderm, markedly promoting the differentiation of extra-embryonic structures of the embryo (Hamashima, 1982). The absence of HA leads to the gradual degeneration and loss of ICM cells and to the end of cell proliferation of primary trophoblast giant cells (Hamashima, 1982).

The interaction of HA with matrix HABPs (hyaladherins) and cell surface HA receptors (CD44) regulates not only cell behaviour, such as cell migration, cell to cell adhesion, cell differentiation and proliferation, but also plays a role in cell metabolism. After endocytosis, HA is transported to lysosomes, degraded to glucuronic acid and *N*-

Fig. 5. Semithin sections of frozen-thawed 8-day-old bovine expanded blastocysts developed in (a) synthetic oviduct fluid–bovine serum albumin (SOF–BSA) or (b) synthetic oviduct–hyaluronic acid (SOF–HA). The cells of the embryos cultured with BSA always lose contact (+) with the zona pellucida after freezing (a); however, this is not the case in embryos cultured with HA (b). ZP: zona pellucida; n: nuclei; *lipid vacuoles. Scale bars represent 50 µm.

acetylglucosamine. These monosaccharides are transferred to the cytosol, where the glucuronic acid is degraded via its regular pathway. *N*-acetylglucosamine becomes phosphorylated to *N*-acetylglucosamine 6-phosphate, which can be deaminated to fructose 6-phosphate, a glycolytic substrate (Laurent, 1992). The mechanism by which HA influences embryonic metabolism is not clear, but in the plasma membrane of cells from the rat liver, HA reduces

Fig. 6. Ultrastructure of frozen 8-day-old expanded bovine blastocysts cultured with (a) synthetic oviduct fluid-bovine serum albumin (SOF-BSA) or (b) synthetic oviduct fluid-hyaluronic acid (SOF-HA) immediately after thawing. Cryopreservation of embryos cultured with BSA results in extended vacuolization of the cytoplasm of the embryonic cells and the formation of large intercellular spaces (+). The accumulation of cell debris (*) is markedly increased. There are wide areas of cytoplasm without organelles (arrowhead). Embryonic cells reveal irregular forms and show signs of deviation. In contrast to the (a) embryos cultured with BSA, (b) embryos cultured with HA possess morphologically intact cells. Scale bars represent 2 μ m.

adenylate cyclase activity, which is necessary for maintaining ATP concentrations (Dittmann *et al.*, 1998). In the present study, embryos cultured with SOF–HA or SOF–BSA and *in vivo*-recovered embryos contained less total ATP than did embryos cultured with SOF–ECS. The possible explanations for these findings are: (i) impaired enzymatic conversion of ATP due to the reduced adenylate cyclase activity in the presence of HA; (ii) developmental status and density of mitochondria; and (iii) high ATP content of blastocysts developed in medium with serum could be attributable to broad metabolic disturbances in ATP degradation and carriers of energy. An increased accumulation of ATP in bovine embryos could be a sign of poor metabolic turnover, that is, stress and non-functional biosynthesis and cellular activity. It was suggested that mouse embryos with high ATP content had low ATP synthesis, and decreased nucleic acid and protein synthesis (Ginsberg and Hillman, 1973).

After thawing, ATP content was low but then increased in all *in vitro*-produced groups, and reached a peak similar to that observed in fresh *in vitro*-produced embryos after 3–6 h. This finding demonstrates that *in vitro*-produced and cryopreserved embryos require some time to recover and to reach the ATP content of fresh embryos. These data are in agreement with the studies reported by Vajta *et al.* (1997), which demonstrated that *in vitro*-produced bovine embryos require at least 4 h to regenerate and reorganize damaged ultrastructural configuration after cryopreservation. In the present study, *in vivo*-derived frozen-thawed embryos do not completely reach the ATP content of fresh embryos. This finding could be caused by the *in vitro* culture conditions used after thawing of *in vivo*-derived embryos. It is important to state that the ATP curves of SOF–BSA and SOF–HA embryos at different measurement times were synchronized with *in vivo*-recovered frozen-thawed embryos, indicating that serum-free embryos are metabolically more similar to *in vivo*-derived embryos than embryos recovered in the presence of serum. Embryos cultured with SOF–BSA showed low rates of development after thawing. The addition of HA to the culture medium significantly increased survival of embryos after thawing, and therefore HA is warranted as a supplement for *in vitro* production of bovine embryos, particularly for those embryos that are to be cryopreserved. Further studies are necessary to confirm the improved viability of *in vitro*-produced bovine embryos cultured in the presence of HA by embryo transfer.

Palasz *et al.* (1993, 2000) demonstrated that the presence of HA in the freezing medium increases survival of frozen-thawed bovine blastocysts. These authors postulated that this may be due to a reduced toxicity of cryoprotectant by HA. It is possible that the high water binding capacity of HA increased not only viscoelastic properties of the culture medium, but also cytosolic viscosity of embryos, which may bolster cell survival by decreasing the growth of lethal intracellular ice crystals (Stojkovic *et al.*, 2001c). Introduction of intracellular trehalose improves the survival of cryopreserved mammalian cells in a similar way: increasing

cytosolic viscosity and decreasing the growth of lethal intracellular ice crystals (Eroglu *et al.*, 2000). Similarly, plants synthesize oligosaccharides of the raffinose family in response to low temperature (Crowe and Crowe, 2000). The accumulation of oligosaccharides of the raffinose family is believed to play an important role in the stabilization of structures and function of cellular liquids and the freezing tolerance. Again, HA has strong water-binding capacity, the ability to interact with water to obtain a gel-like structure and, hence, the ability to reduce water mobility. It is highly likely that HA decreases the lethal consequences of crystallization and, at the same time, increases the resistance of membranes and organelles to the shock of cryopreservation. This finding is confirmed by our ultrastructural studies in which HA was demonstrated to reduce the extent of cellular damage caused by cryopreservation. Thus, the vacuolization of the embryonic cells, the degradation of cell organelles and the formation of intercellular spaces after cryopreservation were markedly reduced by the addition of HA to the culture medium. In embryos cultured with SOF–HA, morphologically intact cells were observed after cryopreservation; this was not observed in the SOF–BSA embryos. The presence of dense and prominent microvilli in the embryos cultured with SOF–HA indicates the preservation of metabolic activity in these embryos. Although the mitochondria of embryos cultured in the presence of HA had very few cristae or even ballooned cristae, the ATP content was not significantly altered in these embryos as compared with control (BSA) embryos. These results indicate that the morphology of mitochondria is clearly influenced by culture conditions and origin of the embryos (Abe *et al.*, 1999; Crosier *et al.*, 2000; Fair *et al.*, 2001), but that mitochondrial morphology does not necessarily affect energy metabolism.

In summary, the results from the present study demonstrate that a high concentration of HA increases the viscosity of culture medium and improves *in vitro* development and the number of cells of bovine embryos. The survival rate after freezing of embryos cultured with HA supplementation before freezing was also increased. HA probably affects the development, proliferation, vitality and ultrastructure of *in vitro*-produced bovine embryos via different pathways acting through its complex chemical and physical properties. Further studies are necessary to explain the molecular mechanism and biological-physiological roles of HA in early bovine embryogenesis.

The authors are grateful to A. Koppi from the Fraunhofer Institute in Munich for the measurements of viscosity. This study was supported by grants from the Bayerische Forschungsförderung and from the Deutsche Forschungsgemeinschaft.

References

- Abe H, Yamashita S, Itoh T, Satoh T and Hoshi H (1999) Ultrastructure of bovine embryos developed from *in vitro*-matured and -fertilized oocytes: comparative morphological evaluation of embryos cultured

- either in serum-free medium or in serum-supplemented medium *Molecular Reproduction and Development* **53** 325–335
- Bavister BD** (1995) Culture of preimplantation embryos: facts and artifacts *Human Reproduction Update* **1** 91–148
- Campbell S, Swann HR, Aplin JD, Seif MW, Kimber SJ and Elstein M** (1995) CD44 is expressed throughout preimplantation human embryo development *Human Reproduction* **10** 425–430
- Crosier AE, Farin PW, Dykstra MJ, Alexander JE and Farin CE** (2001) Ultrastructural morphometry of bovine blastocysts produced *in vivo* or *in vitro*. *Biology of Reproduction* **64** 1375–1385
- Crowe JH and Crowe LM** (2000) Preservation of mammalian cells: learning nature's tricks *Nature Biotechnology* **18** 145–146
- Dittmann J, Keller C and Harisch G** (1998) Inhibition of adenylate cyclase of rat hepatic membranes by glycosaminoglycans *Life Sciences* **63** 2199–2208
- Eppig JJ** (1979) FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles *Nature* **281** 483–484
- Eroglu A, Russo MJ, Bieganski R, Fowler A, Cheley S, Bayley H and Toner M** (2000) Intracellular trehalose improves the survival of cryopreserved mammalian cells *Nature Biotechnology* **18** 163–167
- Fair T, Lonergan P, Dinnyes A, Cottell DC, Hyttel P, Ward FA and Boland MP** (2001) Ultrastructure of bovine blastocysts following cryopreservation: effect of method of blastocyst production *Molecular Reproduction and Development* **58** 186–195
- Fraser JRE, Laurent TC and Laurent UBG** (1997) Hyaluronan: its nature, distribution, functions and turnover *Journal of Internal Medicine* **242** 27–33
- Furnus CC, de Matos DG and Martinez AG** (1998) Effect of hyaluronic acid on development of *in vitro* produced bovine embryos *Theriogenology* **49** 1489–1499
- Gardner DK, Rodriguez-Martinez H and Lane M** (1999) Fetal development after transfer is increased by replacing protein with the glycosaminoglycan hyaluronan for mouse embryo culture and transfer *Human Reproduction* **14** 2575–2580
- Ginsberg L and Hillman N** (1973) ATP metabolism in cleavage-stage mouse embryos *Journal of Embryology and Experimental Morphology* **30** 267–282
- Hamashima N** (1982) Effects of hyaluronic acid on the peri-implantational development of mouse embryos *in vitro*. *Development, Growth and Differentiation* **24** 353–357
- Hoae K, Savani RC, Wang C, Yang B and Turley EA** (1993) Identification of hyaluronan binding proteins using a biotinylated hyaluronan probe *Connective Tissue Research* **30** 117–126
- Jackson RL, Busch SJ and Cardin AD** (1991) Glycosaminoglycans: molecular properties, protein interactions and role in physiological processes *Physiological Reviews* **71** 481–539
- Kano K, Miyano T and Kato S** (1998) Effects of glycosaminoglycans on the development of *in vitro*-matured and -fertilized porcine oocytes to the blastocyst stage *in vitro*. *Biology of Reproduction* **58** 1226–1232
- Laurent TC and Fraser JRE** (1992) Hyaluronan *Journal of the Federation of American Societies for Experimental Biology* **6** 2397–2404
- Lee CN and Ax RL** (1984) Concentration and composition of glycosaminoglycans in the female bovine reproductive tract *Journal of Dairy Science* **67** 2006–2009
- Marquant-Le Guenne B, Guyader-Joly C, Ponchon S, Delalleau N and Humblot P** (1998) Effect of hyaluronic acid in a serum free maturation medium on bovine embryo development *Theriogenology* **51** 386 (Abstract)
- Massip A, Mermillod P and Dinnyes A** (1995) Morphology and biochemistry of *in vitro* produced bovine embryos: implications for their cryopreservation *Human Reproduction* **10** 3004–3011
- Miyoshi K, Umezumi M and Sato E** (1999) Effect of hyaluronic acid on the development of porcine 1-cell embryos produced by conventional or new *in vitro* maturation/fertilization system *Theriogenology* **51** 777–784
- Nagyova E, Vanderhyden BC and Prochazka R** (2000) Secretion of paracrine factors enabling expansion of cumulus cells is developmentally regulated in pig oocytes *Biology of Reproduction* **63** 1149–1156
- Nishida K, Nakakoga Y, Sato N, Kawakami S, Mukai T, Sasaki H, Sakaeda T and Nakamura J** (2000) Effect of viscous additives on drug absorption from the liver surface in rats using phenol red as a model *European Journal of Pharmacy and Biopharmacology* **50** 397–402
- Ohboshi S, Fujihara N, Yoshida T and Tomagane H** (1998) Ultrastructure of bovine *in vitro*-produced blastocysts cryopreserved by vitrification *Zygote* **6** 17–26
- Palasz A, Alkemade S and Mapletto RJ** (1993) The use of sodium hyaluronate in freezing media for bovine and murine embryos *Cryobiology* **30** 172–178
- Palasz AT, Thundathil J, de la Fuente R and Mapletto RJ** (2000) Effect of reduced concentration of glycerol and various macromolecules on the cryopreservation of mouse and cattle embryos *Cryobiology* **41** 35–42
- Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH and First NL** (1986) Bovine *in vitro* fertilization with frozen-thawed semen *Theriogenology* **25** 591–600
- Rodriguez-Martinez H, Larsson B, Pertoft H and Kjellen L** (1998) GAGs and spermatozoa competence *in vivo* and *in vitro*. In *Gametes: Development and Function* pp 239–272 Eds A Lauria, G Enne, F Gandolfi and L Gianaroli. Sero Symposium, Roma
- Scott JE** (1992) Supramolecular organization of extracellular matrix glycosaminoglycans, *in vitro* and in the tissues *Journal of the Federation of American Societies for Experimental Biology* **6** 2639–2645
- Stojkovic M, Wolf E, Büttner M, Berg U, Charpigny G, Schmitt A and Brem G** (1995a) Secretion of biologically active interferon tau by *in vitro*-derived bovine trophoblastic tissue *Biology of Reproduction* **53** 1500–1507
- Stojkovic M, Willemsen D, Büttner M and Wolf E** (1995b) Development and interferon production by frozen-thawed *in vitro* derived bovine trophoblastic vesicles *Theriogenology* **43** 326 (Abstract)
- Stojkovic M, Zakhartchenko V, Brem G and Wolf E** (1997) Support for the development of bovine embryos *in vitro* by secretions of bovine trophoblastic vesicles derived *in vitro*. *Journal of Reproduction and Fertility* **111** 191–196
- Stojkovic M, Büttner M, Zakhartchenko V, Brem G and Wolf E** (1998a) A reliable procedure for differential staining of *in vitro* produced bovine blastocysts: comparison of tissue culture medium 199 and Menezes's B2 medium *Animal Reproduction Science* **50** 1–9
- Stojkovic M, Boxhammer K, Zakhartchenko V and Wolf E** (1998b) Plant extracts (Guar gum) support development of bovine embryos *in vitro*. *Theriogenology* **49** 219 (Abstract)
- Stojkovic M, Thompson JG and Tervit HR** (1999a) Effects of hyaluronic acid supplementation on *in vitro* development of bovine embryos in a two-step culture system *Theriogenology* **51** 254 (Abstract)
- Stojkovic M, Büttner M, Zakhartchenko V, Riedl J, Reichenbach HD, Wenigerkind H, Brem G and Wolf E** (1999b) Secretion of interferon-tau by bovine embryos in long-term culture: comparison of *in vivo* derived, *in vitro* produced, nuclear transfer and demi embryos *Animal Reproduction Science* **55** 151–162
- Stojkovic M, Westesen K, Zakhartchenko V, Stojkovic P, Boxhammer K and Wolf E** (1999c) Coenzyme Q₁₀ in submicron-size dispersion improves development, cell proliferation, and hatching of *in vitro* produced bovine embryos *Biology of Reproduction* **61** 541–547
- Stojkovic M, Kölle S, Zakhartchenko V, Stojkovic P, Sinowatz F and Wolf E** (2001a) Effects of estrous cow serum/bovine serum albumin on early cleavage, blastocyst rate, cell number, and ultrastructural configuration of *in vitro* produced bovine embryos *Advances in Reproduction V* **35**–44
- Stojkovic M, Machado SA, Stojkovic P, Zakhartchenko V, Hutzler P, Goncalves PB and Wolf E** (2001b) Mitochondrial distribution and adenosine triphosphate-content of bovine oocytes before and after *in vitro* maturation: correlation with morphological criteria and developmental capacity after *in vitro* fertilization and culture *Biology of Reproduction* **64** 904–909
- Stojkovic M, Peinl S, Stojkovic P, Zakhartchenko V, Thompson JG and Wolf E** (2001c) High concentration of hyaluronic acid in culture medium increases the survival rate of frozen/thawed *in vitro* produced bovine embryos *Theriogenology* **55** 317 (Abstract)
- Suchanek E, Simunic V, Juretic D and Grizelj V** (1994) Follicular fluid contents of hyaluronic acid, follicle-stimulating hormone and steroids

- relative to the success of *in vitro* fertilization of human oocytes *Fertility and Sterility* **62** 347–352
- Tervit HR, Whittingham DG and Rowson LEA** (1972) Successful culture *in vitro* of sheep and cattle ova *Journal of Reproduction and Fertility* **30** 493–497
- Toole BP** (1997) Hyaluronan in morphogenesis *Journal of Internal Medicine* **242** 35–40
- Vajta G, Hyttel P and Callesen H** (1997) Morphological changes of *in vitro* produced bovine blastocysts after vitrification, in-straw direct rehydration, and culture *Molecular Reproduction and Development* **48** 9–17
- Valcarcel A, de Matos DG and Furnus CC** (1999) The hyaluronic acid receptor (CD44) is expressed in bovine oocytes and preimplantational stage embryos *Theriogenology* **51** 193 (Abstract)

Received 8 November 2001.

First decision 15 January 2002.

Revision received 1 February 2002.

Accepted 26 February 2002.